Exchange of Heme among Hemoglobins and between Hemoglobin and Albumin*

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SUMMARY

Intact heme groups undergo exchange between molecules of human hemoglobin under physiological conditions. This phenomenon requires the presence of ferrihemoglobin and is blocked by heme ligands or the prior binding of hemoglobin to haptoglobin. The kinetics of heme exchange between hemoglobins A and F allows a calculation of the rate constants for the dissociation of heme and globin. The rate of exchange of hemes between the non-α chains of ferrihemoglobins A and F is approximately 8 times that between the α chains. Mixtures of ferrihemoglobins and oxyhemoglobins exchange hemes at a rate greater than predicted from the rates of exchange observed in mixtures containing only oxyhemoglobin or terrihemoglobin.

Ferriheme groups also undergo exchange between hemoglobin not bound to haptoglobin and methemalbumin, although at equilibrium the affinity of human albumin for ferriheme is only about one-fifteenth that of globin. Only primate albumins possess appreciable affinity for ferriheme; albumins from the nonprimate species examined appeared to be incapable of forming methemalbumin. Depletion of hemes from ferrihemoglobin by excess human albumin leads to the accumulation of free, precipitable globin.

EXPERIMENTAL PROCEDURE

Preparations of Hemoglobin—Hemoglobin A was prepared as follows. Three volumes of cold distilled water were added to packed human red cells which had been washed three times. A physiological salt concentration (0.15 M) was then restored by the addition of 5% NaCl, after which a clear hemoglobin solu-
tion was separated from red cell stroma by centrifugation at 2500 × g at 4°. Hemoglobin A was isolated by cation exchange chromatography (Amberlite IRC-50), according to the method of Allen, Schroeder, and Balog (9); the buffer was identical in pH and ionic strength with their "developer No. 2," but lacked cyanide. In like manner hemoglobin F was prepared from normal umbilical cord blood cells.

59Fe-labeled hemoglobin F was prepared by incubating reticulocyte-rich cord blood cells with isologous, heparinized plasma to which had been added 59FeCl3 (Abbott Radiopharmaceuticals), and glucose sufficient to give a final concentration of 2.0 mg per ml. The amount of iron added never exceeded the measured iron-binding capacity of the plasma transferrin. After incubation at 37° for 3 hours with gentle agitation, the cells were washed five times with large volumes of 0.15 ml. The amount of iron added never exceeded the measured iron-binding capacity of the plasma transferrin. After incubation at 37° for 3 hours with gentle agitation, the cells were washed five times with large volumes of 0.15 m NaCl solution. The labeled hemoglobin F was then isolated as described above. In like manner 59Fe-hemoglobin A was prepared from red cells of patients with elevated reticulocyte levels. In parallel incubations glycine-2-14C (New England Nuclear Corporation) was labeled hemoglobin F was then isolated as described above.

Ferrihemoglobin was prepared by adding 1.2 moles of K3Fe(CN)6 per mole of oxyhemoglobin heme at pH 6.8, followed by dialysis against the incubation buffer (see below). Iron determinations on the dialysates indicated that, contrary to the experience of others with water (10), dialysis against the incubation buffer removed all the ferro- and ferricyanide from the ferrihemoglobin solution. Ferrihemoglobin cyanide was formed by the addition of 1.2 moles of neutral cyanide per mole of ferrihemoglobin heme, again followed by dialysis. Carboxyhemoglobin was prepared by exposing solutions of oxyhemoglobin for several minutes to carbon monoxide. Deoxyhemoglobin was prepared by passing oxygen-free, water-saturated nitrogen over a thin layer of oxyhemoglobin solution, pH 6.8, at 37°, in a thermostat for 90 min; about 85% of the hemoglobin was deoxygennated. A fresh, heparinized blood sample from a patient heterozygous for hemoglobin M Boston was generously provided by Dr. Park S. Gerald. The relative amount of hemoglobin M in the hemolyzate was determined spectrophotometrically (11) to be about 17%. In this instance, the hemolysate was not further purified prior to its incubation with 59Fe-labeled hemoglobin F. Following incubation, the mixture of hemoglobins A, F, and M Boston was separated by cation exchange chromatography (9). It was necessary to elute with 0.02 m Na2HPO4 to recover hemoglobin M Boston from the column. The molar extinction coefficient of this compound at 500 mμ was confirmed by iron content.

Incubations—Unless stated otherwise, labeled hemoglobin F was incubated with an equal quantity of unlabeled hemoglobin A at 37° in phosphate buffer of pH 7.18, ionic strength 0.09. This is the same buffer that was used in purifying the hemoglobins. It was determined that the reaction to be described could be terminated by cooling to 4° or by adding an excess of neutral cyanide. After dialysis in the cold against developer No. 2 containing 0.01 m cyanide, the hemoglobin mixtures were separated by cation exchange chromatography (9). In one pair of experiments, hemoglobin solutions were dialyzed for 24 hours, at 4°, against solutions of varying pH and constant ionic strength prior to their admixture. In another, hemoglobin solutions were dialyzed against solutions of varying ionic strength but constant pH prior to their admixture.

