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HEME TRANSPORT AND INCORPORATION INTO GLOBIN

Rice University

Ph.D. 1982

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HEME TRANSPORT AND INCORPORATION INTO GLOBIN

by

Melanie Y. Rose

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

Houston, Texas

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Melanie Y. Kose

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similar. The combination of globin with membrane bound heme is much slower \((t_{1/2} = 200 \text{ ms})\) than with free heme \((t_{1/2} = 1 \text{ ms})\) due to partitioning between the various phases. A theoretical expression for \(k_{\text{obs}}\) was derived and evaluated using constants obtained from independent experiments.

Our results demonstrate the rapid exchange of heme between liposomes and extraction by apoproteins which occurs by a rate limiting first order step of heme dissociation into the aqueous phase. A carrier protein is not necessary for heme transport but may be required for compartmentalization.
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Chapter I

INTRODUCTION

Various aspects of hemoglobin structure and function have been extensively researched since the early part of this century, but little is known about the transport and combination of heme with globin during the last stages of hemoglobin biosynthesis. Insertion of iron into protoporphyrin occurs in the mitochondrial inner membrane, whereas globin subunits are synthesized in the aqueous cytoplasmic compartment (Rabinovitz and Olson, 1956, and Allen, 1960). Since the apoprotein, globin, is highly unstable at body temperature and heme is subject to aggregation, dimerization, and oxidation, a number of questions can be raised. In what chemical state does biological heme exist, how does it interact with various cell membranes, how is it transported through different cell compartments, and in what manner does it react with cell proteins, particularly globin?

Heme is a metalloporphyrin which consists of a highly conjugated, tetrapyrrole macrocycle. As shown in figure 1.1 each of the four nitrogen atoms is coordinated to iron in a square planar environment. The iron center may exist in either the oxidized or reduced state and is capable of binding a fifth and sixth ligand. The presence of various
Figure 1.1 The structure of four hemes based on porphyrin IX.

PROTO \( R = \text{CHCH}_2 \)
DEUTERO \( R = \text{H} \)
MESO \( R = \text{CH}_2 \text{CH}_3 \)
HEMATO \( R = \text{CHCH}_3 \text{OH} \)
substituent groups on the porphyrin ring may alter its chemical and physical properties. The most important naturally occurring heme is the iron chelate of protoporphyrin IX which has vinyl groups in the second and fourth positions and propionic acid groups in the sixth and seventh positions. Deuteroheme, mesoheme, and hematoheme have hydrogen, ethyl, and ethanol groups, respectively, substituted at the vinyl group position of protoheme. These related hemes may be substituted for protoheme in hemoglobin, but they alter the oxygen binding characteristics of the protein. All hemes and porphyrins exhibit an intense Soret absorption band around 400 nm which is due to the conjugation of the tetrapyrrole nucleus. In addition, porphyrins generally have four minor bands in the visible region, while hemes have two, called alpha and beta.

Physiological reactions involving heme or hemin are difficult to study in vitro because of problems with heme solubility and aggregation. Solubility in aqueous alkali is conferred by ionization of the carboxylic acid side chains. However, a colloidal suspension is actually obtained rather than a true solution. Even at concentrations of $10^{-7}$ molar, hemin exists mainly as oxo-bridge dimers which form clusters of molecular weight 50,000 or more (Granick and Beale, 1978, and Brown, Dean, and Jones, 1970). For kinetic studies, a monomeric form of heme, such as the carbon monoxide complex, must be used. Carbon monoxide heme is believed to be mono-
meric since its absorbancy is proportional to concentration over a wide range, various solvents produce little change in its extinction coefficient or wavelength of maximal absorption, the ratio of iron bound to carbon monoxide is 1:1 even in strong millimolar solutions, and the rate of heme binding to globin is independent of carbon monoxide concentration (Smith, 1959, and Gibson and Antonini, 1960, 1963).

Obviously, the cell must satisfy its requirement for free, monomeric heme in some way other than with the carbon monoxide form. As heme is synthesized it would probably accumulate in the lipid phases of the cell and polymerize unless bound to one or more carrier proteins. In fact, when globin synthesis is inhibited by cyclohexamide, heme accumulates in the mitochondria and does not saturate other cell membranes (Ponka, Borova, and Neuwirt, 1973). Hemo-
plexin and serum albumin bind heme and are known to participate in the extracellular transport of heme (Granick and Beale, 1978, Peters, 1970, and Muller-Eberhard and Morgan, 1975). Certain glutathione-S-transferases have been found to bind heme tightly with a $K_d$ on the order of $10^{-7}$ molar and have been suggested as possible intracellular heme carriers. Such carriers may also function as cell reservoirs of monomeric heme (Jakoby, 1978, and Sinclair and Granick, 1976).

The interaction of heme with cell membranes and proteins can be studied in a model system using liposomes.
Liposomes are the enclosed, lamellar structures formed when amphiphilic lipids are dispersed in aqueous media. They may exist as multilamellar aggregates or as large or small single bilayer vesicles. The smaller liposomes range from 250 to 300 Å in diameter whereas the less stable larger vesicles may be 1000 Å or more in diameter. Each vesicle is constructed of two layers of lipid oriented so that the charged head groups face a water-lipid interface at either the interior or exterior of the liposome while the hydrocarbon tails inhabit the mid-bilayer region as shown in figure 1.2.

Gorter and Grendel (1925) first proposed the lipid bilayer structure of membranes, but many others have suggested a dominant structural role for protein since most membranes contain about 50% or more protein by weight. However, a review of the experimental evidence leads to the conclusion that the basic structure of cell membranes is similar to that formed by the interaction of lipids alone (Stoeckenius and Engelman, 1969, and Tanford, 1973). Naturally, proteins play an important part in the function of membranes and must physically account for a large part of the actual structure, but it is reasonable to expect that lipid bilayers represent a useful model system for the study of biological membranes.

Another criticism of the use of liposomes as model systems has been the heterogeneity in size and number of
Figure 1.2  A structural representation of a cross-section through a small (25-35 nm), single bilayer liposome. The inner layer accounts for approximately 30% of the total lipid. The phospholipid and heme molecules each contain a polar region oriented toward the solvent, and a hydrophobic portion facing the interior of the bilayer.
AQUEOUS SOLVENT

PHOSPHOLIPID

HEME
bilayers in the vesicles produced by mechanical agitation. Such a population of vesicles is unsuitable for kinetic and diffusion studies. However, recent methods have introduced a number of ways to form and fractionate liposomes to produce homogeneous preparations of small, single bilayer vesicles. Both heterogeneous and homogeneous populations of liposomes have been used in a wide variety of experimental studies. These include ion transport, permeability, lysis, interaction and entrapment of proteins and other biomolecules, effects of membrane composition, electron transport, and the structure and properties of lipid molecules in a membrane system (Bangham, Hill, and Miller, 1974).

The final reaction of free heme within the cell is the combination with an apoprotein. In the case of developing red cells, the most important of these is globin. The tetrameric hemoglobin molecule is composed of two alpha globin subunits and two beta subunits which are distinguished by different amino acid sequences. However, the subunits do have similar three-dimensional shapes. Each heme lies in a pocket surrounded by hydrophobic amino acid residues and has its carboxylic acid groups oriented toward the solvent. Direct attachment to the protein occurs through a bond between the nitrogen of a histidine residue in the pocket and the fifth coordination position of the heme iron. The sixth iron coordination position is
available for binding to oxygen, water, carbon monoxide, or any of a number of other ligands.

Hemoglobin binds oxygen reversibly and cooperatively. The binding of oxygen by one or more of the hemes increases the oxygen affinity of the remainder, and the converse is also true. This cooperative effect is accompanied by a conformational change in the protein which is generally designated as a transition from the T state to the R state. The unliganded, or T state, is characterized by the presence of a number of salt bridges and the ability to bind carbon dioxide, chloride ions, protons, and 2,3-diphosphoglycerate or inositol hexaphosphate (inositol-P₆). The liganded, or R state, is produced by a slight rearrangement of the sub-units which breaks the salt bridges, allows tighter binding of oxygen, and releases the anions mentioned above. This conformational change is thought to be triggered by the movement of the iron into the plane of the porphyrin ring as it binds oxygen.

A greater conformational change occurs during the binding of heme to globin. Unlike hemoglobin, globin is very unstable. This suggests that in the absence of heme the protein is more loosely organized with more hydrophobic residues exposed to the solvent. This exposure increases the likelihood of further protein unfolding and aggregation with other globin molecules to produce a denatured precipitate. Furthermore, since globin exists as alpha-beta dimers, heme
binding must promote rearrangement to form tetramers as well as individual changes in each monomer. Clearly, the reaction between globin and its prosthetic group is of fundamental importance in characterizing the hemoglobin molecule and its function.

An understanding of the reactions between heme and cell proteins and membranes is relevant to many different aspects of physiology. Hemes, heme-related compounds, and their associated apoproteins are found in both plants and animals where they function in various capacities. They are associated with oxygen transport and storage in hemoglobin, myoglobin, erythrocruorin, and chlorocruorin, and with oxidation and electron transfer reactions in peroxidase, catalase, and the cytochromes. The porphyrin-like chlorin rings when complexed with magnesium form the chlorophylls which are important in photosynthesis, and a corrin nucleus coordinated to cobalt constitutes vitamin B_{12}. Some of the proteins, such as hemoglobin and myoglobin, are water soluble while others, like the cytochromes and chlorophylls, are membrane components. The apoprotein may drastically alter the functional and solubility properties of the heme-like molecule. Knowledge of the interactions between apoprotein, prosthetic group, and cell membrane is important for an understanding of the biological processes in which these components take part.
In this study carbon monoxide protoheme, human globin, and phosphatidyl choline bilayer vesicles were used as model components for such a system. Before considering the overall scheme, it was first necessary to look separately at the reactions between heme and globin and between heme and liposomes. We first investigated the kinetics of heme and globin recombination and then looked at uptake and release of heme by liposomes. Finally, a mechanism was proposed for the system containing all three components in which heme partitioned between the protein, lipid, and aqueous phases.
Chapter II

MATERIALS AND METHODS

Many of the different experiments to be discussed employ the same techniques and reagents. These are described in the following text.

A. Preparation of Hemoglobin Solutions

Hemoglobin was prepared from whole blood obtained from the Institute of Hemotherapy, Houston, Texas. Red cells were separated from plasma by centrifuging at 1000 x g and 4° C for twenty minutes. The erythrocytes were washed three times with isotonic saline (0.9% w/v sodium chloride) and then lysed with an equal volume of distilled water at room temperature for thirty minutes. Solid sodium chloride was added (3 gm/100 ml) to precipitate stroma and cell debris. The hemolysate was centrifuged at 10,000 x g and 4° C for thirty minutes to remove cell debris, and then spun two more times with a pinch of celite added each time to seed the precipitation. Salt free hemoglobin was prepared by dialyzing against numerous changes of distilled water at 4° C for three days. The heme concentrations were determined by measuring the absorbances of oxyhemoglobin at 576 nm (\( \epsilon = 15.15 \text{ mM}^{-1}\text{cm}^{-1} \)) and 542 nm (\( \epsilon = 14.25 \text{ mM}^{-1}\text{cm}^{-1} \)).

B. Preparation of Globin

Globin was prepared by the method of Rossi-Fannelli,
Antonini, and Caputo (1958). Salt free hemoglobin at 4° C was diluted with cold distilled water to yield a concentration between 1.0 and 1.5 mM. One volume of this solution was added dropwise to 20–30 volumes of vigorously stirring acetone which was kept at -20° C and contained 3 ml/l of 2 N hydrochloric acid. After addition, the solution was kept stirring at -20° C for fifteen minutes. The precipitate was collected by centrifuging at 1500 x g and -15° C for fifteen minutes, and then dissolved in distilled water at 4° C. To remove residual acetone, the globin solution was dialyzed at 4° C against distilled water for five or six hours. Denatured globin was precipitated by dialyzing against 0.0016 M sodium bicarbonate at 4° C for twenty hours. Complete precipitation of inactive material was then achieved by replacing the bicarbonate with 0.02 M phosphate buffer at pH 7 and 4° C. The precipitate was removed by centrifuging at 11,000 x g and 4° C for thirty minutes. The clear, straw-colored solution of globin was kept for two to three weeks at 4° C and was filtered in the cold immediately before each use. Concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 12.7 mM\(^{-1}\)cm\(^{-1}\) per subunit.

