Rates of Reaction of Native Human Globin with Some Hemes*

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The reaction of native globin with several protoheme derivatives has been shown to be both rapid and complex, and a scheme involving the reversible formation of an intermediate complex has been suggested (1).

\[
\text{Heme} + \text{globin} \xrightarrow{k_1} \text{complex} \xrightarrow{k_2} \text{hemoglobin} \ldots \quad (1)
\]

It was possible to account for the time course of the reactions of both carboxyheme and monocyanide heme with globin by use of suitable values for \(k_2\) and the ratio \(k_1/k_2\); the two latter constants being too great to measure directly by the stopped flow method.

It has been known for more than 30 years that meso- and deuterohemes can combine with globin to form compounds able to react reversibly with oxygen and other ligands (2). Some of the properties of these unnatural hemoglobins and myoglobins have been studied in detail (3-6) and are generally similar in kind to those of protohemoglobin, although showing considerable quantitative differences.

In this paper the kinetic experiments with protoheme and globin are extended to meso-, deuter-, hemato-, and some other hemes, and the effects of chemical substitution in the heme on the rate and course of the reaction are examined. The results are consistent with the mechanism already proposed, and the considerable differences in rates can be correlated with the chemical modifications in the heme and with the structure of the protein as revealed by x-ray analysis.

As a necessary preliminary, the absorption spectra and extinction coefficients of the heme derivatives were determined in different solvents, both to provide the data required for quantitative treatment of the kinetic results and to give information about the degree of polymerization in the solutions.

**EXPERIMENTAL PROCEDURE**

**Globin**—Native human globin, prepared by the method of Rossi-Fanelli, Antonini, and Caputo (3), was lyophilized and stored at \(-20^\circ\)C. It was dissolved in 0.1 M phosphate buffer, pH 7.4, or 0.025 M borax, pH 9.1, immediately before use. The concentration of globin solutions was determined spectrophotometrically at 280 nm by taking \(A_{\text{cm}}^\infty = 8.5\).

**Hemins**—Protohemin was obtained from B.D.H. Ltd., Poole, Dorset, England, and from Merck A.G., Darmstadt, Germany.

Deuterohem in was prepared by the method of Fischer and Orth (7). Meso-hem in was made by the method of Erdmann and Corwin (8). Hemato-hem in was made from hematoporphyrin (California Corporation for Biochemical Research) by introducing iron according to the method of Paul (9). Spirographis hem in was prepared from chlorocruorin by the procedure of Warburg, Negelein, and Haas (10). Porphobilin a hemin was made by introducing iron into porphobilin a (Fluka A.G., Buchs, St. Gallen, Switzerland) according to Fischer and Orth (11). The dimethyl ester of deuterohem in disulfonate was a gift from Dr. R. L. J. Lyster, National Institute for Dairy Research, Reading, England.

**Preparation of Solutions of Heme Derivatives**—The hemes were dissolved in enough 0.1 N NaOH to give about \(5 \times 10^{-5}\) M. This stock was diluted with at least 9 volumes of the buffers required; the spectra of the hemes were recorded on this material. To prepare heme solutions, portions of the diluted hemin solutions were deoxygenated in glass tonometers by repeated evacuation and shaking. The hemin was reduced by injecting 1% by volume of a 1% solution of sodium dithionite into the tonometer from a syringe. CO-hemins were obtained by equilibrating the heme solutions with a partial pressure of 200 mm of Hg of CO. Solutions in 90% ethylene glycol (volume for volume) were prepared by diluting the NaOH stock solution with it instead of with buffer; the other details were the same.

**Spectra** were recorded with a double beam spectrophotometer (Optica U.K. Ltd., Team Valley, Newcastle-upon-Tyne, England). This is a grating instrument, and it was used also to determine the positions of the absorption maxima.

**Stopped Flow Kinetic Determinations** were made with the equipment already described (1).

**RESULTS**

**Spectrophotometric Findings**—The positions of the Soret (\(y\)-band) maxima and the corresponding extinction coefficients \((E = A/Cb, A = \text{absorbancy}, C = \text{concentration}, b = \text{path length, units cm}^2 \text{per mmole})\) are given in Table I. Some of the figures call for comment. The value for CO-protoheme of 1.45 \(\times 10^4\) in borate, pH 9.1, agrees well with values previously reported (12, 13), but applies only to the B.D.H. hemin sample; the Merck sample gave consistently lower values both when examined immediately on preparation, and after standing for 24 hours at room temperature. Both preparations gave the same value for CO-heme in ethylene glycol, and the values for protohemin and protoheme also agreed within experimental error. The value \(e = 2.1 \times 10^4\) for CO-heme in 90% ethylene glycol.
glycol is substantially greater than that (1.5 x 10^5) reported earlier (13). The difference may be due to the care taken to exclude oxygen as far as possible, for when the measurements were repeated by adding a few crumbs of dithionite to a solution of hemin in 90% ethylene glycol saturated with CO in an open cuvette, lower extinctions were observed.

