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Isolation of Intermediate Compounds between Hemoglobin and Carbon Monoxide*

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A human hemoglobin solution partially saturated with carbon monoxide was rapidly quenched at −25 °C into a hydro-organic buffer containing ferricyanide. Under the experimental conditions of pH, ionic strength, and buffer composition used in this work, it was found that the deoxy hemes were rapidly transformed into their met form, whereas practically no carbon monoxide-bound hemes were oxidized before the separation of the mixture from the oxidizing agent. As a preliminary step to the analysis of the resulting solution, carbonylhemoglobin solutions partially oxidized with ferricyanide were studied by isoelectric focusing at −25 °C under identical conditions. The relative position in the gel of all nine possible valence hybrids was established as follows (going from the anodic to the cathodic side of the gel) $\alpha_2^{CO}\beta_2^{CO}$, $(\alpha^{CO}\beta^{+})(\alpha^{CO}\beta^{CO})$, $(\alpha_2^{CO}\beta_2^{+})$, $(\alpha^{+}\beta^{CO})(\alpha^{CO}\beta^{CO})$, $(\alpha^{+}\beta^{+})$-$(\alpha^{CO}\beta^{CO})$, $(\alpha^{+}\beta^{+})(\alpha^{CO}\beta^{+})$, $(\alpha_2^{+}\beta_2^{CO})$, $(\alpha^{+}\beta^{+})(\alpha^{+}\beta^{CO})$, $\alpha_2^{+}\beta_2^{+}$. When carbonylhemoglobin and methemoglobin were mixed in equal proportion at −25 °C and then analyzed by isoelectric focusing at the same temperature, it was found that the contribution of valence hybrids other than $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta^{+}{}_2$ to the total amount of hemoglobin in the gel was no more than 6%. When carbonylhemoglobin and deoxyhemoglobin were mixed in the same proportion and incubated at 20 °C so to allow the redistribution of the carbon monoxide molecules between all possible binding sites to occur, a substantially higher amount of valence hybrids, derived from the oxidation of intermediate compounds of hemoglobin with carbon monoxide, was found.

The isoelectric focusing separation indicated the presence in the original solution of intermediate species other than carbonylhemoglobin and deoxyhemoglobin at a concentration of about 10% of the total.

We have previously reported a technique to obtain electrophoretic and isoelectric focusing separations of macromolecules at −20 to −40 °C (1) and have applied this technique to study the kinetics of formation of the asymmetric carbonylhemoglobin hybrid $(\alpha\beta)^{A}(\alpha\beta)^{C}$[1] from the parent molecules $\alpha_2\beta_2^{A}$ and $\alpha_2\beta_2^{C}$ (2). More recently, we have developed a method to rapidly quench aqueous hemoglobin solutions into a hydro-organic solvent at subzero temperatures and applied it to the identification and isolation of all valence hybrids of different charge between carbonyl- and methemoglobin (3). In the present study, we report the application of the quenching and the isoelectric focusing techniques at subzero temperatures to isolate the intermediate compounds between carbonyl- and deoxyhemoglobin.

The general strategy is as follows. A human hemoglobin solution, in equilibrium with CO at 50% saturation, is rapidly quenched into a cold (−25 °C) hydro-organic solvent containing a suitable concentration of ferricyanide. Under appropriate conditions, the deoxy hemes are rapidly oxidized. To oxidize the liganded hemes, the ligand has first to dissociate from the iron atom. Since Douzou (4) and Mosca *et al.* (5) have shown that the rate of dissociation of CO from hemoglobin is drastically reduced at subzero temperature, the removal of excess ferricyanide can be accomplished in the separation process before an appreciable amount of CO-bound hemes are oxidized. Since each oxidized Fe^{3+} heme carries one extra charge, it is then theoretically possible to separate by isoelectric focusing at low temperatures the intermediate compounds between deoxyhemoglobin and CO originally present in the hemoglobin solution. Our previous work has shown that in the temperature range −25 to −30 °C other slower reactions such as (i) the association-dissociation reactions of hemoglobin dimers and tetramers, (ii) the exchange of hemes, and (iii) the transfer of electrons between hemoglobin molecules (6) are slowed down and do not produce a significant alteration in the distribution of charges or CO molecules.