C. Preparation of Hemes

Protohemin was prepared according to the method of Fischer (1955). One volume of concentrated hemoglobin solution was dripped into three volumes of glacial acetic
acid at 100-102°C which was saturated with solid sodium chloride and stirred. After addition the temperature was maintained at 100°C for fifteen minutes. Hemin crystals formed upon cooling and were filtered when the solution had reached 60°C to reduce the amount of protein contamination. The hemin crystals were washed with 50% acetic acid, water, alcohol, and ether.

Deuterohemin was prepared using the method obtained from Smith (1975). One gram of protohemin was mixed and ground in a mortar with three grams of resorcinol and then heated in an oil bath at 150-160°C under a condenser for forty-five minutes. The resulting melt was cooled, ground, and washed with ether or ethyl acetate until the extract was almost colorless. The deuterohemin residue was dried and recrystallized from acetic acid.

Mesohemin and hematohemin were prepared using the method from Smith (1975) for insertion of iron into porphyrins. Mesoporphyrin (Calbiochem) or hematoporphyrin (Sigma) was dissolved in one volume of pyridine and fifty volumes of glacial acetic acid at 80°C. One volume of saturated iron sulfate in water was added immediately while a stream of nitrogen was passed over the solution. After ten minutes, the mixture was exposed to air to allow auto-oxidation to the ferric complex. Residual porphyrin was removed by extraction with 3 N hydrochloric acid.

The purity of the prepared hemins was checked by
reversed phase chromatography on silicone coated paper using samples dissolved in 0.1 N sodium hydroxide. The solvent proportion was 5.1 water to 0.1 propanol to 0.8 pyridine by volume. Ascending chromatography was carried out in a five liter tank containing a water-soaked paper liner and a two milliliter well of pyridine.

D. Assay of Heme Concentration

Stock heme solutions were made on the day of the experiment by dissolving an appropriate amount of solid hemin in 0.01 N sodium hydroxide to make a 0.5-1.5 mM solution. Exact concentrations were determined by measuring reduced minus oxidized pyridine hemochrome spectra (Smith, 1975). To 0.15 ml of the heme stock were added 0.25 ml of 1 N sodium hydroxide, 0.6 ml of pyridine, and 2.0 ml of distilled water. This mixture was divided between two cuvettes, and a few grains of dithionite were added to one. Both cuvettes were capped and the visible absorption spectrum taken immediately. Difference extinction coefficients used for protoheme, deuteroheme, mesoheme, and hematoheme were 20.7, 15.3, 21.7, and 21.7 mM⁻¹cm⁻¹ respectively, using the wavelengths given by Smith (1975).

E. Preparation of Liposomes

Liposomes were made by the method of Batzri and Korn (1973) with some modifications. Egg lecithin (Lipid Products, South Nutfield, England) in chloroform-methanol was evaporated to dryness, dessicated under vacuum for
twenty-four hours, and redissolved in absolute ethanol to give 25 mg/ml phospholipid stock solution. Two milliliters of this solution were forcefully injected through a gas tight Hamilton syringe into 28 ml of rapidly stirring buffer at 4°C. Unless indicated otherwise, all buffers for preparing and using liposomes were 0.05 M Tris and 0.05 M sodium chloride at pH 8.0.

The syringe tip was blunted to produce a fine mist of lipid upon injection, and small bilayer vesicles were produced spontaneously. To stabilize the small vesicles ethanol was quickly removed by passing the liposome solution through a 2.5 x 30 cm Sephadex G-25 column which had been pre-equilibrated with lipid and buffer. The liposome fractions were recovered and concentrated to about one milliliter volume in an Amicon ultrafiltration device with rapid stirring and 10 psi pressure of argon. The concentrate was immediately loaded onto a 1.5 x 40 cm, lipid equilibrated Sepharose 4B column to fractionate the vesicles by size. Column fractions were assayed by absorbance measurement at 300 nm and phosphate analysis. Vesicle preparations were stored under nitrogen or argon at 4°C to minimize oxidation. Liposomes containing heme, cholesterol, or stearyl amine were prepared with columns pre-equilibrated with the appropriate lipid mixture. In these cases, the lipid stock was made by dissolving the added component in the chloroform-methanol lecithin solution and then
proceeding as usual. However, heme containing liposomes were generally prepared by adding a heme solution to the preformed vesicles unless otherwise indicated in the text.

F. Analysis of Lipid Phosphorus

The phosphate content of liposome fractions was assayed by the method of Chen, Toribara, and Warner (1956). Aliquots were ashed by heating in a test tube with four drops of concentrated sulfuric acid until white fumes of sulfur trioxide appeared. Three drops of 60% perchloric acid were added and heating continued until the liquid was clear. The contents of each tube were adjusted to a volume of 25 ml with distilled water. Aliquots of ashed sample solution or phosphorus standard were pipetted into a test tube and the volume adjusted to four milliliters. The contents of each tube were then combined with 4 ml of the assay reagent, mixed, and incubated at 37° C for 1.5 to 2 hours. After cooling, absorbances at 820 nm were read against a blank, and a standard graph was constructed for reading unknown phosphorus samples. The assay reagent was made immediately before use by mixing three volumes of 2 N sulfuric acid with one volume of 2.5% ammonium molybdate and one volume of 10% ascorbic acid.

G. Kinetic Measurements and Analyses

Rapid mixing experiments were performed in a Gibson-Durrum (Dionex model 110) stopped flow spectrophotometer interfaced to an A/D converter (OLIS, Inc., Athens,
Georgia) and a Nova 2/10 computer equipped with software for converting data transmittance voltages into absorbance traces. The time courses could be stored on floppy disks and analyzed by fitting to a one or two exponential process with BASIC programs employing the CURFIT nonlinear least squares algorithm described by Bevington (1969).

Solutions used in stopped flow experiments were made by bubbling carbon monoxide through syringes containing buffer, injecting an appropriate amount of stock solution, and adding a few grains of dithionite to scavenge residual oxygen. Buffers containing stock solutions plus different amounts of glycerol were made up individually to prevent dilution errors. Solutions were mixed in the stopped flow device in a 1:1 ratio and observed through a 2 cm path length cuvette with light of a wavelength set by a diffraction grating monochromator.

H. Spectrophotometric Measurements

Heme-liposome titrations and dilution experiments were carried out in a Cary 118 spectrophotometer attached to the OLIS A/D converter and Nova 2/10 computer. Anaerobic conditions in stoppered cuvettes were achieved by bubbling with carbon monoxide and adding a few grains of dithionite. Stock solutions were added by syringe. Absorbance traces for titrations were stored on floppy disks, and fractional saturations determined by fitting spectra to a linear combination of the absorption spectra of free heme in solution.
and heme completely bound to lipid. The program employed for fitting used the REGRES algorithm described by Bevington (1969).

I. Electron Microscopy

Electron micrographs of liposomes were obtained through the cooperation and assistance of Dr. Barry Van Winkle at the University of Texas Medical School in Houston. One drop of liposomes containing 100-300 uM phospholipid was placed on a Formvar coated grid, and immediately a drop of 1% sodium phosphotungstate staining solution was added. After 15-30 seconds, the solution was drained off with filter paper and the grid allowed to air dry. Instrument magnification of 70,000-100,000 was used.

J. Fluorescence

Fluorescence emission spectra were recorded on a SLM Series 400 polarization fluorometer interfaced to a Hewlett Packard 9825A calculator. An excitation wavelength of 319 nm and scanning range of 350-600 nm was used. Various volumes of saturated N-(1-pyrene sulfonyl)-dipalmitoyl-L-\( \alpha \)-phosphatidyl-ethanolamine (PSDPPEA from Calbiochem) in hot ethanol were injected into a cuvette containing liposome solution at room temperature. The pyrene stock was kept in solution throughout the experiment by means of a boiling water bath. Its concentration was 0.5 mg/ml. Stock pyrene in dioxane was made to the same concentration and dissolved at room temperature.
Fluorescence rapid mixing experiments were performed on the previously described Gibson-Durrum stopped flow spectrophotometer equipped for fluorescence measurements. The pyrene labelled compound was excited at 280 nm, and all fluorescence emission at wavelengths greater than 350 nm was measured by using an appropriate cut off filter. Solutions were made anaerobic as previously described, and pyrene label was added by injection into the liposome solution.
Chapter III
THE REACTIONS OF HEME WITH GLOBIN

Reactions between isolated globin and its prosthetic group have been reported as early as 1926 by Hill and Holden. These studies were later expanded by Rossi-Fanelli and co-workers (1958, 1959) and Antonini and Gibson (1960) to show that the characteristics of such artificially derived hemoglobins are indistinguishable from those of the natural material. The rate of the recombination reaction was then further investigated by Gibson and Antonini (1960, 1963). The reaction of globin with ferric heme was found to be slow, non-reproducible and evidently dependent on the extent and rate of hemin dispersion. In contrast, the recombination of carbon monoxide heme with protein was both rapid and relatively simple. Gibson and Antonini suggested the mechanism given in equation 3.1 to explain anomalous spectral changes and deviations of this reaction from true second order behavior.

(3.1) Heme + Globin $\xrightarrow{k_1}$ Complex $\xrightarrow{k_2}$ $\xrightarrow{k_3}$ Hemoglobin

In this scheme, globin and heme are postulated to be in rapid equilibrium with an intermediate complex which then reacts to form hemoglobin. Time courses computed using
this mechanism fit reasonably well with their experimental observations.

Gibson and Antonini (1960) and Gibson (1964, a, b) suggested that the formation of the intermediate represents reversible binding of heme to a specific site on the globin molecule. This process is followed by an irreversible step involving a refolding of the globin chain around the heme to generate the molecular conformation of hemoglobin. They assumed that the intermediate had the same absorption spectrum as hemoglobin and concluded from work done using porphyrins that iron plays an insignificant role in the binding of heme to globin.

More specifically, we propose that the intermediate consists of heme dissolved into the hydrophobic pocket of globin. The second, slower step is then either conformational changes or iron-histidine bond formation. The expected conformational changes may occur in response to the mere physical presence of heme in the pocket or may be triggered by iron-histidine bond formation. After heme adsorption to globin, it is necessary for certain conformational changes to take place in order to correctly position the heme and protein for iron-histidine binding. If this general interpretation is correct, then we suggest that the first complex has a Soret absorption peak similar but intermediate to that for CO-heme and CO-hemoglobin. In fact, the spectrum for the intermediate might be simulated
by the absorbance of heme bound to liposomes. In this model, heme is dissolved in a lipid phase with the same orientation to the solvent as might be expected when it is bound to globin. The absorbance spectra of CO-heme, heme in liposomes, and heme in globin (hemoglobin) are shown in figure 3.1 for purposes of comparison. The spectrum of CO-heme shifts to longer wavelengths when dissolved in a lipid phase, and shifts even more when bound to globin. The following experiments were undertaken to find kinetic and spectral proof of the suggested intermediate, to more narrowly define the rate constants, and to acquire further information about the exact molecular mechanisms involved.

A. Basic Features of the Reaction of CO-heme with Globin

The reaction between CO-heme and globin is essentially second order and biphasic as shown in figure 3.2. The fast phase accounts for 90% or more of the absorbance change and represents the major reaction being studied. The slow phase accounts for only a very small part of the total absorbance change, and represents a number of secondary reactions of lesser significance. These include binding of heme to other histidine residues, to nonspecific hydrophobic areas, and to denatured protein. Nonspecific binding was investigated by reacting CO-heme with CO-hemoglobin. A comparison of this time course with that for globin plus heme is shown in figure 3.2. It can be seen that nonspecific binding may account for much of the slow
Figure 3.1  The wavelength dependence of the extinction coefficient for CO-hemoglobin (Hb), CO-heme (FREE), and lipid bound heme (LIPID). Absorbance measurements were performed in the Cary 118 spectrophotometer with stoppered anaerobic cuvettes. The spectrum for lipid bound heme was obtained by adding increments of liposome stock to both the reference cuvette and the sample cuvette containing a heme solution, until no further absorbance change was noted. Samples were in 0.05 M Tris, 0.05 M NaCl, pH 8.0, and 24°C buffer equilibrated with carbon monoxide.
Figure 3.2  The reactions of CO-heme with globin and CO-hemoglobin. Heme (3 µM) was mixed with 6 µM globin (Gb) or 3 µM hemoglobin (Hb) in the stopped flow device and observed at 419 nm. All buffers were 0.01 M phosphate at pH 7.2 and 10°C. About 60% of the absorbance change due to the reaction of heme with globin is not observed because it occurs within the dead time of the apparatus.
phase but contributes little to the overall faster reaction. As might be expected, the rate for this slower reaction increases with heme concentration. We have kept this process to a minimum in most of the following studies by using excess protein.