Similar sensitivity to traces of oxygen was observed with other hemes, but was particularly marked in the case of dimethyl deuteroheme disulfonate and Spirographis heme. In the latter case, admission of oxygen was associated with the appearance of a shoulder on the long wave length side of the Soret band, which in the former case, was never observed. In the second, the concentration of globin was held constant at about 6 x 10^-4 M and the concentration of CO-heme was systematically varied. For reasons dealt with in “Discussion,” it is difficult to summarize the results in terms of the three constants of Equation 1, and they are therefore presented graphically for meso-, deuter-, and hematohemes (Figs. 1 to 4). In view of the instability of the solutions of Spirographis CO-heme, no results are shown; it did, however, react with globin in a manner analogous to that of the three hemes for which results are given.

There was no evidence of reaction when the dimethyl ester of deuteroheme disulfonate was mixed with low concentrations of globin. With high concentrations of globin a spectroscopic change was observed, but this was apparently nonspecific, since similar spectroscopic changes could be obtained with bovine serum albumin.

Effect of Varying Wave Length of Observation—In earlier work the reaction between CO-protoheme and globin was followed at the wave lengths of maximal absorption, both of the heme and of the hemoglobin. The apparent course of the reaction at these wave lengths agreed well, and this, together with other evidence (1), was interpreted as showing that the complex (see Equation 1) and the hemoglobin have similar absorption spectra. The generally slower reactions of the hemes studied here have allowed a slightly more detailed analysis, and the difference spectrum for CO-mesoheme — CO-mesohemoglobin obtained in the stopped flow apparatus is given in Fig. 5. The apparent progress of the reaction between CO-mesoheme and globin as followed with light of various wave lengths is shown in Fig. 6. The reaction appears to go faster at 390 m\(\mu\) and 405 m\(\mu\) and to lag at 415 and 420 m\(\mu\). If only two spectroscopically distinct species (CO-heme and CO-hemoglobin) were present, the apparent rate of progress would be independent of the wave length of the observing light, and at least one other molecular species must be present to account for the curves of Fig. 6.

Effects of Temperature and pH—The effect of changing the temperature on the overall rate of reaction as measured at the absorption maximum of the CO-hemoglobin was the same for both deutero- and meso-CO-heme, and gave a Q_10 at both high and low globin concentrations, of 2.0 per 10^\(\circ\)C, corresponding to an apparent activation energy for the rate-limiting step of about 10 kcal. This is the same result as was previously obtained with proto-CO-heme (1). Attempts to carry out experiments at pH 7.4 in phosphate buffer were made with both meso- and deuterohemes, but no satisfactory results were obtained owing to instability of the CO-heme solutions.

**Discussion**

Detailed kinetic analysis of the heme-globin reaction requires that either a monomeric heme derivative be used, or a polymer whose behavior is accurately known. The CO-hemes were chosen because CO-protoheme is believed to be monomeric in dilute aqueous solution. The main reasons for this belief are that absorbancy is proportional to concentration over a wide range, that the CO-dissociation curve is close to a hyperbola, and that there is little change in the wave length of maximal absorption or extinction coefficient on changing solvent from water to ethylene glycol (13). Additional evidence from kinetic studies (1) of the CO-protoheme-globin reaction is that even in strong (millimolar) solutions the ratio Fe:CO is 1:1; thus a dimer must have the formula CO—II—II—CO, where II is the heme nucleus. The rate of formation of heme-globin bonds would be expected to depend on the concentration of CO, but this was not found experimentally. Further, if CO-protoheme...
Fig. 1 (left). Reaction of CO-meso-heme with native human globin in borate buffer, 0.025 M, pH 9.1. The reaction was followed at 410 μm (wave length of maximal absorption of CO-meso-hemoglobin) in a path length of 2 cm; temperature = 22.5°. A, Initial heme, 2.6 × 10⁻⁴ M; globin, 3.0 × 10⁻⁴ heme equivalent. B, Heme, 1.2 × 10⁻⁴ M; globin, 1.5 × 10⁻⁴. C, Heme, 6.4 × 10⁻⁷ M; globin, 7.5 × 10⁻⁷. D, Heme, 2.9 × 10⁻⁶ M; globin, 3.8 × 10⁻⁷. The points are experimental observations, the lines were calculated from the scheme of Equation 1 with \( k_1 = 4.9 \times 10^7 \) M⁻¹ sec⁻¹, \( k_2 = 160 \) sec⁻¹, \( k_3 = 203 \) sec⁻¹.