Specific Activity Measurements and Calculations—In most experiments the hemoglobin concentration of each fraction was measured by the absorbance at 540 mμ after conversion to ferrihemoglobin cyanide.

In hemoglobin mixtures containing only oxyhemoglobin and ferrihemoglobin cyanide, the relative amount of each was determined after appropriate dilution with water by measuring the optical density at 580 mμ and at 550 mμ. The percentage of ferrihemoglobin cyanide was calculated as follows:

\[
\% = \frac{2.08 - \frac{A_{580} - A_{550}}{1.05}}{\text{counts per min per mg of hemoglobin}}
\]

This formula gave good approximations of the relative amounts of oxyhemoglobin and ferrihemoglobin cyanide in a set of standardized mixtures. When indicated, ferrihemoglobin was determined by the method of Evelyn and Malloy (12). The 59Fe activity of each fraction was measured with a well-type scintillation counter. The 14C activity was measured with a low background gas flow counter on planchet-dried aliquots. When appropriate, heme activity of individual fractions was measured by crystallization of hematin (13) after the addition of a known amount of unlabeled carrier. The measured activity was corrected for the percentage recovered. In like manner the globin activity of individual fractions was determined (14). All 14C data were corrected for self-absorption. The specific radioactivity of each fraction was expressed as counts per min per mg of hemoglobin. A weighted mean of the specific activities of neighboring hemoglobin-rich fractions was calculated. Specific activities of neighboring tubes generally agreed within ±5%.

If heme groups exchanged freely among hemoglobin molecules in equimolar mixtures of the labeled hemoglobin (e.g. hemoglobin F) and the unlabeled hemoglobin (e.g. hemoglobin A), then at equilibrium the specific activity of the newly labeled hemoglobin would be 50% of the initial specific activity of the originally labeled hemoglobin, which represents 100% exchange. At time t the quotient of the specific activity of the newly labeled hemo-

globin over one-half the specific activity of the originally labeled hemoglobin gives the fraction of heme exchanged. Thus, if heme-labeled hemoglobin F is incubated with an equal amount of unlabeled hemoglobin A

\[
\text{Percentage heme exchange} = \frac{2 \times S.A. \text{Hb} A_f}{S.A. \text{Hb} F} \times 100
\]

where S.A. represents specific activity. This expression will be used throughout when the exchange of hemes between hemoglobins is reported. Similarly, for the exchange of hemes between methemalbumin containing 1 heme per mole and hemoglobin (4 hemes per mole), the heme exchange is calculated as follows.

\[
\text{Percentage heme exchange} = \frac{5 \times S.A. \text{Hb} heme}{S.A. \text{methemalbumin hemes}} \times 100
\]

Miscellaneous Procedures—Subunit hybrids were prepared by dialyzing a solution containing equal amounts of 59Fe-carboxyhemoglobin F and unlabeled carboxyhemoglobin A (and vice versa) against 0.1 m acetate buffer, pH 4.5, for 48 hours at 4°, followed by dialysis against the incubation buffer for 24 hours.
The mixture was then separated as described above. Hybrids were thus formed, in which only the α chains were labeled. The intact α and β chains of carboxyhemoglobin A were separated on a carboxymethylcellulose column by the method of Bucci and Fruticelli (15). In order to achieve separation we found it necessary to convert solutions of ferrihemoglobin cyanide to carboxyhemoglobin by adding an excess of sodium dithionite under 1 atm of nitrogen followed by gassing with carbon monoxide.

Haptoglobin-bound hemoglobin A was separated from free hemoglobin A by chromatography on Sephadex G-200 (16).

Human serum albumin (Nutritional Biochemicals) was dialyzed against several changes of incubation buffer prior to use. After mixtures of hemoglobin and albumin had been incubated at 37° for varying periods, the two proteins were separated by electrophoresis and by chromatography. Following electrophoretic separation on cellulose acetate, the hemoglobin was localized with a peroxidase stain, o-dianisidine, and the relative distribution of heme between the two proteins was estimated by densitometric scanning. For quantitative studies, the hemoglobin-methemalbumin mixtures were separated on a carboxymethylcellulose column by the method of Bucci and Fruticelli (15). In order to achieve separation we found it necessary to convert solutions of ferrihemoglobin cyanide to carboxyhemoglobin by adding an excess of sodium dithionite under 1 atm of nitrogen followed by gassing with carbon monoxide.
FIG. 2. Rate of exchange between methemalbumin and ferrihemoglobin A. Methemalbumin, containing 1 mole of $^{59}$Fe-heme per mole of albumin, was incubated for various periods at 37°, pH 7.18, with a heme equivalent amount of ferrihemoglobin, and then separated by cation exchange chromatography. Heme exchange was calculated from the specific activities of the isolated hemoglobin and methemalbumin, as described in "Experimental Procedure." The curve was fitted to the points visually.