High globin concentrations were used not only to minimize the slow phase but also to maintain first order conditions during experiments. Lower concentrations of globin were used to construct some of the absolute absorbance spectra, but in no case did the heme concentration ever exceed the protein concentration. Heme concentrations between one and five micromolar were chosen as a compromise between minimizing the reaction rate and maximizing expected absorbance change.

Time courses for hemoglobin formation were observed at different wavelengths and then fitted to a two exponential expression. Only the kinetic properties of the first phase were considered in detail since it comprised almost all of the absorbance change. The rate for this phase varied from 211 s\(^{-1}\) at 408 nm to 213 s\(^{-1}\) at 421 nm, showing no significant change as a function of wavelength. This homogeneous behavior suggests that \(k_2\) and \(k_1\) (globin) in equation 3.1 are much greater than \(k_3\) and that the overall reaction consists of a rapid equilibrium followed by a slower first order step. This two-step mechanism is analogous to the Michaelis-Menten concept of enzyme action,
and standard methods of enzyme kinetics may be used for its analysis.

The overall reaction can be described by the differential equations 3.2 and 3.3, where Hb is hemoglobin and gb is globin.

\[
\frac{d(Hb)}{dt} = k_3(gb)
\]

\[
\frac{d(gb)}{dt} = k_1(gb)(Heme) - (k_2 + k_3)(Heme-gb)
\]

Since \( k_1(gb) \) and \( k_2 \) are much greater than \( k_3 \), the amount of intermediate is determined by:

\[
\frac{(Heme)(gb)}{K_d} = (Heme-gb)
\]

where \( K_d \) is the equilibrium dissociation constant for the formation of the heme-globin complex. Under these conditions, equations 3.2 and 3.3 reduce to:

\[
\frac{d(Hb)}{dt} = k_3(gb)(Heme) + (Heme-gb) \frac{(gb)}{(gb) + K_d}
\]

If the concentration of globin is kept significantly greater than that of free heme the time courses should exhibit first order behavior with:

\[
k_{observed} = k_3(gb) \frac{(gb)}{(gb) + K_d}
\]

This equation indicates that the observed rate should reach a limit at the value of \( k_3 \) as the globin concentration is increased to an amount which is high relative to \( K_d \). In earlier work Gibson and Antonini (1960, 1963) suggested a value of \( K_d \) equal to 36 uM using borate buffer at pH 9.1.
and 21°C. The proposed reaction mechanism was tested by reacting globin with several different concentrations of heme. Regardless of heme concentration, the observed rates appear to level off with excess amount of globin. This behavior supports the theory discussed above, providing kinetic evidence for the existence of an intermediate. From several different experiments, the apparent $K_d$ for the formation of this complex was found to be 22 μM at pH 7.4, 20°C.

B. Spectral Evidence for the Intermediate

Since the reaction has a short half time (1-2 ms), at high globin concentrations much of the absorbance change occurs in the dead time of the apparatus. Furthermore, the relatively high $K_d$ indicates that very little of the intermediate may exist at any one time, particularly at lower protein concentrations. Several experiments were undertaken to demonstrate this and to obtain absolute absorbance spectra of the intermediate and other optical species at the dead time and at various time intervals during the course of the reaction. Transmittance voltages were measured in the stopped flow device for buffer, heme, and final hemoglobin solutions at eight to ten different wavelengths. These values together with the time courses at each wavelength were used to calculate absolute absorbance spectra at various times after mixing. The experiments in figures 3.3 and 3.4 were carried out with a slight excess of
Figure 3.3  Absolute spectra for the reaction of heme with a 2-fold excess of globin. Heme (5 μM) was mixed with 10 μM globin in the stopped flow device. The absolute absorbances of the initial heme (■), the final hemoglobin product (◆), and the reaction mixture existing at the dead time (△) were calculated from transmittance voltages. For heme or reference buffer transmittances, the appropriate solution was flushed through the system and the voltage measured. The average of several measurements was used, and the absolute heme absorbance was calculated from \[ \log \left( \frac{V_{\text{ref}}}{V_{\text{heme}}} \right) \]. Hemoglobin absorbance was calculated in a similar manner using the final voltage given by the reaction time course. The change in absorbance obtained from the time course at various times after mixing was added to the hemoglobin absorbance to obtain spectra for the reaction at these intermediate times. All reactants were in 0.01 M phosphate buffer, pH 7.2, 10°C.
Figure 3.4 Absolute spectra for the reaction of heme with a 100-fold excess of globin. Heme (5 μM) was mixed with 500 μM globin in the stopped flow device. Absorbances for heme (■), hemoglobin (♦), and the reaction at the dead time (△) were measured as described and with the conditions given in figure 3.3.
globin and with a very large excess of globin to see if the detectable amount of intermediate could be increased.

The results indicate that by the dead time, the absorbance maximum of the heme groups has shifted toward an intermediate wavelength. With increasing time this shift progresses until the spectrum of hemoglobin is reached. These results are compatible with the kinetic parameters and the existence of an intermediate with an absorbance maximum between that of heme and hemoglobin. In the experiment in figure 3.3 less than half of the heme is initially included in the intermediate complex, so that at the dead time, much of the absolute absorbance is still due to unreacted heme with a smaller fraction due to hemoglobin product and an even smaller but detectable amount due to the intermediate. The conditions of the experiment in figure 3.4 are a bit more favorable but still do not suffice to isolate the spectrum of the complex. With a large excess of protein, almost all of the heme combines with globin initially to form the intermediate. By the dead time most of this has formed product which results in a large contribution from hemoglobin to the absolute spectrum. In each experiment, the spectrum of the intermediate is partially camouflaged by free heme or hemoglobin absorbance. Nevertheless, at wavelengths in the region of 414 nm the absorbance clearly rises and then falls during the course of the reaction, indicating the presence of a
third spectral species with properties resembling those of free heme dissolved in lipid (see figure 3.1).

This behavior contrasts with experiments carried out at low concentrations of heme and globin (i.e., very little intermediate formed) where the reaction proceeds through an apparent isosbestic point. Under these conditions (1 μM CO-heme, 1 μM globin), the amount of intermediate formed at any one time is so low that there are essentially only two spectral species. There is no shifting of absorbance toward intermediate wavelengths such as that observed for the previous experiments.

C. The Effects of pH, Sodium Chloride, and Organic Phosphate

The preceding results suggest the existence of an intermediate which could be further characterized if the kinetic parameters were more favorable for its formation. If the theories about the nature of the intermediate are correct, then it should be possible to perturb the kinetics of the reaction by varying such experimental conditions as pH, salt, inositol-P₆, and glycerol concentrations.

If iron-histidine bond formation is involved in the formation of hemoglobin from the intermediate, then the pH of the solution might be expected to influence k₃ by varying the protonation of the proximal imidazole and hence, its availability for binding to the heme iron. Unfortunately, both heme and globin are unstable at the
proton concentration desirable for studying this effect, but experiments were performed at pH values ranging from 6.5 to 9.2 as shown in figure 3.5. At four pH values, the computer fitted observed rates are plotted versus excess globin concentration, and a double reciprocal plot for these data is shown. The constant, \( k_3 \), appears to remain unchanged, while the \( K_d \) decreases, particularly at pH 6.5. The value for \( k_3 \) cannot be more narrowly defined, because at higher globin concentrations too much of the reaction occurs within the dead time to allow accurate analysis. The experimental results indicate that a change in pH from 9.2 to 6.5 does not inhibit iron histidine bond formation, but does increase the affinity of heme for globin, probably through a greater charge attraction. Since the reagents are only marginally stable at pH 6.5 a compromise pH of 7.2 was used for the remaining experiments.

If the formation of the intermediate involves binding of aqueous heme to a hydrophobic phase, then an increase in ionic strength of the solution might be expected to favor formation of the complex. The results of an experiment using three different concentrations of sodium chloride are given in table 3.1. No significant changes in rate are observed for these salt concentrations. Unfortunately, very high amounts of salt seriously affect globin stability.

As discussed previously, a conformational difference exists between globin and hemoglobin. Since inositol-P_6
Table 3.1  The effect of salt concentration on the observed rate. Heme (1.5 μM) was reacted with two different concentrations of globin in the stopped flow apparatus and observed at 419 nm, 10° C. The buffers were 0.01 M phosphate, pH 7.2, and contained the indicated amount of sodium chloride.

<table>
<thead>
<tr>
<th>salt M</th>
<th>globin μM</th>
<th>observed rate s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>6</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>291</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>264</td>
</tr>
<tr>
<td>0.2</td>
<td>6</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>249</td>
</tr>
</tbody>
</table>
Figure 3.5 The effect of pH on the observed rate of the reaction of heme with globin. Heme (3 μM) was mixed with varying concentrations of globin in the stopped flow device and observed at 419 nm, 10°C. The different 0.025 M buffers used were borate, pH 9.2 (●), Tris, pH 8.3 (▲), phosphate, pH 7.2 (+), and Bis Tris, pH 6.5 (■). The values for $K_d$ were found to be 25 μM, 24 μM, 22 μM, and 9 μM, respectively, and $k_3$ was about 710 s$^{-1}$. 
is known to influence conformational changes that occur in hemoglobin during ligand binding, it is reasonable to question whether this organic phosphate might also affect the rate of heme binding to globin. Figure 3.6 describes an experiment using various concentrations of inositol-P₆. There is a definite decrease in the observed rate with increasing concentrations of inositol-P₆ up to about 500 μM, after which there is negligible change. The double reciprocal plot for these results (figure 3.7) indicates that k₃ for the reaction remains about the same (~700 s⁻¹) while the K₆ changes considerably. It varies from about 20 μM in the absence of inositol-P₆ to about 90 μM in the presence of 500-1000 μM inositol-P₆. This suggests that inositol-P₆ binding to globin lowers the affinity of the protein for heme, but does not influence greatly the first order step producing hemoglobin from complex. Since the net effect is to decrease the amount of intermediate present, the use of inositol-P₆ certainly does not provide more favorable experimental conditions for examining the spectral properties of the heme-globin intermediate.

The increase in the value of the dissociation constant with increasing organic phosphate concentration is probably due to a charge effect. At pH 7, each inositol-P₆ contributes about nine extra negative charges when bound to the protein. This change in charge on the protein causes it to have less affinity for heme with its negatively charged
Figure 3.6  The effect of inositol-P$_6$ on the observed rate of the reaction of heme with globin. Heme (3 µM) was mixed with varying concentrations of globin in the stopped flow device and observed at 419 nm, 10°C. The buffers used were 0.01 M phosphate, pH 7.2 and contained the following concentrations of inositol-P$_6$: 20 µM (▲), 100 µM (●), 500 µM (■), and 1000 µM (◆). Solid lines were calculated from equation 3.6 using constants obtained from figure 3.7.
Figure 3.7  The double reciprocal plot for the effect of inositol-P$_6$ on the observed rate. All conditions were the same as for figure 3.6. From this plot, values of 740 s$^{-1}$ for $k_2$, and 21 μM, 33 μM, and 91 μM for the dissociation constants were obtained for 20 μM (▲), 100 μM (●), and 500 and 1000 μM (■)(●), IP$_6$. 
acid groups. This effect is similar to that observed when the pH is lowered. The lack of any large change in $k_3$ suggests that bound inositol-P$_6$ exerts little influence on the first order conformational change which produces hemoglobin.