Fig. 2 (right). Continuation of the experiment shown in Fig. 1. Concentration of globin = 3 × 10⁻⁶ heme equivalent throughout. A, Heme = 2.3 × 10⁻⁴ M; B, heme = 1.15 × 10⁻⁴ M; C, heme = 5.8 × 10⁻⁷ M; D, heme = 2.9 × 10⁻⁷ M. All other conditions were the same as for Fig. 1. In this experiment, as in other experiments of Figs. 1 to 4, the extinction change with the concentrations of globin was followed for times longer than shown in the figure.

The results of Fig. 6 support the mechanism of Equation 1. On mixing CO-heme with globin there would be a fairly rapid formation of complex, and the appearance of CO-hemoglobin should follow with a slight lag. If the complex has a spectrum intermediate between that of CO-heme and CO-hemoglobin, the effects of changing solvent, a shift of absorption maximum is more important than change of extinction. If this is true, all of the hematin, except deuterohematin disulfonate, are polymeric, together with proto-, Spirographis, and deuterohemes.

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The marked deviations from the general mean are at 390 μm, 405 μm, and 420 μm, wave lengths where the total absorbanoy changes are small (cf. Figs. 5 and 6). At 390 μm, the overlap of the γ-bands of CO-heme and CO-hemoglobin is least, and the conversion of CO-heme to complex weights the observations, giving a more rapid change than the average. At 405 μm near the isosbestic point for CO-heme and CO-hemoglobin, the formation of the complex is similarly important, again giving a more rapid change. At 420 μm the changes in the hemoglobin concentration are more important, and the spectrophotometric change lags behind the average. At the wave lengths of maximal absorption of CO-heme and CO-hemoglobin (400 and 410 μm, respectively) the changes are closely correlated and intermediate in rate, as would be expected if the spectrum of the complex were intermediate between that of CO-heme and CO-hemoglobin. The absolute absorption spectrum of the complex could be obtained from the data of Fig. 6 either if the concentration of any one species (CO-heme, CO-hemoglobin, or complex) could be determined independently or if the CO-heme and complex could justifiably be regarded as in equilibrium with each other. Although the assumption of equilibrium may well be sufficiently nearly true, four points from Fig. 6 are needed to start the calculation, and the accumulation of experimental errors, together with possible systematic errors arising from insufficiently monochromatic observing light, mean that the idea is more attractive in theory than in practice.

Inspection of the results (Figs. 1 to 4) shows that all of the hemes studied react considerably less rapidly than does protoheme, and that hematoheme reacts less rapidly than meso- and deuterohemes. Estimates of the numerical values are collected...
in Table II. Although there are some difficulties in assigning these values, which are discussed later, the general significance of the results is in no way affected. They may be explained in terms of the structural findings of Perutz et al. (14) and Kendrew et al. (15), who have shown that the vinyl groups of protoheme lie deeply buried in the hydrophobic interior of the molecule. Replacement of the unsaturated side chains by the ethyl groups of mesoheme, or their removal, as in deuteroheme, is associated with slower binding, whereas the changes that follow the replacement of the vinyl groups by the hydroxyethyl groups of hematoheme are still more marked. The latter effect may be correlated with the hydrophilic nature of these hydroxyl groups, which is sufficiently marked to cause the porphyrin ring to lie flat on a water surface instead of standing on edge with the vinyl groups in the air, as does protoheme (16). The inability of the very hydrophilic dimethyl ester of deuteroheme disulfonate to combine at all also fits well into this simple picture. The variation in the apparent progress of the reaction with the wave

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**Fig. 3.** Reaction of CO-deuteroheme with native human globin in borate buffer, 0.025 M, pH 9.1; temperature = 22.8°. The reaction was followed at 408 mUw (wave length of maximal absorption of CO-deuterohemoglobin) with a path length of 2 cm. The figure shows only part of an experiment. A, Heme = 2.6 × 10^{-4} M; globin = 3.0 × 10^{-4} heme equivalent. B, Heme = 1.3 × 10^{-4} M; globin = 1.5 × 10^{-4}. C, Heme = 6.5 × 10^{-4} M; globin = 7.5 × 10^{-4}. D, Heme = 3.3 × 10^{-4} M; globin = 3.8 × 10^{-4}. Curves were calculated with \( k_1 = 6.9 \times 10^7 \text{sec}^{-1}, k_2 = 230 \text{sec}^{-1}, k_3 = 130 \text{sec}^{-1} \).

**Fig. 4.** Reaction of CO-hematoheme with native human globin in borate buffer, 0.025 M, pH 9.1; temperature = 21°. The reaction was followed at 411 mU (wave length of maximal absorption of CO-hematohemoglobin) with a path length of 2 cm. Only a small part of the experiment is plotted to allow comparison with Figs. 1 to 3. A, Heme = 1.48 × 10^{-4} M; globin = 3 × 10^{-4} heme equivalent. B, Heme = 7.3 × 10^{-5} M; globin = 1.5 × 10^{-4}.