FIG. 3. Distribution of radioactivity after incubation of labeled ferrihemoglobin F (80 $\mu$M) with an equal amount of unlabeled ferrihemoglobin A for 14 hours at 37°, pH 7.18. The mixtures were then separated on cation exchange chromatography. Upper portion, $^{59}$Fe-labeled heme; lower portion, $^{14}$C-labeled globin. HGB A, hemoglobin A; HGB F, hemoglobin F.

FIG. 4. Distribution of radioactivity after incubation of $^{14}$C-labeled ferrihemoglobin F (80 $\mu$M) with an equal amount of unlabeled ferrihemoglobin A for 14 hours at 37°, pH 7.18, followed by separation on a cation exchange column. The hemoglobin F had been labeled during biosynthesis with glycine-2-$^{14}$C, so as to label the heme ($O$) as well as the globin ($\Delta$). Only the $^{14}$C-heme underwent exchange. HGB F, hemoglobin F; HGB A, hemoglobin A.
been shown to react specifically and quantitatively with heme haptoglobin. Exchange with the unlabeled ferrihemoglobin initially bound to haptoglobin (Fig. 5). In a similar but reversed experiment, the heme remained fixed to the ferrihemoglobin initially bound to labeled hemes of free ferrihemoglobin did not transfer to or the free and bound hemoglobins were separated. The labeled A to the haptoglobin of normal human serum (Fig. 5). To this when added in near equivalent amounts under the conditions imperfect separation of F and A hemoglobins or to a limited amount of cubin hybrid formation. There was slightly more exchange in mixtures containing oxyhemoglobin and deoxyhemoglobin (7.5% and 5.5%, respectively). This may be attributable in part to the slight autoxidation that occurred during incubation.

Effect of Haptoglobin—The influence of haptoglobin on heme exchange was evaluated by binding 56Fe-labeled ferrihemoglobin A to the haptoglobin of normal human serum (Fig. 5). To this serum, containing bound, labeled hemoglobin, was added an excess of free, unlabeled ferrihemoglobin A. After incubation, the free and bound hemoglobins were separated. The labeled heme remained fixed to the ferrihemoglobin initially bound to haptoglobin (Fig. 5). In a similar but reversed experiment, the labeled hemess of free ferrihemoglobin did not transfer to or exchange with the unlabeled ferrihemoglobin initially bound to haptoglobin.

Studies with "Endogenous" Ferrihemoglobins—Ferricyanide has been shown to react specifically and quantitatively with heme when added in near equivalent amounts under the conditions used (19, 20). Nevertheless, experiments were performed with "natural" ferrihemoglobin A obtained from a patient with congenital methemoglobinemia secondary to a deficiency of the NADH-linked methemoglobin reductase (diaphorase). For evaluating the effect of natural ligands for ferrihemoglobin, hemoglobin was also obtained from a patient who had comparable concentrations of ferrihemoglobin as the result of an inherited anomaly, hemoglobin MBoston, Homoglobin M Boston comprises about 20% of the patient's total hemoglobin, and possesses normal β chains but structurally abnormal α chains, the hemess of which are predominantly in the oxidized state. The substitution of tyrosine for histidine on Residue 58, directly opposite the heme moiety, is believed to permit formation of an internal ligand which interferes with ferriheme reduction (21). These two spontaneous ferrihemoglobins, one normal and one abnormal, were admixed with 56Fe-ferrihemoglobin F and were compared with ferrihemoglobin A produced in vitro with ferricyanide. As shown in Table I, 56Fe-heme transferred similarly from ferrihemin F to ferrihemoglobin A, whether the latter was generated spontaneously in vivo (diaphorase deficiency) or artificially in vitro. Hemoglobin M Boston, on the other hand, took up less of the labeled heme; indeed, there was more transfer of labeled heme to the hemoglobin A present in this patient's blood (Table I), although only a small portion of this was present as ferrihemoglobin. When all of the hemes of the latter patient's hemolysate were converted to ferrihemes by appropriate treatment with ferricyanide, the uptake by hemoglobin M Boston of

![Graph](http://example.com/graph.png)

**Fig. 5.** Effect of haptoglobin on heme exchange. Upper, 56Fe-ferrihemoglobin A was added to normal plasma in an amount that did not exceed the binding capacity of its haptoglobin. Unlabeled (free) ferrihemoglobin A was then added in excess. Lower, unlabeled ferrihemoglobin A was added to normal plasma in amounts sufficient to exceed the haptoglobin-binding capacity. 56Fe-Labeled ferrihemoglobin was then added. These two mixtures were incubated at 37°, pH 7.18, for 100 min, a period during which 46 ± 4% of hemes would exchange among ferrihemoglobins that are unbound to haptoglobin (see text). The haptoglobin-bound hemoglobin and the free hemoglobin were then separated by chromatography on Sephadex G-200. HGB-A, hemoglobin A; HP-HGB A, hemoglobin A bound to haptoglobin.