D. The Effects of Glycerol and Viscosity

A final set of experiments was carried out using various amounts of glycerol in the buffers. It was thought that glycerol could be used to test for any effects due to diffusion or solvent solubility. As shown in figure 3.8 the observed rate is considerably affected by glycerol in concentrations from 0-50%. The double reciprocal plot (figure 3.9) indicates that $k_3$ as well as the $K_d$ is changed. Increasing amounts of glycerol lower $k_3$ but simultaneously raise $K_d$. These effects cancel each other with respect to favoring formation of the intermediate. However, it is interesting to note that glycerol was the only material tested which substantially affected both the initial equilibrium and the first order rate.

We had initially hoped that increasing the solvent viscosity by adding glycerol would slow down the first bimolecular step in hemoglobin formation so that it could be visualized in rapid mixing experiments. This did not happen. However, glycerol did increase the apparent $K_d$ for the formation of the heme-globin intermediate. It is well known that glycerol has a stabilizing effect on some
Figure 3.8  The effect of glycerol on the observed rate of the reaction of heme with globin. Heme (3 μM) was mixed with varying concentrations of globin in the stopped flow device and observed at 419 nm, 10°C. The buffers used were 0.01 M phosphate, pH 7.2 and contained the following % by volume concentrations of glycerol: 0 (■), 5 (△), 20 (●), 30 (□), 40 (▲), and 50 (◇) having the viscosities of 1.3, 1.5, 2.4, 3.5, 5.4, and 9.0 centipoise, respectively. Solid lines were calculated from equation 3.6 using constants obtained from figure 3.9.
Figure 3.9 The double reciprocal plot for the effect of glycerol on the observed rate. All conditions were the same as for figure 3.8. From this plot the following values for $k_3$ and $K_d$ were obtained at each % glycerol concentration: 670 s$^{-1}$ and 22 μM at 0% (■), 526 s$^{-1}$ and 29 μM at 5% (△), 345 s$^{-1}$ and 44 μM at 20% (♦), 222 s$^{-1}$ and 67 μM at 30% (□), 105 s$^{-1}$ and 77 μM at 40% (▲), and 56 s$^{-1}$ and 125 μM at 50% (◊).
proteins. This may be caused by a tighter folding of the polypeptide chain with a minimum exposure of hydrophobic residues to the aqueous medium. Shielding of the hydrophobic regions of globin at higher glycerol concentrations would be expected to cause a decreased affinity of the protein for heme. This idea is depicted in figure 3.10. The apparent $K_d$ is given by $(1 + K_1)K_2$ where $K_1$ is the equilibrium constant for the formation of the folded protein conformation which is increased by glycerol concentration, and $K_2$ is the equilibrium dissociation constant for heme binding to the unfolded conformation.

The decrease in $k_3$ is most readily interpreted as a viscosity effect. The major refolding of the protein in response to heme binding appears to be affected markedly by glycerol concentration. The value of $k_3$ decreases from about 700 s$^{-1}$ to 60 s$^{-1}$ in going from 0 to 50% glycerol. Frauenfelder and co-workers have suggested that solvent viscosity exerts a profound influence on the rates of large protein conformational changes (Beece et al., 1980). The expected dependence is given by:

$$k_{\text{observed}} = k_o (\eta/\eta_o)^{-\kappa}$$

where $k_o$ is the rate observed in water, $\eta$ and $\eta_o$ are the viscosities of the glycerol solution and water, respectively, and $\kappa$ is a number which reflects the degree of coupling between the solvent and the protein change. For bimole-
Figure 3.10  A scheme for proposed conformational states for the reaction of heme with globin. Dissolved globin (A) partially exposes its hydrophobic residues (dotted area) to the solvent. CO-heme rapidly adsorbs to the hydrophobic area (B) and elicits a conformational change (C) and histidine binding. The competing reaction involves a folded conformation with partial shielding of hydrophobic groups (D). This folded state makes the hydrophobic pocket less accessible to dissolved heme.
cular reactions where two molecules must diffuse up to each other, $\kappa$ is around one; for small conformational changes where the amino acid movements are small $\kappa$ approaches zero. A plot of $\log k_3$ versus $\log (\eta/\eta_0)$ is shown in figure 3.11. The slope of this curve, $\kappa$, is about 1.3 which suggests that the protein conformational change is substantial and greatly influenced by the viscosity of the aqueous medium.

We had originally thought that $k_3$ represented the rate of histidine-iron bond formation which occurred after the protein folded around the heme. Clearly, the formation of this bond results in the final spectral change during the formation of hemoglobin. However, the results with glycerol and inositol-P$_6$ suggest very strongly that $k_3$ represents a large protein conformational transition which orients the iron atom and proximal imidazole so that bond formation is extremely rapid and does not limit the observed rate. Inositol-P$_6$ is known to destabilize the iron-proximal histidine bond in the presence of a sixth ligand such as NO or CO (Maxwell and Caughey, 1976). However, it has little or no influence on the value of $k_3$. The large effect of solvent viscosity on $k_3$ also argues strongly that this rate does not represent the rate of binding of histidine to the iron atom since the latter reaction occurs deep within the protein molecule. Thus, in figure 3.10 protein folding and imidazole binding are written as a concerted one step process.
Figure 3.11  The logarithmic dependence of the observed rate on the relative buffer viscosity. Values for the observed rates and viscosities were taken from figures 3.8 and 3.9. The points were fitted to a straight line, and the slope \((-\kappa)\) was found to be \(-1.28\).
E. The Effects of Modified Hemes

Gibson and Antonini (1963) have studied the reaction of globin with various hemes. It was hoped that these hemes would be a useful tool for mechanistic studies since they react with globin less rapidly than does protoheme. Although an intermediate complex was postulated for the reaction with protoheme, its spectral identity remained elusive, and it was assumed that the absorption spectra of the complex and hemoglobin are similar. In contrast, the reaction with CO-meso-heme exhibited spectral irregularities. These were interpreted as an indication of a complex having a spectrum intermediate between CO-heme and hemoglobin, but no definitive evidence was obtained.

We have investigated these puzzling results in greater detail. With concentrations similar to those used by Gibson and Antonini (1963), absolute absorption spectra were constructed for the reaction of CO-deuteroheme with globin using the method described for figures 3.3 and 3.4. Since the reaction involving deuteroheme is even slower than that for mesoheme, we thought that an intermediate spectrum might be obtained. However, this was not the case. The results for deuteroheme (figure 3.12), mesoheme, and hemato-
Figure 3.12 Absolute spectra for the reaction of CO-deutero-heme with an equivalent amount of globin. Heme (1 µM) was mixed with 1.1 µM globin in the stopped flow apparatus. The absolute absorbances of the initial heme (■), the final hemoglobin product (♦), and the reaction mixtures existing at the dead time (◇), at 50 ms (□) and at 290 ms (△) were calculated from transmittance voltages as described for figure 3.3. Some unreacted heme remains after the reaction has ended.
heme exhibited apparent isosbestic points with little or no indication of an intermediate species with unique spectral properties. The conflicting results of Gibson and Antonini could be due to differences in buffer, pH, temperature, or the possible presence of other contaminating hemes.

The double reciprocal plot in figure 3.13 shows the dependence of the observed rates on globin concentration. Each heme exhibits a similar value for $K_g$, but the $y$ intercepts ($k_3$) vary. Evidently, the adsorption affinity of globin for heme is mostly due to the hydrophobicity of the overall porphyrin ring structure and is little affected by the substituent groups. However, greater specificity is required for inducing the conformational change represented by $k_3$. Evidently, the hydrophobic pocket is arranged to conform to the structural characteristics of protoheme, so that changes in the size or polarity of substituent groups may exert a large influence on the protein transition. When the vinyl groups of protoheme are replaced by ethyl substituents (mesoheme) the observed rates remain about the same (figure 3.13). However, when the vinyl groups are removed entirely (deuteroheme), the rates become slower. An even greater effect is seen for the hydroxyethyl groups of
Figure 3.13 The double reciprocal plot for the effect of modified hemes on the observed rate. Varying concentrations of globin were mixed in the stopped flow apparatus with 3 μM hematoheme (○), deuteroheme (■), or mesoheme (▲), and observed at 403 nm, 397 nm, and 393 nm, respectively. From this plot, $K_d$ was found to be about 26 μM and $k_3$ was about 320 s$^{-1}$, 520 s$^{-1}$, or 770 s$^{-1}$, respectively. Values for protoheme (○) are shown for comparison. Buffers were 0.01 M phosphate, pH 7.2, 10 °C.
hemato-heme. However, in general, the use of modified hemes does not alter the reaction kinetics drastically enough to allow the spectral isolation of the intermediate complex.

F. Conclusion

A comparison of the values for $K_d$ and $k_3$ obtained under different conditions is shown in table 3.2. None of the conditions tested caused a variation in kinetic parameters sufficient to completely isolate the intermediate spectrum. Nevertheless, absolute spectra can be constructed for the reaction mixture which exists at various time intervals after combination in the stopped flow device. Clearly, the intermediate exists and exhibits spectral characteristics similar to that of heme dissolved in a lipid bilayer. The effects of pH, glycerol, and organic phosphate suggest that heme is rapidly adsorbed onto hydrophobic areas of the globin molecule to form an intermediate complex. Heme correctly orients itself within the hydrophobic pocket area and induces a conformational change in the rate limiting step of the reaction. Iron histidine bond formation follows very rapidly, producing the final spectral change. Our results suggest that hydrophobic interactions with the porphyrin ring structure are responsible for folding the
Table 3.2 A comparison of the dissociation constants and \( k_3 \) for the reaction of heme with globin under various conditions. Unless indicated otherwise, all conditions are 1.5 \( \mu \text{M} \) CO-protoheme, pH 7.2, 0.01 M phosphate buffer, 10\(^\circ\) C. with no organic phosphates or glycerol.
<table>
<thead>
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<th>CONDITION</th>
<th>$K_d$ μM</th>
<th>$k_3$ s$^{-1}$</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>pH 7.2</td>
<td>22</td>
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</tr>
<tr>
<td>pH 8.3</td>
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</tr>
<tr>
<td>pH 9.2</td>
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<td>710</td>
</tr>
<tr>
<td>20 μM inositol-P$_6$</td>
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</tr>
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<td>1000 μM inositol-P$_6$</td>
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</tr>
<tr>
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</tr>
<tr>
<td>hematoheme</td>
<td>26</td>
<td>320</td>
</tr>
</tbody>
</table>
protein into the native functional conformation. This idea is supported by the results of porphyrin binding studies (Gibson, 1964 b) which indicate that iron exhibits very little effect on the reaction of heme with globin.
Chapter IV

THE REACTIONS OF CO-HEME WITH LIPOSOMES

The biosynthesis of heme originates within the mitochondrial matrix where glycine condenses with succinyl-CoA to form δ-aminolevulinic acid (ALA). This step is controlled by a regulatory enzyme, ALA synthetase, and is the only reaction which requires vitamin cofactors and added energy. The enzyme is inducible and susceptible to product inhibition (Granick, 1964, 1966, Calissano et al., 1966, and Gallo, 1967). This reaction is followed by a complex series of steps catalyzed by cytoplasmic enzymes. Two molecules of ALA are linked together to form the small ring compound, porphobilinogen, and then four of these are assembled to yield the tetrapyrrole, uroporphyrinogen. Further decarboxylation and oxidation reactions occur, the last of which again require mitochondrial enzymes. The final product, heme, is formed in the mitochondrial inner membrane by the enzyme, protoheme ferrolyase, which catalyzes the insertion of ferrous iron into protoporphyrin IX.

The utilization of heme within the cell is complicated by several problems. In some manner, the membrane bound heme must be extracted by apoproteins formed in the aqueous cytoplasmic compartment. To overcome this barrier, a carrier protein may be necessary for solubilization and
transport. Furthermore, free heme is potentially unstable and toxic. It may polymerize into insoluble aggregates or react with oxygen to form radicals. The presence of heme also affects other cell processes. It is known to influence both its own synthesis as well as that of globin (Granick, 1964, 1965, Grayzel et al., 1966, and Steiner and Baldini, 1967). Clearly, some mechanism is necessary to compartmentalize heme as well as to maintain it in a reduced, monomeric form. It is possible that intrinsic components of some membranes may associate with heme to localize and stabilize it, while the mitochondrial electron transport chain may provide the necessary reducing environment. Alternatively, a carrier protein could serve one or more of these functions. Certain glutathione-S-transferases have been tentatively assigned this role in hepatocytes (Jakoby, 1978).