MATERIALS AND METHODS

Preparation of Hemoglobin—Saline-washed and packed erythrocytes from normal human blood were lysed with an equal volume of distilled water. Ghosts were removed by centrifugation after the addition of solid NaCl to 5% (w/v) concentration. Hemoglobin was stripped of organic phosphates and equilibrated with 0.05 M KCl by gel filtration on Sephadex G-25. Oxyhemoglobin was stored in liquid nitrogen at a concentration of 10 to 15 g/dl.

Deoxygenation of Buffers and Hemoglobin Solutions—Hydro-organic solvents were deoxygenated prior to cooling to subzero temperatures by equilibration with pure nitrogen for 1 h at 20 °C in glass tonometers of varying capacity (3 to 30 ml) (3). Alternatively, to remove residual traces of oxygen, the hydro-organic buffer was shaken in a nitrogen atmosphere in a glass bottle. The gas phase was replaced several times, and dithionite was then added to the buffer (0.05 mg/ml) before storage under a positive pressure of nitrogen. The tonometer was thoroughly purged with nitrogen before being filled with the deoxygenated solvent. Oxyhemoglobin solutions were deoxygenated by equilibration with nitrogen in the same tonometer and traces of oxygen were removed by the addition of dithionite (0.5 mg/ml). Catalase and superoxide dismutase were added to the hemoglobin solution prior to deoxygenation. Deoxygenated hydro-organic solvents were cooled to −20 °C (or lower temperatures) directly in the

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[1] The abbreviation used is $(\alpha\beta)^{A}(\alpha\beta)^{C}$, the hemoglobin asymmetric hybrid obtained from the parent hemoglobins, HbA($\alpha_2\beta_2^{A}$) and HbC($\alpha_2\beta_2^{C}$).

same tonometer, as previously described (3).

Preparation of Hemoglobin Solutions Partially Saturated with CO—A hemoglobin solution from a deoxygenated sample was drawn into a gas-tight Hamilton syringe. The remaining sample was saturated with CO in a tonometer. Unbound CO was removed by a short equilibration with nitrogen before the carbonylhemoglobin solution was drawn into another Hamilton syringe. Anaerobic mixing of appropriate volumes of the solutions contained in the two syringes gave the required degree of CO saturation in the mixed solution. The mixture was then incubated at 20 °C for as long as necessary. The CO saturation of the mixture was determined spectrophotometrically by measuring at 540 and 555 nm the absorbance of 100 μl each of the three solutions, deoxyhemoglobin, carbonylhemoglobin, and their mixture, dissolved in 10 ml of 50 mM phosphate buffer, pH 7.0, in a Thunberg cuvette. The measured CO saturation agreed to within ±2% with that calculated from the mixing ratio of the deoxy- and the carbonylhemoglobin solutions.

Spectrophotometric Measurements at Subzero Temperatures—The rate of oxidation by ferricyanide of samples of deoxyhemoglobin, carbonylhemoglobin, oxyhemoglobin, and of their mixtures, at subzero temperatures, was followed at 630 nm in a Beckman Acta III spectrophotometer fitted with a thermostatted cuvette (5). The procedure was as follows. Samples (4 ml) of phosphate buffer (10 mM) containing 50% (v/v) ethylene glycol, pH 8.5, at −20 °C (4) and ferricyanide were deoxygenated before cooling at subzero temperature. Samples (100 μl) of hemoglobin solutions (10 to 15 g/dl) were injected into the cold buffer under constant shaking. The protein is rapidly dissolved under these conditions (3). Shaking was continued for 40 s to obtain a homogeneous concentration of the solution. After 2 min rest in the tonometer to eliminate nitrogen bubbles entrapped during mixing, the solution was transferred into the spectrophotometric cuvette with a glass syringe thermostatted at the same temperature as the tonometer and the cuvette. The minimum time required to start reading the absorbance of the solution was about 3 min. Anaerobic handling of hemoglobin solutions was carried out by the use of gas-tight Hamilton syringes.