**Table I**

<table>
<thead>
<tr>
<th>Source of unlabeled hemoglobin</th>
<th>Ferrihemoglobin concentration</th>
<th>Exchange of 56Fe-heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled Hb</td>
<td>Unlabeled Hb</td>
<td>To ferrihemoglobin A</td>
</tr>
<tr>
<td>% hemoglobin fraction</td>
<td>%</td>
<td>%</td>
</tr>
</tbody>
</table>

**Experiment A**

- Normal subject: 17 % 21 % 15 % 15 %
- Diaphorase deficiency: 15 % 21 % 15 % 16 %
- Hemoglobin M Boston: 17 % 21 % 15 % 8%

**Experiment B**

- Normal subject: 86 % 92 % 58 % 28%
- Hemoglobin M Boston: 100 % 92 % 58 % 28%

*Estimated on the basis of the optical density ratio, A562: A480.*
TABLE II
Lack of effect of hemoglobin concentration on rate of heme exchange
Mixtures of equal amounts of "Fe-labeled ferrihemoglobin F and unlabeled ferrihemoglobin A were incubated at 37°, pH 7.18, ionic strength 0.09, for 100 min and then separated by cation exchange chromatography. Percentage of heme exchange was calculated from the specific activities of the isolated hemoglobins, as described in the text.

<table>
<thead>
<tr>
<th>Hemoglobin concentration</th>
<th>Heme exchange %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 g/ml</td>
<td>51</td>
</tr>
<tr>
<td>9.3 g/ml</td>
<td>49</td>
</tr>
<tr>
<td>15.0 g/ml</td>
<td>45</td>
</tr>
<tr>
<td>33.0 g/ml</td>
<td>49</td>
</tr>
</tbody>
</table>

"Fe-heme from methemoglobin F was less than half that by the hemoglobin A derived from the same patient’s blood (Table I). The exchange to hemoglobin M_ Boston that did occur was presumably to the normal β chains of that molecule. In all mixtures involving only partially oxidized hemoglobins A and F, heme transfer was greater than predicted from that observed in mixtures in which about 90% of both hemoglobins was in the ferrihemoglobin form.

Kinetics of Heme Exchange—The rate of exchange of labeled heme groups between ferrihemoglobins A and F was quite consistent, regardless of which hemoglobin was initially labeled. In 12 experiments done on separate days, the mean exchange in a period of 100 min was 46 ± 4% (1 S.D.).

Varying the hemoglobin concentration over a 10-fold range had no effect on the rate of exchange (Table II). This suggests that the rate-limiting step in heme exchange is a first order process. The most straightforward way of representing this would be the following reactions, in which H is heme, H* is labeled heme, GF is the globin of hemoglobin F, and GA the globin of hemoglobin A.

\[
\begin{align*}
H^*G_F & \rightarrow \frac{k_1}{k_2} G_F + H^* \\
HG_A & \rightarrow \frac{k_3}{k_4} G_A + H \\
H^* + G_A & \rightarrow \frac{k_5}{k_6} H^*G_A \\
H + G_F & \rightarrow \frac{k_7}{k_8} HG_F
\end{align*}
\]

It is assumed that no more than one heme group is detached from an intact molecule at a given time. Thus, the symbols \(G_F\) and \(G_A\) represent heme acceptor sites on the respective apoproteins with three of the four heme groups attached. Since the rate of association on between heme and globin \((k_0)\) has been shown to be extremely high (17, 22), the formation of the heme-globin hybrid \((H^*G_A)\) would be limited by the rate of dissociation of the intact molecules \((k_1)\). If it is assumed that ferrihemoglobins A and F are identical in their rates of dissociation, then the following derivation of \(k_1\) can be set up.

Let \(C = \) total concentration of hemoglobin in system; \(F^* = \)

concentration of heme-labeled hemoglobin F at time \(t\); and \(A^* = \)

concentration of heme-labeled hemoglobin A at time \(t\). At time 0, \(F^* = C/2\); at equilibrium, \(F^* = A^* = C/4\). In a first order process

\[-\frac{dF^*}{dt} = \frac{k_1}{2} F^* - \frac{k_2}{2} A^*\]

since (a) rate of change depends not only on loss of label from \(F^*\) but also on gain of label from \(A^*\); and (b) only one-half of the dissociations of labeled heme groups will result in formation of newly labeled hemoglobin.

\[-\frac{dF^*}{dt} = \frac{k_1}{2} \left[ F^* - \left( \frac{C}{2} - F^* \right) \right] = k_1 \left( F^* - \frac{C}{4} \right)\]

On integrating

\[-t = \frac{1}{k_1} \ln \left( \frac{F^* - \frac{C}{4}}{ \frac{C}{2} - F^*} \right) + \frac{1}{k_1} \ln \frac{A^*}{C}\]

Rearranging

\[-t = \frac{1}{k_1} \ln \left( \frac{4F^* - C}{C - 1} \right)\]