Only in recent years have the interactions between various lipid particles and hydrophobic organic compounds received much attention. Numerous physical studies have investigated the role of cholesterol as a membrane component. These include the positioning of cholesterol within the bilayer (Darke et al., 1971 and Brockerhoff, 1974), its condensing effect (Lee et al., 1972), its molecular rigidity (Kroon et al., 1975) and the rate of transbilayer interchange (Poznansky and Lange, 1976). In spin label experiments, the rates of exchange of phosphatidylcholine between
small vesicles, of bilayer interchange, and of lateral
diffusion of phospholipid in the plane of the membrane have
been measured (Kornberg and McConnell, 1971 a, 1971 b, and
Devaux and McConnell, 1972). More recently, the mechanism
of transfer of pyrene and a pyrene labelled diglyceride
between high density lipoproteins (Charlton et al., 1976,
1978) and the transfer of a pyrene labelled fatty acid
between liposomes have been investigated (Doody et al.,
1980). These experiments are beginning to define the pro-
cesses by which hydrophobic organic molecules are absorbed
and transferred throughout biological membranes. The fol-
lowing studies examine the properties of heme in similar
model systems using liposomes.

A. Characterization of the Membrane Vesicles

In order to make meaningful kinetic measurements, the
homogeneity, size, and lamellar nature of the vesicles must
be determined. The liposomes were characterized by elec-
tron microscopy, molecular sieve chromatography, light
scattering, and phosphate content.

Electron micrographs of fractionated liposomes show a
reasonably homogeneous population of small bilayer vesicles
about 15-20 nm in diameter (figure 4.1). These results
agree well with the literature (Batzri and Korn, 1973 and
Enoch and Strittmatter, 1979). Liposomes containing heme
or cholesterol and stearylamine showed no detectable dif-
ference in size or character. Micrographs of the fraction
Figure 4.1  Electron micrograph of small, bilayer liposomes. Vesicles separated on a Sepharose 4B column (fraction 54 in figure 4.3) were stained with 1% sodium phosphotungstate and micrographed. Magnification is 460,000 times.
of large, multilamellar liposomes indicate heterogeneity in size and multiple bilayers (figure 4.2).

The difference in size and character of the two fractions of liposomes is also demonstrated by their behavior with Sepharose 4B chromatography. The large liposomes elute in the void volume while the small ones are retained by the Sepharose. A column profile for vesicles containing cholesterol and stearylamine is shown in figure 4.3. Similar results were obtained for simple liposomes and those prepared with heme. The absorbance at 300 nm is a measure of light scattering and is therefore proportionately greater for the large vesicles than for the small ones. The actual quantity of each fraction present is better represented by the phosphate content. As shown in figure 4.3, the amount of phospholipid incorporated into the large liposomes is negligible. The small bilayer vesicles account for almost all of the phospholipid present but are only faintly opalescent because of their small particle size. When this fraction was pooled, concentrated, and rechromatographed on Sepharose 4B, the liposomes eluted in the same place and showed insignificant formation of large vesicles. This demonstrated that the small bilayer vesicles could be manipulated and stored for at least several days without greatly changing their properties. The small liposomes were considered suitable for kinetic measurements and were used in all the following experiments.
Figure 4.2 Electron micrograph of multilamellar liposomes. Vesicles eluting in the void volume of a Sepharose 4B column (fraction 33 in figure 4.3) were stained with 1% sodium phosphotungstate and micrographed. Magnification is 390,000 times.
Figure 4.3 Column profile for separation of liposomes on Sepharose 4B. Vesicles containing 10 moles % stearamine and 20% cholesterol were fractionated on pre-equilibrated Sepharose 4B. The large, multilamellar vesicles elute first (fraction 33) and exhibit a high ratio of absorbance at 300 nm (•) to lipid phosphate content (▲). The small liposomes elute later (fraction 54) and show less light scattering.
B. Equilibrium Association of Heme with Membranes

When phospholipid vesicles are added to a CO-heme solution, the Soret absorption maximum of the porphyrin gradually shifts from 408 nm to 414 nm (figure 4.4). The fractions of bound and unbound heme in these mixtures were obtained by fitting the intermediate spectra to a linear combination of standard spectra for free CO-heme and completely bound heme. Observed and calculated curves are shown in figure 4.5. A series of titrations were carried out in which the concentration of CO-heme was fixed and the lipid phosphate concentration was varied. These experiments allow the greatest variation in conditions since the lipid concentration can be varied over a large range without changing the magnitude of the absorbance spectrum. Light scattering is not a problem since the same amount of lipid was added to both the reference and sample cuvettes. As shown in figure 4.6, the ratio of lipid phosphate to heme which is required to remove all of the heme from the aqueous phase increases with decreasing heme concentration. In an alternative experiment a fixed amount of lipid was titrated with heme. Again, the concentrations of free and bound heme were obtained by fitting the observed spectra to the sum of the standards. As shown in figure 4.7, the ratio of bound heme to total phosphate begins to limit off at high heme concentrations suggesting that the membrane vesicles have a fixed capacity for binding this compound.
Figure 4.4 The spectral shift observed upon titrating free CC-heme with a liposome stock solution. Indicated μl amounts of liposomes (1.7 μM lipid phosphorus) were added to 5 μM heme (dotted line) to give these spectra. Buffers were 0.05 M Tris, 0.05 M NaCl, pH 8.0, 24°C, anaerobic, and bubbled with carbon monoxide. Liposomes were added to both sample and reference cuvettes to minimize error due to light scattering.
Figure 4.5 The comparison of observed and fitted values for spectra of CO-heme in lipid bilayers. The observed data (solid lines) were fitted to a linear combination of standard spectra for 5 μM free CO-heme and completely bound heme. Fitted values are shown for the presence of 30 μl of liposome stock (♦) and for 115 μl liposomes (■). Other conditions were the same as for figure 4.4.
Figure 4.6 The effect of heme concentration on the equilibrium binding ability of liposomes. The ratio of lipid bound heme to total concentration of heme was plotted versus the ratio of total lipid phosphorus per total heme. The amount of bound heme was obtained from fitted spectra as described in figure 4.5. Data were obtained for 1 μM (O), 5 μM (Δ), and 10 μM (□) CO-heme solutions. All other conditions were the same as for figure 4.4.
Figure 4.7 The relationship between concentrations of lipid bound heme and the total heme in an equilibrium system containing 27 μM total liposomes. All other conditions and the amounts of bound heme were determined as described in figures 4.4 and 4.5.
All of these data can be analyzed in terms of a simple binding reaction. The equilibrium dissociation constant for this process can be defined as:

\[(4.1) \quad K_d = \frac{(\text{Heme})_f(P)/n}{(\text{Heme})_b}\]

where \((\text{Heme})_f\) is the concentration of free heme, \((\text{Heme})_b\) is the concentration of heme bound in the lipid phase, \((P)\) is the concentration of lipid phosphorus, and \(n\) is the number of phospholipid molecules per heme binding site (ie. \(1/n\) is the maximum number of heme molecules per phospholipid which can be dissolved in the membrane). This analysis considers that each heme binding site is comprised of \(n\) phospholipid molecules so that the free concentration of binding sites, \((P)/n\), is given by \((P)_t/n - (\text{Heme})_b\) where \((P)_t\) is the total concentration of lipid phosphorus. Substituting this expression into equation 4.1 and rearranging:

\[(4.2) \quad \frac{(P)_t}{(\text{Heme})_b} = n + \frac{nK_d}{(\text{Heme})_f}\]

A plot of \((P)_t/(\text{Heme})_b\) versus \(1/(\text{Heme})_f\) for all the data in figures 4.6 and 4.7 is shown in figure 4.8. The true equilibrium situation where the concentration of binding sites, bound heme, and free heme are all about equal only occurs for the lipid titration of 1 μM heme (open circles, figure 4.8). These data were fit to equation 4.2 and the resultant values for \(n\) and \(K_d\) are 7.8 lipid molecules per
Figure 4.8 The dependence of the ratio of total lipid per bound heme on the reciprocal free heme concentration. The data were collected from the titrations of 1 μM (○), 5 μM (△), and 10 μM (□) heme, and 27 μM lipid (●) as described in figures 4.6 and 4.7. The y intercept and Kd are 7.8 lipid molecules per binding site and 0.7 μM, respectively.
binding site and 0.7 μM, respectively. Careful analysis of the data at higher heme concentration suggests that more heme can be dissolved in the lipid phase, but the apparent affinity is decreased. As shown in figure 4.9, the data above a free CO-heme concentration of about 2 μM can be fitted to a line in which n = 4.8 and Kd = 2.7 μM. Thus, although more heme is dissolved in the membrane, the affinity of the lipid for these additional molecules is at least 4-5 fold less than when the lipid phosphorus to bound heme ratio is greater than eight. This result is readily explained in terms of charge repulsion between the propionate side chains of the bound heme molecules. As the density of heme groups in the membrane increases beyond about one heme per eight phospholipid molecules, further binding is inhibited by unfavorable electrostatic interactions. Under our conditions the maximum solubility of heme is about one molecule per 4-5 lipid molecules (figure 4.9).

C. Heme Flipping in the Lipid Bilayer

The orientation of heme in a bilayer vesicle is assumed to be similar to that of the phospholipid molecule since it is also amphiphilic with acid substituents at one end and a hydrophobic region at the other. The propionate groups are presumably associated with the water-lipid interface at either the inside or outside surface of the liposome (figure 1.2). We felt that before the kinetics of
Figure 4.9  Detail of figure 4.8. The data were collected from the titrations of 1 μM (○), 5 μM (△), and 10 μM (□) heme, and 27 μM lipid (●) as described in figures 4.6, 4.7 and 4.8. The y intercept and $K_d$ of the solid line are 4.8 lipid molecules per binding site and 2.7 μM, respectively.
heme uptake and release by liposomes could be properly interpreted, the rate of heme flipping should be examined.

Kornberg and McConnell (1971 b) measured the rate of phospholipid flipping by using a nitroxide spin label attached to the head group of phosphatidylcholine. Bilayer vesicles were prepared from lipid containing the label which was distributed randomly between the inner and outer surfaces. Only 65% of the initial electron paramagnetic resonance intensity could be removed by adding external ascorbate to reduce the spin label. The gradual appearance of more reducible label corresponded to the rate of trans-bilayer interchange. This process was found to have a half-time of 6.5 hours at 30 °C. An even slower half-time on the order of days was found by later investigators using a specific exchange protein (Rothman and Davidowicz, 1975).

A comparable slow rate for heme flipping could complicate kinetic measurements or render part of the bound heme inaccessible to reaction with globin. However, there seems to be considerable variation in flipping rates, especially for smaller molecules. Doody et al. (1980) concluded that the flipping of a pyrene labelled fatty acid was much faster than its rate of transfer to the aqueous phase which was 16 s⁻¹ at 33 °C and pH 7.4. The reaction kinetics for the transfer process exhibited single exponential behavior, and the extents of transfer and fluorescence intensity change were identical for liposomes containing label added
before or after vesicle preparation. In order to obtain an estimate for the rate of heme flipping, we performed two different experiments. The possibility of a slow rate occurring on the order of minutes or more was first checked by chromatographic studies, and a faster rate was then looked for in stopped flow experiments.

Liposomes were prepared from egg lecithin containing 2.5% by weight of heme to insure that heme would be randomly distributed in the vesicle bilayer. Columns used in the experiment were pre-equilibrated with both lipid and heme. The fraction of small, unilamellar vesicles was incubated with a five fold excess of globin for two minutes and applied to a Sephadex G-200 column. As shown in figure 4.10 the lipid phosphorus was detected only in the void volume. All of the heme originally present in the liposomes reacted with globin to form hemoglobin which was retained by the column and eluted in a later fraction. Therefore, all of the lipid bound heme was available for reaction, and flipping must occur within at least several minutes. As a control, similar heme containing liposomes were incubated with only a half-equivalent of globin and chromatographed as before (figure 4.11). In this case heme appears in both the liposome and hemoglobin fractions as shown by absorbances at 300 nm and 405 nm.