**Fig. 5.** Difference spectrum (meso-CO-heme) - (meso-CO-hemoglobin): 1.5 × 10^{-4} M meso-CO-heme + 1.5 × 10^{-4} heme equivalent of native human globin; 2-cm path, 23.8°, 0.025 M borate buffer, pH 9.1.

**Fig. 6.** Progress of the reaction between 1.5 × 10^{-4} M CO-meso-heme and 1.5 × 10^{-4} heme equivalent of globin as followed at different wavelengths. Conditions were the same as for Fig. 5. The figure shows the percentage of the total AE at each wave length which has taken place at the times indicated on the curves.
length of the observing light is clear evidence that the reaction involves at least one molecular species other than the CO-heme and the CO-hemoglobin. If it is accepted that \( k_1 \) and \( k_2 \) of Equation 1 describe the formation of this new species and its conversion into hemoglobin, respectively, it seems clear from Table II that both processes are markedly affected by structural changes in the heme group.

There is a close correlation between the rate of formation of the various hemoglobin complexes and their stability. In experiments in which various combinations of hematin and hemoglobins were mixed together, it was found (17) that protoporphyrin could displace meso-, deutero-, and hematohemin. The affinities are in all cases very great and may be estimated roughly as follows. When apomyoglobin was mixed with protoporphyrin-hemoglobin at neutral pH there was a transfer of hematin to the apomyoglobin with a half-time of the order of hours (corresponding to a rate constant of \( 10^{-4} \) sec\(^{-1} \)). Taking the overall rate constant for the combination as \( 7 \times 10^{-8} \) sec\(^{-1} \), an apparent dissociation constant of the order of \( 10^{-12} \) M is indicated if the combination rates for protoporphyrin and protoporphyrin-hemoglobin are similar. Although admittedly crude, this figure gives some idea of the order of affinity involved.

Evaluation of the constants in Table II proved unexpectedly difficult; where \( k_2 \gg k_3 \) as is true for CO-protoporphyrin, Gibson and Antonini (1) have described simple approximate methods for obtaining \( k_3/k_2 \) and \( k_2 \), but this procedure is not applicable to the cases studied here, where \( k_2 \) may not be much greater than \( k_3 \).

Trial and error experiments with an electronic analogue computer (Solartron Ltd., Farnborough, Essex) quickly showed that with three arbitrary constants, many sets of three would give a good fit to any one experimental curve. As analogue methods do not lend themselves especially well to dealing with a family of curves, recourse was had to digital methods. Mr. P. M. Blundell of the Department of Applied Mathematics, University of Sheffield, kindly prepared a program for a "Pegasus" computer (Ferranti, Ltd., Manchester) which allowed the best fitting set of three constants to be computed by a "least squares" procedure.

Even with this much more powerful method, difficulties remained. One is illustrated in Figs. 1 and 2. In this group of eight kinetic curves taken from a single experiment, seven show a reasonable fit, but the eighth (Fig. 2A) is widely discrepant and contributes 6 times as much to the final sum of squared residuals as the other seven curves put together. It is clear that an accidental error of some kind has occurred, and that this record should be rejected. In addition, with meso CO-heme the formation of the complex was sufficiently rapid so that several pairs of values of \( k_3 \) and \( k_2 \) gave similar sums of squares of residuals, although in this case the ratio \( k_3/k_2 \) did not change much. As in many "least squares" treatments, there is also a faint possibility that good fits could be obtained with sets of constants radically different from those quoted, representing effectively a different scheme; e.g. if \( k_3 = 0 \), best fitting values for \( k_1 \) and \( k_3 \) could be obtained. In spite of the difficulties and qualifications which remain, it seems that without the digital computer, no detailed analysis at all could have been made, although even without such an analysis the interesting correlation between the location and orientation of the heme groups, as shown by x-ray analysis, and the kinetic behavior of the different hemes would still have been evident.

**Summary**

1. The rate of reaction of native human globin with the carbon monoxide compound of various hemes is in the order proto- \( > \) meso- \( > \) deutero- \( >> \) hematoheme. The dimethyl ester of deuteroheme disulfonate does not react.

2. These results correlate well with the known structure of hemoglobin in which the vinyl groups lie deeply in the hydrophobic interior of the molecule, the sharpest break in rate occurring when the hydroxycetyl groups of hematoporphyrin are substituted for the vinyl groups of protoporphyrin.

3. Spectrophotometric evidence of the formation of an intermediate heme-globin complex in the reaction between heme and globin is given.

4. Spectrophotometric data are presented for the \( \gamma \)-bands of the hematin, hemes, and CO-hemes of proto-, meso-, deutero-, hemato-, Spirogruphis, and phaeophorbid \( a \) hemes and for the dimethyl ester of deuteroheme disulfonate.

**REFERENCES**