The experimental data are most readily expressed as specific activities of originally labeled and newly labeled hemoglobin. The above equation can be restated as

\[-t = \frac{1}{k_1} \ln \left( 1 - \frac{2S.A.} {S.A. F^*} \right)\]

A mixture of equal amounts of "Fe-labeled ferrihemoglobin F and unlabeled ferrihemoglobin A was incubated under standard conditions (37°, pH 7.18, ionic strength, 0.09); at varying times thereafter, samples were withdrawn, the exchange reaction was stopped with cyanide in the cold, and separations were carried out. The percentage of heme exchange was calculated from specific activities as explained earlier. In Fig. 6 the values for (100 – percentage heme exchange)/100 have been plotted as a function of time on a semilogarithmic scale. It is evident that the rate of heme exchange was similar whether the initially labeled hemoglobin was F or A.

If the rate of heme exchange were dependent upon a single dissociation constant common to the two hemoglobins used, the experimental points should follow a straight line function when plotted in the above manner. Instead the points formed a complex curve which appeared to represent two main phases: initially the rate of heme exchange was relatively rapid; after the first 100 min the reaction rate declined considerably. As the two hemoglobins appeared not to differ with respect to the rate of transfer of label, one of several possible explanations for the two (or more) phases was that heme dissociated more readily from one type of globin subunit than from the other, and thus yielded a composite curve defining two different first order processes. For example, the rate of exchange between the α chains of F and A hemoglobins could be quite different than that between the non-α chains. In order to test this hypothesis, hemoglobins F and A were selectively labeled in the α chain with "Fe by subunit hybridization. Analysis of isolated intact α and β chains
The complex kinetics in Fig. 6 may represent a large number of elementary steps and the gradual approach to equilibrium. On the other hand, the points depicted in Fig. 6 may be analyzed in line with the assumption that there are two rate constants. The resultant of two such independent simultaneous first order processes, when plotted semilogarithmically against time, would be a biphasic curve. The initial slope would reflect primarily the rapid exchange of hemes between non-α chains, and the latter slope would approximate exchange between α chains. As would be expected if the different slopes represent the behavior of the two kinds of peptide chains, the intercept of the lower slope at the ordinate is very close to 0.50 (Fig. 6). As each slope in Fig. 6 represents half of the hemo of the entire molecule, both were plotted for analysis starting at 0.50 on the ordinate. On regression analysis the slopes were calculated from the data in Fig. 6 to be $2.9 \times 10^{-4}$ and $3.5 \times 10^{-5}$, respectively. The curved solid line in Fig. 6 is the calculated resultant of these two slopes. It is apparent that the observed points fit this curve within the limits of experimental error. Thus, the rate of dissociation of heme from globin for the non-α chains of ferrihemoglobin F and A is estimated to be $2.9 \times 10^{-4}$ sec$^{-1}$, and that for the α chains $3.5 \times 10^{-5}$ sec$^{-1}$. The ratio of these two rates, 8:1, is very close to that of 7:1 that was estimated above on the basis of subunit analysis.

Incubation Conditions—The effects of certain alterations of the incubation medium were explored in the following experiments.

In each experiment temperature, pH, or ionic strength was altered. The other two variables were kept constant (temperature $37^\circ$, pH 7.18, ionic strength 0.09). The rate of heme exchange was quite dependent upon temperature. At $28^\circ$, for example, the percentage of exchange was only 17%, compared to 43% at $37^\circ$. We have postulated that the percentage of heme exchange can be expressed in terms of a rate constant for the dissociation reaction as follows: $k_t = \ln \left( \frac{100 - \text{percentage heme exchange}}{100} \right)$. By use of the experimentally obtained values for percentage of heme exchange at varying temperatures ($t = 6000$ sec), a series of $k$ values can be readily obtained. Fig. 8 is an Arrhenius plot relating the negative log of the rate constant to the inverse of the absolute temperature. From these data the activation energy was calculated as 19.8 kcal per mole.

Heme exchange was affected only slightly when pH was varied between 5.8 and 7.2; however, it was enhanced approximately 50% when pH was increased to 7.5 or higher or decreased below 5.8 (Table III). Heme exchange was enhanced only moderately when ionic strength was increased (Table III).