A flipping rate on the order of milliseconds to several seconds was looked for in rapid mixing experiments.
Figure 4.10 The separation of hemoglobin and liposomes on Sephadex G-200. A mixture of liposomes containing heme and excess globin was fractionated on a 1.5 x 60 cm column in 0.05 M Tris, 0.05 M NaCl, pH 8.0, 4°C. Lipid phosphate (•••) was analyzed as discussed in Chapter II, and hemoglobin (■■■) was detected by absorbance at 412 nm.
Figure 4.11 The separation of hemoglobin and liposomes containing heme on Sepharose 4B. A mixture of a limited amount of globin and liposomes containing heme was chromatographed on a 1.5 x 60 cm column in 0.05 M Tris, 0.05 M NaCl, pH 8.0, 4°C. Lipid (△) was detected by optical density measurements at 300 nm, and heme or hemo-
globin (■) absorbance was followed at 405 nm.
As will be discussed in the following sections, when slow phases were observed they did not appear to be associated with a first order molecular transition but rather appeared to be due to second order binding of another ligand to the CO-heme complex. Thus, we believe that transbilayer flipping of heme occurs too rapidly to be observed by stopped flow techniques and does not limit the release of heme from membranes.

D. Slow Phases Associated with Heme Uptake into Membranes

The rates of association of CO-heme with lipid bilayers were measured directly by observing the absorbance changes which occur when heme dissolves in the membrane. Experiments were carried out with large multilamellar liposomes as well as with small unilamellar vesicles. These were composed of egg lecithin and in some cases contained 20 mole % cholesterol and/or 10% stearylamine. Heme was mixed with varying amounts of lipid in the stopped flow device. Sample time courses for the reaction with simple lecithin vesicles and those containing stearylamine and cholesterol are given in figure 4.12. In the absence of stearylamine, the reaction of CO-heme with liposomes exhibits a large exponential phase which is rapid ($t_{1/2} \approx 10$ ms) and a very small, slower phase which comprises, at most, no more than about 5-10% of the total absorbance change at 417 nm.

We were initially worried that the slow phase might represent transmembrane flipping of the heme, particularly
Figure 4.12 Time courses for the reaction of heme with simple liposomes (SIMPLE) and with those containing 10 moles 3% stearylamine and 20% cholesterol (SA). Heme (5 μM) was mixed with 150 μM liposomes in the stopped flow device and observed at 417 nm. The buffers used were 0.05 M Tris, 0.05 M NaCl, pH 3.0, and 10°C. The fitted rates and amplitudes for the fast phases of the simple and stearylamine reactions are: 90 s⁻¹, -0.303, 340 s⁻¹, and -0.305, respectively.
at high heme to lipid phosphorus ratios. Under these conditions, the outside layer of lipid would become saturated (0.1–0.2 heme molecules per lipid molecule), and further uptake could be limited by migration of the heme from the outside to the inside lipid layer. However, if this were the case, much more absorbance change would be expected for the slow phase. At low liposome concentrations 30% of the heme uptake should be governed by the rate of flipping. This phase should disappear at high liposome concentrations when all of the heme present can readily dissolve in the outer layers. In addition, the rate of this phase would be expected to be independent of either heme or liposome concentration.

None of these predictions for the slow phase are observed. Figure 4.13 shows the dependence of the rate of the slow phase on lipid phosphorus concentration for an experiment in which 5 μM CO-heme was mixed with various amounts of simple, lecithin vesicles. The time courses at 417 nm were fitted to two exponentials. The fast phase amplitude ranged from -0.05 at 5 μM lipid phosphorus to a maximum of -0.30 absorbance units above 100 μM lipid. The amplitude of the slow phase also increased with increasing lipid concentration but was never greater than 10% of the total change. The opposite result would be expected if this phase represented flipping. In addition, the rate of the slow phase increases from 0.1 s⁻¹ to about
Figure 4.13 The dependence of the rate and amplitude of the slow phase on liposome concentration. Stopped flow data were obtained for heme uptake by simple (●) liposomes and those containing 10 moles % stearylamine and 20% cholesterol (○) at 419 nm. Unless indicated otherwise, conditions were the same as for figure 4.12.
1 s\(^{-1}\) with a change in lipid phosphorus concentration from 20 to 200 \(\mu\)M. Again, this result is not compatible with flipping which should be a first order process.

An alternative interpretation is that the slow phase represents aggregation of heme molecules which have accumulated in the membrane. However, the results in figure 4.13 are also incompatible with this idea since a decrease in both rate and amplitude would be expected with an increasing lipid to heme ratio. An indication of the nature of the slow phase was obtained when experiments with membranes containing stearylamine (or stearylamine plus cholesterol) were carried out. When corrections for the 3 ms dead time of the stopped flow apparatus are taken into account, the rapid phase at 417 nm for the amine containing vesicles exhibits the same amplitude as that observed for simple lecithin vesicles, although the rate for these modified liposomes is 3-4 fold greater. However, a large slow phase is observed which exhibits an absorbance decrease at 417 nm. Note, the change in absorbance for this phase is positive. The dependence of the rate and amplitude of this change is also shown in figure 4.13. The rate is proportional to lipid concentration and is 10-30 fold greater than the rate observed for simple lecithin vesicles. The difference spectrum for the slow phase observed at high lipid concentrations is indicative of a red shift of the Soret peak. This suggests that the amine is binding to the pentaco-
ordinate CO-heme complex. Thus, the higher the total amine concentration, the faster its rate of chelation with the heme iron atom. The complex dependence of the slow phase amplitude on lipid concentration is also explicable (second panel, figure 4.13). At high heme to lipid ratios (≥ 0.2), the reaction of stearylamine with dissolved CO-heme allows more of the pentacoordinate species to dissolve in the membrane. The spectral change which occurs as the heme goes from the aqueous to lipid phase is much greater than that due to amine binding. Under these conditions the slow phase has the same sign as the more rapid association reaction. However, at high lipid concentrations, all the CO-heme dissolves rapidly into the membrane, and the binding of amine is observed directly as a distinct spectral change which, at 419 nm, exhibits a change opposite in sign to that for the more rapid dissolution reaction.

By analogy with the stearylamine reaction, we assume that the slow phases seen for the simple vesicles are also a reflection of secondary chelation reactions. A variety of ligands is possible, chiefly contaminating ethanolamines or free fatty acids. However, it should be noted that the magnitude of the slow phases is quite small even in the case of the stearylamine containing vesicles. The large appearance of this phase in figure 4.12 is due to the fact that a large portion of the rapid absorbance change is lost
in the dead time of the apparatus. Thus, at least in the case of simple, lecithin vesicles, we felt that no further study was warranted.

E. Rates of Heme Association and Dissociation with Liposomes

The large absorbance change associated with the reaction of heme with liposomes can be described by the following equation:

\[
(4.3) \quad (\text{Heme})_a + (P)/n \xrightarrow{k_1} \text{Heme}_b
\]

The symbols are the same as those defined for equation 4.1. The observed association rate is independent of heme concentration as long as the lipid concentration is high (figure 4.14). As expected, the observed binding rate does vary with liposome concentration and should be defined as:

\[
(4.4) \quad k_{\text{observed}} = k_1(P) + k_2
\]

An approximate value of 1.36 \( \mu \text{M} \cdot \text{P}^{-1} \cdot \text{s}^{-1} \) was found for \( k_1 \) from the slope in the plot of \( k_{\text{observed}} \) versus liposome concentration (figure 4.15). As shown, the data do not exhibit an exact linear relationship. This may be due to the negative cooperativity demonstrated in the equilibrium binding studies (figures 4.7 and 4.8). At higher concentrations of lipid there is less charge repulsion between heme molecules as they bind, so that the apparent second order rate is greater. The curvature is definitely real.
Figure 4.14 The dependence of the observed rate of the uptake reaction on heme concentration. Liposomes (400 µl) were mixed with varying concentrations of heme in the stopped flow device and observed at 417 nm. The buffers used were 0.05 M Tris, 0.05 M NaCl, pH 3.0, 10°C.
Figure 4.15 The dependence of the observed rate of heme uptake on liposome concentration. Data were obtained from two different stopped flow experiments (●, △) following heme absorbance and from fluorescence measurements (◊). Standard composition liposomes were reacted with 2.5 µM heme and observed at 419 nm using 0.05 M Tris, 0.05 M NaCl, pH 8.0, 10°C buffers. The fluorescence data was obtained using pyrene labelled liposomes as described in sections F and G.
Values for observed rates were accurately duplicated in experiments performed on separate days with different preparations of liposomes.

The dependence of CO-heme uptake on lipid concentration is different for large multilamellar liposomes (figure 4.16). The larger vesicles exhibit an uptake rate of 0.64 \( \mu M P^{-1} s^{-1} \) which is about half that of the unilamellar vesicles. This difference is most likely due to the smaller exposed surface area per amount of phospholipid. The heme quickly binds to the outer surfaces of the liposomes and then slowly passes through the aqueous interlamellar spaces to the interior bilayers. The rate limiting step is the dissociation of heme from the outer membranes since bilayer heme flipping appears to occur very rapidly. The initial reaction rates for multilamellar and single bilayer liposomes are about the same as heme is quickly bound to the exposed lipid surfaces. The rate for the large vesicles then gradually becomes less than that for the small ones as heme is slowly taken up by the inner layers. Although fitted to a single exponential to obtain an overall rate constant, the multilamellar time course is probably better thought of as the sum of many slower phases due to heme uptake and release by the inner lipid layers.

Small liposomes containing either cholesterol or stearylamine also exhibit different heme uptake rates. Those containing cholesterol alone have the same or
Figure 4.15 The dependence of the rate of heme uptake on various types of liposomes. Heme (2.5 μM) was reacted in the stopped flow device with small, standard composition liposomes (△), those containing 10 moles % stearylamine and 20% cholesterol (▲), stearylamine alone (□), or cholesterol alone (■), and with multilamellar vesicles (♦). Conditions were the same as for Figure 4.15.
slightly lower rates than the standard lecithin liposomes while those containing stearylamine exhibited rates which are at least twice as fast. Observed rates for liposomes containing both stearylamine and cholesterol were about the same or slightly less than those obtained for vesicles containing stearylamine alone. Therefore, no dramatic change in the rate of heme uptake occurs in the presence of cholesterol. The higher rates seen with stearylamine are probably due to enhancement of the initial attraction between anionic heme and the positively charged liposome surface. This electrostatic effect may also lower the energy barrier for passage of the hydrophobic ring of the heme through the charged bilayer interface to the interior hydrocarbon region of the membrane. Once bound, the heme molecules would also have less charge repulsion for each other due to neutralization by stearylamine. A less cooperative binding effect would result. Some evidence for this is given by the data in figure 4.16 where the rates for liposomes containing stearylamine approach a more linear dependence on lipid concentration than do rates for the standard vesicles.

A value of 2-5 s\(^{-1}\) for the rate of heme release from vesicles (\(k_2\) in equation 4.3) was obtained from the \(y\) intercept of the plot in figure 4.16. A linear relationship is assumed between the observed rate of uptake and lipid phosphate concentration. Similar results were ob-
tained from rapid dilution experiments. Solutions containing 5 μM heme and 10, 20, or 40 μM phospholipid were mixed with an equal amount of buffer in the stopped flow apparatus. At these low concentrations of lipid, a measurable amount of heme dissociates from the liposomes into the aqueous phase upon dilution, and an absorbance decrease at 419 nm is observed. The resultant time course exhibits exponential behavior, and the observed rate appears to represent the rate of heme dissociation from the membrane vesicles. Rates of heme release from standard, multilamellar, and mixed composition liposomes were all about the same value (Table 4.1).