Mixtures of Ferrihemoglobin and Oxyhemoglobin—Although there was negligible heme exchange between oxyhemoglobins, mixtures of ferrihemoglobin A or F and oxyhemoglobin F or A showed considerable exchange. For example, admixing $^{59}$Fe-labeled ferrihemoglobin F and unlabeled oxyhemoglobin A led to 22% exchange of heme in a 100-min incubation, as compared with 51% in a completely oxidized mixture (Table IV). The
combination of labeled oxyhemoglobin F and unlabeled ferri-
hemoglobin A exchanged even more rapidly (35%). Spectro-
photometric analysis of the relative amounts of oxyhemoglobin
and ferrihemoglobin cyanide after chromatographic separation
indicated that considerable electron exchange occurred during
the incubation: about one-third of the original ferrihemoglobin
was reduced to ferrohemoglobin. In addition, there was a small
amount of autoxidation (i.e. a net increase in ferrihemoglobin)
during the process of separation. The electron exchange between

![Fig. 8. Arrhenius plot, relating the negative logarithm of the rate constant for heme exchange to the inverse of absolute temperature. Mixtures containing 59Fe-labeled ferrihemoglobin F (80 μM) and an equivalent amount of ferrihemoglobin A were incubated at pH 7.18, ionic strength 0.09, for 100 min, at varying temperatures. They were then separated by cation exchange chromatography. Heme exchange was calculated from the specific activities of the isolated hemoglobins.](image)

The possibly more extensive oxidation of hemoglobin F in Mix-
trate 3 than of hemoglobin A in Mixture 2 may bear out reports
in the text 35%.

**Table III**

<table>
<thead>
<tr>
<th>pH</th>
<th>Ionic strength</th>
<th>Heme exchange</th>
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<tbody>
<tr>
<td>5.6</td>
<td>0.00</td>
<td>62</td>
</tr>
<tr>
<td>5.9</td>
<td>0.00</td>
<td>36</td>
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<tr>
<td>6.0</td>
<td>0.00</td>
<td>37</td>
</tr>
<tr>
<td>6.4</td>
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<tr>
<td>6.7</td>
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</tr>
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<td>51</td>
</tr>
<tr>
<td>7.2</td>
<td>0.30</td>
<td>57</td>
</tr>
</tbody>
</table>

**Table IV**

**Heme exchange and electron transfer in mixtures of ferrihemoglobin F and oxyhemoglobin A.**

Equal amounts (80 μM) of 59Fe-labeled hemoglobin F and un-
labeled hemoglobin A were incubated at pH 7.18, ionic
strength 0.09, for 100 min and then separated by cation exchange
chromatography. Prior to admixture, one or both of the hemog-
lobins were in some cases (indicated below) oxidized with
K₃[Fe(CN)]₆, and then dialyzed against the incubation buffer
overnight at 4°C. Percentage of heme exchange was calculated from the specific activities of the isolated hemoglobins as described in the text.

<table>
<thead>
<tr>
<th>Hemoglobin mixture</th>
<th>Ferrihemoglobin concentration</th>
<th>Increment in ferrihemoglobin</th>
<th>Transfer of e⁻</th>
<th>Heme exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before incubation</td>
<td>After incubation</td>
<td>% mixture</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>F</td>
<td>A</td>
<td>F</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
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<td>2</td>
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<td>2</td>
<td>0</td>
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<tr>
<td>4</td>
<td>80</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* A minimal value representing the percentage of the original ferrihemoglobin fraction reduced during incubation (see text).

the hemoglobins A and F was gradual rather than instantaneous.
Sequential sampling of mixtures of oxyhemoglobin A and ferri-
hemoglobin F indicated that complete oxidation-reduction equil-
ibrium was not reached for at least 60 min, although there
was considerable variability in these determinations. In mix-
trates of ferrihemoglobin A and oxyhemoglobin F (Mixture 3 in Table IV), at least 30% of the former was reduced during incub-
ulation, albeit there was a net increase in the ferrihemoglobin
levels of the mixture of 14%. With mixtures of oxyhemoglobin A and ferrihemoglobin F (Mixture 2 in Table IV), 28% of the latter
was reduced despite a net increase in ferrihemoglobin of 11%.

The possibly more extensive oxidation of hemoglobin F in Mix-
trate 3 than of hemoglobin A in Mixture 2 may bear out reports
(23) of the slightly greater oxidizability of hemoglobin F. How-
ever, if one estimates electron transfer during the incubation
period by the increment in ferrihemoglobin in the fraction
initially lacking ferrihemoglobin, and then subtracts the incre-
ment of ferrihemoglobin observed in the mixture (autoxidation)
entirely from the originally reduced hemoglobin, the result is the
same: 28% in Mixture 2 and 30% in Mixture 3. With either
calculation, it appears that heme exchange was roughly propor-
tional to the transfer of electrons from the ferrohemoglobin
fraction to the ferrihemoglobin fraction. However, it cannot be
determined from these data whether or not an electron transfer
necessarily precedes each heme exchange in such mixtures.

Heme exchange was enhanced by the addition of N-ethyl-
maleimide. Adding sufficient N-ethylmaleimide to block the
two "reactive" thiol groups of hemoglobin increased the rate of
exchange by 35%. The addition of 6 moles of N-ethylmaleimide
per mole of hemoglobin had little added effect. On the other
hand, the addition of a 2-fold molar excess of bis-(N-maleimido-
 methyl) ether, a reagent which reduces the physiological disso-
ciation of hemoglobin tetramers into αβ dimers (24), had no
effect on heme exchange.