F. Incorporation of Pyrene Label into Phospholipid Vesicles

In order to examine the kinetics of heme transfer between liposomes and to check the previously determined rates for heme uptake, experiments were performed using pyrene labelled phospholipid. Pyrene fluorescence has been studied and discussed in the work by Charlton et al. (1976, 1978). After absorption of light, pyrene and many pyrene substituted compounds can either exhibit excited singlet state (monomer) fluorescence emission at 365-425 nm or collide with a ground state molecule to form an excited state dimer (excimer) which exhibits a broad emission band centered around 470 nm. The ratio of excimer to monomer fluorescence intensity is dependent on pyrene concentration, temperature, and viscosity as described by equation 4.5:
Table 4.1  The rates of heme release from simple, mixed composition, and multilamellar liposomes. Liposomes containing 5 μM heme were rapidly diluted with an equal amount of buffer in the stopped flow device and observed at 419 m. The buffers used were 0.05 M Tris, 0.05 M NaCl, pH 8.0, 10°C.

<table>
<thead>
<tr>
<th>LIPOSOME TYPE</th>
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<th>(LIPOSOME)</th>
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<tr>
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<td>9</td>
<td>20 μM</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>40 μM</td>
</tr>
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</table>
\[(4.5) \quad E/M = (\text{Pyrene})TK/\eta\]

where \(T\) is the absolute temperature, \(\eta\) is the viscosity, and \(K\) is a constant incorporating theoretical and instrumental parameters. At constant temperature and viscosity, the ratio of excimer to monomer fluorescence is linearly dependent on pyrene concentration. This property can be used to follow microscopic concentration changes of pyrene or its derivatives when localized in lipid phases.

The kinetics of transfer of free pyrene and a pyrene substituted diglyceride between high density lipoproteins has been reported by Charlton et al. (1976, 1978). They found that transfer rates were very rapid for the free label (\(\sim 200 \text{ s}^{-1}\)) and considerably slower for the diglyceride (0.2-4 s\(^{-1}\)). In all cases, the transfer rates were independent of pyrene label and lipid concentration. The rate of dissociation of pyrene label from the lipoprotein into the buffer as measured in rapid dilution experiments was equal to that observed for the transfer reaction. These results suggested a mechanism involving rate limiting dissociation of the pyrene label from the lipoprotein followed by rapid diffusion and uptake by another lipid particle.

In a similar study, Doody et al. (1980) investigated the transfer of a pyrene labelled fatty acid between phospholipid, single bilayer vesicles. They observed the transfer to be a first order process independent of lipo-
some concentration or chemical composition of acceptor vesicles. Rate constants for the transfer process and for dissociation of labelled fatty acid into buffer were also the same. They reached the same conclusion as Charlton et al. (1976, 1978) that transfer of a fatty acid between liposomes occurs by dissociation into the aqueous phase rather than by formation of collisional complexes. When similar experiments were carried out with pyrene labelled phospholipid the rates of transfer were found to be extremely small, exhibiting half times on the order of several hours. Evidently, the polar head group prevents rapid dissociation from the membrane surface.

Like pyrene, heme is a hydrophobic, polycyclic compound, but it also resembles a fatty acid in its amphiphilic properties. We wished to determine if heme exhibits similar behavior in its mechanism of transfer between lipid phases. Experiments were performed using liposomes labelled with N-(l-pyrene sulfonyl)-dipalmitoyl-L-α-phosphatidyl-ethanolamine (PSDPPEA), a pyrene substituted phospholipid. This pyrene label cannot equilibrate between the vesicles as heme can, so that the observed rate of fluorescence quenching should be a measure of the heme transfer rate.

Since pyrene label was added to preformed liposomes, it was necessary to determine whether the pyrene substituted lipid had been incorporated uniformly into the vesi-
cles or had formed its own concentrated micellar structures. Fluorescence emission spectra were recorded of vesicles containing various amounts of added label (figure 4.17). These spectra were normalized to the total fluorescence to illustrate the change in relative intensities of the monomer and excimer emissions. When a small amount of pyrene labelled phosphatidyl ethanolamine is added to a vesicle suspension, very little excimer fluorescence is observed. However, with successive additions there is a monotonic increase in the emission band at 470 nm. This increase in excimer fluorescence reflects the increase in PSDPPEA concentration within the individual lipid vesicles. These data show clearly that the pyrene label is taken up by the liposomes and is not simply forming its own micellar structures when injected into the lipid suspensions. If the latter were the case, the microscopic concentration of PSDPPEA would remain about the same with each addition of label. That is, the total number of label containing micelles would increase, but the concentration of PSDPPEA within each lamellar structure would be constant, and the excimer to monomer ratio would be high. This phenomenon is observed when labelled phospholipid is injected into buffer alone (figure 4.18). As shown, the fluorescence emission at 470 nm is very high even at the lowest lipid concentration, and is practically independent of the amount of pyrene label added.
Figure 4.17  The change in the fluorescence spectra of liposomes as a function of increasing pyrene labelled phospholipid concentration. To one ml of 150 μM liposomes were added 2, 10, 20, 40, or 80 μl of PSDPPEA stock solution (0.5 mg/ml) in hot ethanol. For comparison, the resulting spectra were normalized by dividing the fluorescence by the indicated μl amounts of added pyrene label. With increasing concentration, monomer emission (370-410 nm) decreases and excimer emission (450-550 nm) increases. An excitation wavelength of 319 nm was used, and buffers were 0.05 M Tris, 0.05 M NaCl, pH 8.0 and 24 °C.
Figure 4.18 The dependence of fluorescence on PSDPPBA concentration when injected into buffer alone. The ratio of excimer (490 nm) to monomer (395 nm) emission was calculated for the fluorescence spectra of 150 µM liposomes containing various amounts of added PSDPPBA stock solution. Conditions were the same as for figure 4.17.
Quantitative analysis of label incorporation into liposomes is shown in figure 4.19 where the ratio of excimer to monomer fluorescence is plotted versus total PSDPPEA concentration. As shown, addition of pyrene label in ethanol produces a linear increase in the excimer to monomer ratio with a slope of about one. Again, this suggests that the label is uniformly distributed among the membrane vesicles.

In contrast, when the label was initially dissolved in dioxane rather than ethanol, a relatively high and distinctly curved relationship is obtained between E/M and the amount of PSDPPEA added. Under these conditions, a large amount of the pyrene label is forming its own micelles. This result could be due to the greater solubility of the pyrene substituted lipid in dioxane or the lower solubility of this solvent in water when compared to ethanol. When injected into the liposome solution, the pyrene label in ethanol is dispersed much more rapidly than when it is in the dioxane solution. The PSDPPEA in dioxane appears to dissolve slowly enough to allow formation of micelles containing pyrene labelled phospholipid. Consequently, in all of the following experiments, labelled vesicles were prepared by injecting a warm ethanolic solution of the pyrene containing lipid into a suspension of preformed liposomes.

G. Heme Transfer Between Lipid Particles

The rate of heme binding to pyrene labelled liposomes
Figure 4.19 The dependence of fluorescence on PSDPPEA concentration when injected into a liposome solution. The ratio of excimer (490 nm) to monomer (385 nm) emission was calculated for the fluorescence spectra of 150 μl liposomes containing various amounts of added pyrene label in a stock ethanol solution (■) or in dioxane (▲). Conditions were the same as for figure 4.17.
can be observed in the stopped flow device by measuring changes in fluorescence. As shown in figure 4.20, the time course exhibits a decrease in fluorescence followed by a slight, slower increase. The faster phase is due to quenching of the pyrene label fluorescence by heme as it is taken up into the liposomes. The fitted rate for this phase agrees well with rates obtained from absorbance measurements in previous heme uptake experiments (figure 4.16). There is also close agreement between the fitted rates for the slow phases. Previously, the slow phase was interpreted as the result of chelation of the heme by a lipid soluble base as it was dissolved in the membrane. The slow increase in fluorescence may be similarly explained. If the heme molecules become associated with a long chain alkyl amine, they become less mobile and cannot approach the pyrene molecules as easily or as closely for Forster energy transfer. As a result, their efficiency as quenchers is reduced, and an increase in pyrene fluorescence would be expected. It should be noted that the pyrene label itself contains a primary amine. However, the magnitude of the slow phase is quite small, and only a few percent of the heme molecules become hexacoordinate.
Figure 4.20 A sample time course for heme uptake by pyrene labelled liposomes. Heme (2 μM) was mixed in the stopped flow device with 250 μM liposomes labelled with 10 μl/ml PDPPEA stock. An excitation wavelength of 290 nm was used, and observations were made with a 350 nm cutoff filter. Buffers were 0.01 M phosphate, pH 7.2, 10°C.
The rate of heme exchange between bilayers was examined by mixing liposomes containing heme with those containing pyrene label. As shown in figure 4.21, simple exponential behavior is observed. Several different kinds of experiments were performed (table 4.2). In the first, the amount of acceptor lipid is kept constant, and the concentration of donor liposomes is varied while maintaining the same heme to phospholipid ratio. As shown, the observed rate is independent of the donor to acceptor liposome ratio. When the acceptor concentration is doubled while keeping the donor lipid and heme amounts constant, there is no change in the observed rate. Finally, amounts of donor and acceptor liposomes were held constant while the heme concentration was varied. Again, the observed rate does not significantly change. These results strongly suggest that heme exchange between liposomes does not occur through a second order process involving a collisional complex of the vesicles. If this were true, the concentration of vesicles would definitely influence the rate. If diffusion of heme through the lipid phases of the complex were rate limiting, then the concentration of heme within the donor liposomes would influence the rate. However,
Figure 4.21 A sample time course for heme exchange between lipid bilayers. Liposomes (250 μl) containing 2 μl heme were mixed with 125 μl liposomes containing pyrene label and observed in the stopped flow device. Other conditions were the same as for figure 4.20.
Table 4.2  Rates of heme transfer between liposomes. Donor liposomes containing heme were mixed in the stopped flow apparatus with acceptor liposomes containing pyrene label. Fluorescence was measured, and fitted rates were obtained. Indicated concentrations were before mixing and all other conditions were the same as for figure 4.20.
<table>
<thead>
<tr>
<th>Donor Liposome/μL</th>
<th>Heme μM</th>
<th>donor/acceptor</th>
<th>Liposome μM</th>
<th>Heme/Lipid</th>
<th>Ratio</th>
<th>Observed Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>625</td>
<td>0.008</td>
<td>5</td>
<td>125</td>
<td>1.008</td>
<td>0.5</td>
<td>3.9 s−1</td>
</tr>
<tr>
<td>250</td>
<td>0.008</td>
<td>2</td>
<td>125</td>
<td>0.008</td>
<td>0.5</td>
<td>3.4 s−1</td>
</tr>
<tr>
<td>125</td>
<td>0.008</td>
<td>1</td>
<td>125</td>
<td>0.008</td>
<td>0.5</td>
<td>3.6 s−1</td>
</tr>
<tr>
<td>250</td>
<td>0.008</td>
<td>2</td>
<td>250</td>
<td>0.016</td>
<td>0.5</td>
<td>3.8 s−1</td>
</tr>
<tr>
<td>125</td>
<td>0.008</td>
<td>1</td>
<td>250</td>
<td>0.024</td>
<td>0.5</td>
<td>5.0 s−1</td>
</tr>
<tr>
<td>125</td>
<td>0.008</td>
<td>3</td>
<td>250</td>
<td>0.004</td>
<td>0.5</td>
<td>6.1 s−1</td>
</tr>
<tr>
<td>125</td>
<td>0.008</td>
<td>5</td>
<td>250</td>
<td>0.040</td>
<td>0.5</td>
<td>11 s−1</td>
</tr>
</tbody>
</table>
neither of these effects was experimentally observed. An alternative mechanism is that hemo dissociates from the membrane in a rate limiting first order step and then is rapidly taken up by acceptor liposomes. This view is supported by the observed rate which is the same order of magnitude as \( k_2 \), the off constant in equation 4.3, which was obtained in previous experiments. This idea is, of course, in complete agreement with the mechanism proposed by Smith and co-workers (Charlton et al., 1976, 1978) for the exchange of free pyrene and various pyrene labelled molecules between lipid particles.
Chapter V

THE INTERACTION OF HEME WITH GLOBIN AND LIPID BILAYERS

There are conflicting views about whether attachment of heme to globin occurs at the site of protein synthesis (Gonzalez-Cadavid and Campbell, 1967, and Davidson and Penniall, 1971) or inside the mitochondria (Kadenbach, 1970). The latter seems unlikely since highly unstable globin chains would have to be transported both into and out of the mitochondria. Cline and Bock (1966) have suggested that the presence of heme may be necessary for the proper folding of the globin polypeptide chain, but there is evidence indicating that heme is not added until after completion of the primary structure (Morris and Liang, 1968). Numerous examples have also been reported of the reconstitution of a native compound from inactive apoprotein and heme (Rossi-Fanelli and Antonini, 1959, Burke and Lascelles, 1976, Correia and Meyer, 1975, and Haddock and Schairer, 1973). The transport of heme to the site of combination with globin may be accomplished by means of a carrier protein, or by diffusion through lipid or aqueous phases. Alternatively, the combination reaction may require contact between the protein and the membrane. This mechanism seems reasonable since both hemoglobin and globin have been shown to bind hydrophobically to lipid bilayers (Sweet and Zull, 1970, and Calissano et al., 1972). The
following experiments were carried out to distinguish between these possible mechanisms.

A. Rates of Reaction of Membrane Bound Heme With Globin

In stopped flow experiments, standard and mixed composition liposomes containing 5 μM heme were mixed with varying concentrations of globin. A typical time course, shown in figure 5.1, exhibits a large exponential absorbance change complicated by a small slow phase. The fast phase is attributed to dissociation of the heme from the membrane followed by rapid reaction with globin.

The presence of a slow phase is not surprising since this is also observed in the separate reaction of heme with globin and the dilution of lipid bound heme. As discussed in previous chapters, the slow phase is probably due to a combination of factors such as nonspecific binding of heme to globin and chelation of heme by various lipid contaminants. The effect of one component, stearylamine, is illustrated in figure 5.2. Time courses are shown for the reactions of 10 μM globin with 75 μM liposomes containing 2.5 μM heme alone and those with heme plus stearylamine and cholesterol. It can be seen that the rates for the two reactions are very similar. However, unlike the time course for simple vesicles the reaction with liposomes containing stearylamine consists almost entirely of the slow phase and is much smaller in amplitude. This observation is consistent with our previous explanation of the effect of stearyl-
Figure 5.1  The reaction of 20 μM globin with small, egg lecithin liposomes (125 μM phosphate) containing 2.5 μM heme in 0.01 M phosphate buffer at pH 7.2. The time course was observed in the stopped flow device at 423 nm and 10 °C with a 20 second final delay. The fitted rates for the fast and slow phases are 3 s⁻¹ and 0.1 s⁻¹, respectively.
Figure 5.2  The reaction of 10 μM globin with 75 μM phosphate liposomes containing either 2.5 μM heme alone (SIMPLE) or 2.5 μM heme plus 20 moles % cholesterol and 10% stearylamine (SA). The other conditions were the same as for figure 5.1. The fitted rates for the fast and slow phases of the simple and stearylamine reactions were 2.6 s⁻¹, 0.1 s⁻¹, 2.5 s⁻¹, and 0.2 s⁻¹, respectively.
amine. Much of the lipid bound heme is complexed to the stearylamine and is not as readily available for dissociation from the membrane and binding to globin. Therefore, the large fraction of slow phase represents the rate limiting dissociation of the heme-stearylamine complex. The decrease in amplitude of the time course is due to the red shifting effect of stearylamine upon the Soret spectrum of liposomes containing heme. At 423 nm, there is less difference between the absorbance of hemoglobin and heme complexed with stearylamine than between the absorbance of hemoglobin and simple lipid bound heme. Thus, the slow phase for the simple reaction may be largely the result of chelation phenomena with membrane components.

The fast phase for the simple reaction exhibits a half time on the order of about 200 ms whereas the half time observed for the reaction of globin with free heme is about 1 ms. The slower rate for the membrane reaction can be attributed to a partitioning of the heme between the lipid, protein, and aqueous phases and may be represented by the scheme in equation 5.1.

\[
\begin{align*}
\text{Heme} + \text{gb} & \quad \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{Heme-gb} \quad \underset{k_3}{\rightarrow} \text{Hb} \\
+ \quad \text{Lp} \\
\text{Heme-Lp} & \quad \overset{k_1}{\underset{k_2}{\rightleftharpoons}}
\end{align*}
\]

where \text{Lp} represents liposomes, and Heme-Lp is lipid bound
heme. The apparent rate for formation of hemoglobin under these conditions can be derived by making a steady state assumption for free heme and heme-gb as given in equation 5.2.

\[ \frac{d(\text{Heme} + \text{Heme-gb})}{dt} = 0 = k_2(\text{Heme-Lp}) - k_1K_d(Lp)(\text{Heme} + \text{Heme-gb}) \left[ \frac{(gb) + K_d}{(gb)} \right] - k_3(gb)(\text{Heme} + \text{Heme-gb}) \left[ \frac{(gb) + K_d}{(gb)} \right] \]

This reduces to equation 5.3.

\[ (\text{Heme} + \text{Heme-gb}) = \frac{k_2(\text{Heme-Lp})(K_d + gb)}{k_1(Lp)K_d + k_3(gb)} \]

This expression can be substituted into equation 5.4.

\[ \frac{d(Hb)}{dt} = -\frac{d(\text{Heme-Lp})}{dt} = \frac{k_3(gb)(\text{Heme} + \text{Heme-gb})}{(gb) + K_d} \]

This gives:

\[ \frac{d(\text{Heme-Lp})}{dt} = -\frac{k_2k_3(gb)(\text{Heme-Lp})}{k_1(Lp)K_d + k_3(gb)} \]

and the expression for \( k_{\text{obs}} \) is given by:

\[ k_{\text{obs}} = \frac{k_2}{\frac{k_1(Lp)K_d}{k_3(gb)} + 1} \]

The individual constants were assigned values based on the independent experiments discussed in the previous two chapters.
Figure 5.3 shows a plot of the observed rate versus globin concentration for the reaction with 125 μM liposomes containing 2.5 μM heme. Similar values were obtained for liposomes containing cholesterol and stearylamine. Only a moderate dependence on globin concentration is observed which suggests that a direct collision of globin with the lipid bilayer is not rate limiting for heme transfer. The theoretical curve was calculated from equation 5.6. Although some deviation from theory is observed at the higher globin concentrations, there is a reasonably good correspondence between the experimental data and the simplified, theoretical representation of the reaction mechanism as given in equation 5.6.

These results are supported by experiments in which the amount of phospholipid is varied while the globin concentration is kept constant. As shown in figure 5.4, the observed rate actually decreases slightly with increasing liposome concentration. Liposomes of mixed composition gave similar results. Again, this lack of rate dependence on liposome concentration indicates that the reaction is not a second order process. As before, the calculated theoretical curve fits the experimental data reasonably well.

B. Fluorescence Studies

The validity of the proposed mechanism was tested further by several experiments employing stopped flow fluorescence measurements (table 5.1). In the first set of
**Figure 5.3** The dependence of the observed rate on globin concentration. Globin was reacted with 125 μM phosphate liposomes containing 2.5 μM heme (▲), 400 μM liposomes containing 20 moles % cholesterol, 10% stearylamine and 2.5 μM heme (◇), and 125 μM liposomes containing 1 μM heme and 10 μl/ml PSDPPEA stock solution (●). The experiments were performed under the same conditions as figure 5.1. The reaction with liposomes containing PSDPPEA was followed by stopped flow fluorescence measurements above 350 nm using an excitation wavelength of 280 nm. The theoretical curve (solid line) was calculated from equation 5.6 using values obtained from previous experiments of 1.36 μM⁻¹s⁻¹ for $k_1$, 4 s⁻¹ for $k_2$, 670 s⁻¹ for $k_3$, 22 μM for $K_d$, and 125 μM for liposome concentration.
Figure 5.4  The dependence of the observed rate on liposome concentration. Globin (10 μM) was reacted with liposomes containing 2.5 μM heme alone (▲) or 2.5 μM heme plus 20 moles % cholesterol and 10% stearylamine (◇). Other experimental conditions were the same as for figure 5.1. The theoretical curve (solid line) was calculated from equation 5.6 using values obtained from previous experiments of 1.36 μM⁻¹s⁻¹ for k₁, 4 s⁻¹ for k₂, 670 s⁻¹ for k₃, 22 μM for k₅, and 10 μM for globin concentration.
### Table 5.1
The comparison of observed rates for the extraction of lipid bound heme by globin, liposomes, and buffer. The reactions with liposomes containing pyrene were followed by stopped flow fluorescence measurement above 350 nm using an excitation wavelength of 280 nm. The dilution of liposomes containing heme was followed by absorbance measurement at 417 nm in the stopped flow device. All other unspecified conditions were the same as for figure 5.1.

<table>
<thead>
<tr>
<th>REACTION</th>
<th>NM CONCENTRATIONS (AFTER MIXING)</th>
<th>OBSERVED RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Lp-donor) (Heme) (Lp-acceptor) (gb)</td>
<td></td>
</tr>
<tr>
<td>Lp(Heme)(Pyrene) + gb</td>
<td>125 1 --- 2.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>125 1 --- 25</td>
<td>3.9</td>
</tr>
<tr>
<td>Lp(Heme) + Lp(Pyrene)</td>
<td>62.5 0.5 62.5 ---</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>125 1 62.5 ---</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>312.5 2.5 62.5 ---</td>
<td>3.9</td>
</tr>
<tr>
<td>Lp(Heme) + Buffer</td>
<td>5 2.5 --- ---</td>
<td>6.2</td>
</tr>
</tbody>
</table>
experiments, two different concentrations of globin were reacted with liposomes containing both pyrene labelled phospholipid and heme. An increase in fluorescence was observed as heme left the labelled membrane to react with globin. The rates for this reaction were comparable to those found in similar previous experiments employing absorbance measurements (figure 5.3). As shown in table 5.1 the rate for extraction of lipid bound heme by globin is of the same order of magnitude as the rates for heme transfer between liposomes, and dissociation of heme from the membrane. This invariance in rate is evidence that these widely different transfer processes are all limited by the rate of dissociation of heme into the aqueous phase.

C. Physiological Implications

Heme is formed and solubilized in the cell membranes, but is eventually found in combination with water soluble proteins. In our work, it has been shown that an apoprotein such as globin can easily and irreversibly extract heme from lipid phases. This might not be expected since the simple adsorption affinity of heme for globin ($K_d=22 \text{ M}$) is less than its affinity for lipid ($K_d=0.7 \text{ M}$, see figure 4.8). Formation of hemoglobin does occur, however, because of the covalent attachment of heme to the protein through the iron-histidine bond ($k_3$ in equation 5.1). This process removes heme from the partition system, resulting in a continual shift of the equilibrium concentrations until all of the heme is
incorporated into hemoglobin. The relatively high affinity of heme for lipid explains why hemoglobin formation in this system is much slower than its formation from lipid-free heme plus globin.

In conclusion, it is important to note that both the individual and overall reaction can be analyzed quantitatively. The overall reaction can be described by equation 5.6, and theoretical lines can be computed using constants derived from independent experiments. A five-fold increase in liposome concentration actually causes a 30% decrease in the observed rate. For a sixty-fold change in globin concentration, a two-fold change in rate is theoretically predicted whereas a three-fold change is actually observed. The discrepancy is apparent only at higher globin concentrations. Its cause is unknown but may be due to direct interaction of globin with heme in the membrane. In general, the theory and data agree very well in spite of slight deviations. Furthermore, the tested variations in lipid composition did not have an important effect on the overall reaction, although the presence of stearylamine did increase the amount of observed slow phase.

Perhaps more significantly, our results also indicate that a carrier protein is not essential for heme transfer, although it may be necessary for other functions. In fact, the rapid rate of heme transfer among lipid particles suggests that a carrier protein may be required to prevent
dispersal of heme throughout the entire cell. Such a protein might also be instrumental in maintaining heme in a monomeric, reduced form. However, in the absence of a carrier, heme would still be rapidly cleared from membranes by apoproteins in the aqueous cell compartment.
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