2 Generously supplied by the United States Rubber Company.
The formation of methemalbumin appears to require the presence of free ferrihemoglobin, a finding that confirms some earlier observations by Nyman (25). Of possibly greater interest, the present studies show that the existence of ferrihemoglobin allows a free exchange of heme groups to take place among hemoglobin molecules as well as between hemoglobin and methemalbumin. As free ferriheme is known to combine with free globin with extreme rapidity (29), exchange among intact molecules of hemoglobin must reflect the extent of ferrihemoglobin dissociation. From analysis of the kinetics of exchange between hemoglobins A and F, there appear to be two rate constants, each of which governs the exchange of half of the hemes. The observations with specifically labeled subunits indicate that these two constants represent the rates of dissociation for both α and non-α chains; however, the non-α chains, β and γ, differ in structure, and thus their respective dissociation constants may not be identical.

As noted in "Results" the labeling of hemoglobin A with 57Fe in reticulocytes was not uniform, there being a high specific activity in the β chains. This is in accord with recent pulse labeling experiments which indicate that α chain synthesis proceeds β chain synthesis (26). Although we were unable for technical reasons to measure the subunit distribution of heme labeling in our preparations of hemoglobin F, presumably this disproportion also pertains here. If so, it would cause a minor degree of error in the estimation of the heme-globin dissociation constants. We have derived a constant for the rate of heme dissociation for both α and non-α chains; however, the non-α chains, β and γ, differ in structure, and thus their respective dissociation constants may not be identical.

It is of interest that, while the presence of ferrihemoglobin appears to be essential to the exchange of heme groups among molecules of hemoglobin, exchange among molecules in mixtures of ferrihemoglobin and oxyhemoglobin (as exist in vivo) is usually as great as, and often greater than, would be predicted from the over-all ferrihemoglobin concentration (Tables I and IV). In such mixtures heme exchange parallels electron exchange. The data do not allow one to determine whether heme exchange between ferrihemoglobin and oxyhemoglobin occurs during a single collision between a dissociated ferriheme and an attached ferroheme, or whether two consecutive interactions are required, the first being an oxidation step involving a ferroheme and a ferriheme (whether attached or not) and the second involving the exchange of two dissociated ferrihemes.

The present studies indicate that, at exchange equilibrium, ferrihemes distribute equally between the hemoglobin and albumin fractions when there is approximately a 15-fold molar excess of the latter. In clinical disorders with plasma hemoglobinemia, there is invariably a marked excess of albumin; at the relatively high plasma hemoglobin concentration of 100 mg per 100 ml, the molar excess of intravascular albumin would be over 49:1. It was found that when excess albumin is admixed with ferrihemoglobin in vitro, free globin is generated as methemalbumin forms; much of this globin is then precipitated in characteristic fashion (27).

The dissociability of ferrihemes from globin could be predicted as a possibility because of the ionic nature of the bond with globin. The blocking effect of a ligand such as cyanide conforms to this explanation, for it converts the heme-globin linkage into a more covalent bond, as indicated by a reduction of magnetic moment (3). The ready dissociability of the heme groups of ferrihemoglobin is not solely due to the ionic nature of its heme-globin bond, however, for the ionically bound hemes of deoxyhemoglobin do not exchange significantly. These two hemoglobins do differ in tertiary structure and in a number of physical and chemical properties. Recently Beychok et al. (28) reported considerable differences in the heme-conferring optical activity of each of the β subunits, as studied by circular dichroism spectra. Their finding that the areas under ellipticity bands in the Soret region and near 260 μm are over 3-fold greater in isolated α chains than in β chains is evidence for a considerably firmer binding of heme to the α chain than to the β chain. It is also of interest that ellipticity in the 260 μm region was about 3 times greater for oxyhemoglobin than for ferrihemoglobin, and that this difference largely disappeared in the presence of cyanide. These studies fit very well with the present evidence that heme dissociation is considerably greater in non-α chains than in α chains, and that it is extensive with ferrihemoglobin but not with oxyhemoglobin or ferrihemoglobin cyanide.

Prior studies by others have shown that certain "unsaturated" apoproteins may remove heme groups from other heme proteins. Yéas and Drabkin (29) suggested the possibility that hemes might exchange among the apoproteins of yeast cytochromes. Subsequently, Rossi-Fanelli and Antonini (30) demonstrated "heme transfer" at neutral pH between two types of apoproteins: for example, both horse apomyoglobin and apohemoglobin were able to remove some heme from Aplysia (sea hare) myoglobin. They also showed that the horse apomyoglobin removes hemes from intact ferrihemoglobin at equimolar concentration. We have found that hemes of human ferrihemoglobins transfer to albumin much more rapidly than those of ferrimyoglobin. Banerjee (17) has used an imidazole ligand to displace heme from hemoglobin III and myoglobins, and from these studies has calculated apoprotein-hematin association constants. Teale (31) has studied heme-globin equilibrium by the use of a fluorescent label. These studies all require the use of an artificially prepared apoprotein or ligand which serves as a heme acceptor. Our data show exchange of heme groups between intact hemoglobin molecules under physiological conditions.

Interestingly, the sulphydryl-blocking compound, N-ethylmaleimide, greatly enhanced heme exchange. In previous studies (20, 27) blocking globin thiols by exposure to sulphydryl inhibitors, by direct oxidation, or by causing the formation of mixed disulfides with glutathione was found to increase the anodal mobility of hemoglobin; when more than two of the globin thiol groups were blocked, the rate of autooxidation and precipitation of hemoglobin was markedly enhanced (27). Scheler, Graf, and Scheler (32) showed that the incubation of ferrihemoglobin with N-ethylmaleimide increases the rate of parahematin formation. Both the oxidation of globin thiols and the formation of ferrihemoglobin occur during hemolysis by oxidant compounds, and possibly during normal red cell aging (20, 27). During oxidative processes which affect both thiol and heme groups, the two processes appear to be potential in causing heme dissociation and destabilization of the globin, as occurs during the so-called "Heinz body hemolytic anemia" that are associated with exposure to certain oxidant compounds (20) or with certain abnormal hemoglobins. A specific example of this potentiation has been reported recently in patients with hemoglobin S/M (αβθεMes), in whom a congenital Heinz body type of hemolytic anemia is caused by...
an untractable hemoglobin. With the use of the methods described in our preliminary report (2), Jacob, Brain, and Dacie (33) have found that the hemes readily detach from hemoglobin in a spontaneous manner and transfer to hemoglobin in an equilibrium manner at an excessive rate. The instability of the heme-hemoglobin bonds in hemoglobin is manifest only with the ferrihemoglobin form, and is suppressed by cyanide. This instability is accompanied by a heightened oxidation of the 3α cysteine residues, most of which are "blocked" as mixed disulfides of glutathione analogous to those observed during the oxidative precipitation of normal hemoglobin (27).

The fact that hemoglobins exchange hemes, particularly when ferrihemoglobin concentrations are elevated, complicates the interpretation of experimental studies involving the use of hemoglobin labels. For example, the reported (34) separation of partially oxidized hemoglobin by column chromatography is very unlikely, for kinetic reasons discussed by Guidotti, Konigsberg, and Craig (35), to result merely from an equilibrium of symmetrically dissociated dimers. On the other hand, it is probable that both heme exchange and electron exchange allow the formation and accumulation of tetramers consisting of half-oxidized dimers. Another experimental problem created by the phenomenon of heme exchange is that some isotopic studies of hemoglobin synthesis could be seriously misleading, for it is apparent that labeled hemes, free or bound, would undergo redistribution unrelated to biosynthetic incorporation. This nonsynthetic exchange would occur with particular rapidity among the non-α chains.

It should be noted that the reversible dissociation and exchange of hemes in solutions of intact ferrihemoglobin under physiological conditions is a phenomenon quite distinct from the other "physiological" kind of hemoglobin dissociation described by others, in which hemoglobin tetramer dissociates reversibly into pairs of α2 dimers (35-39). The two processes differ in several key respects, as was pointed out by Benesch, Benesch, and Tyuma (40) in discussing a preliminary report (2) of the present studies. (a) Heme exchange (as well as electron exchange among hemes) proceeds comparatively slowly (equilibrium requires several hours), whereas tetramer-dimer equilibrium is reached within a few minutes (37, 38); accordingly, hemoglobin and ferriheme-hemoglobin hybrids can be separated and isolated readily, whereas the rapidly shifting equilibria cause subunit hybrids to disappear in the process of separation (35). As even unlike dimers may recombine to form a hybrid tetramer (39), it is probable that the hybrid \( \alpha \bar{\alpha} \) was present in our mixtures, even though it could not be isolated. (b) The ligand, cyanide, blocks the dissociation and exchange of ferrihemes, but it somehow enhances, rather than inhibits, the dissociation of tetramers into dimers (40). (c) Dimer formation occurs readily in solutions of oxyhemoglobin but is inhibited by previous treatment of hemoglobin with the compound bis-(N-maleimidomethyl)ether (24), for reasons not entirely understood. On the contrary, heme exchange does not occur in solutions of oxyhemoglobin, and heme exchange among molecules of ferrihemoglobin is unimpaired by prior treatment with bis-(N-maleimidomethyl)ether. (d) Finally, dissociation of hemoglobin into dimers is markedly enhanced by dilution, whereas the rate of heme exchange is independent of the concentration of hemoglobin.

Apart from the technical and biochemical considerations above, one may speculate that heme dissociation and exchange may be a critical functional feature of various heme proteins, both with respect to oxidation-reduction and to the mechanisms controlling the rate of heme synthesis. In the latter mechanism, where the concentration of free heme is of crucial importance, the oxidation-reduction state of the heme proteins would markedly affect the levels of dissociated heme, thereby influencing the feedback control of the initial steps in heme synthesis.

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