Isoelectric Focusing Separations at Subzero Temperatures—The procedure previously described was followed (3). Gels (copolymers of acrylamide and ethylacrylate, with methylenebisacrylamide as the cross-linker) were prepared in 20% (v/v) ethylene glycol, 15% (v/v) MeOH, 65% (v/v) water containing 3% (w/v) Ampholine, pH 6.0 to 8.0. Catholyte was a 1.6% (w/v) solution of Ampholine (pH range 7 to 9 and 8 to 9.5 in a 1:1 ratio) and anolyte was a 0.23% (w/v) solution of Ampholine, pH 6 to 8, in the same cryosolvent as the gels. Under these conditions, at −25 °C, carbonylhemoglobin focuses about 3 cm from the gel top and the distance of separation between carbonyl- and methemoglobin after about 15 h is 9 ± 1 mm. Better resolution is obtained when focusing is continued for 20 to 24 h (12 ± 1 mm). Although improved resolution can be obtained by the use of other slightly different conditions (3), the Ampholine pH ranges and concentrations described give greater sharpness of the focused protein zones.

Preparation of Hemoglobin Samples for Gel Isoelectric Focusing Separations at Subzero Temperatures—Deoxy- or carbonylhemoglobin solutions (30 μl), or hemoglobin solutions partially saturated with CO (60 μl), were injected into 0.7 ml of cold (−25 °C), deoxygenated, 10 mM phosphate buffer containing ferricyanide and 50% (v/v) ethylene glycol, using gas-tight Hamilton syringes. When volumes in excess of 30 μl were used, the sample was divided into aliquots that were injected separately. The maximum hemoglobin concentration in the hydro-organic solvent was 0.7 mEq of Fe/liter, and the ferricyanide:hemoglobin heme ratio was 5:1 or greater. During ferricyanide oxidation of the hemoglobin, the samples were kept anaerobic by flowing a stream of cold (−25 °C) nitrogen through the tonometer vessel. Samples of cold oxidized hemoglobin solution (10 μl) were transferred within 10 min of quenching onto the prefocused gels as previously described (2).

Photographic Recording of Focused Patterns—Color slides were found to provide the most suitable photographic recording of focused hemoglobin samples. Slides were scanned for a semiquantitative analysis by a Cliniscan apparatus (Helena Laboratories, Beaumont, TX). Scans were carried out at 465 nm, since it was found that at this wavelength the colored bands corresponding to methemoglobin and carbonylhemoglobin were nearly isobestic.

Procedure for the Identification of the Intermediates Isolated as Valence Hybrids—The assignment of various valence hybrids to specific positions in the gel was made by projecting on a screen a color slide showing an electrofocusing separation of met- and carbonylhemoglobin and of all their valence hybrids. A color print of a photograph showing valence hybrids to be identified was then placed on the same screen, and the positions of the methemoglobin and the carbonylhemoglobin bands of the two experiments were aligned by adjusting the projection enlargement. The identification of unknown bands was then made using the position of the identified valence hybrids as reference.

RESULTS

Spectrophotometric Observations at Subzero Temperatures—Fig. 1 shows absorbance values at 630 nm *versus* time of solutions of hemoglobin partially saturated with CO and quenched at −20 °C in a hydro-organic solvent, pH 8.5, containing a 10-fold molar excess of ferricyanide. A sample of deoxyhemoglobin quenched under similar conditions was found to be completely oxidized to methemoglobin within the

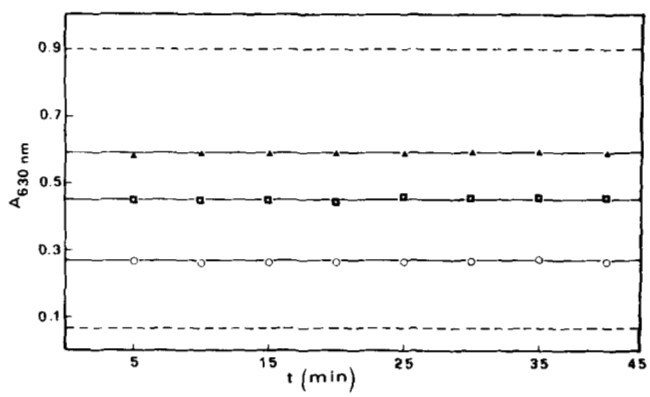

FIG. 1. **Plots of absorbance measured at 630 nm and −20 °C against time when solutions of hemoglobin with different degrees of CO saturation are quenched at subzero temperature in a hydro-organic buffer, pH 8.5, containing a 10-fold molar excess of ferricyanide.** Hemoglobin solutions were equilibrated at 20 °C for 15 to 20 h. ○, hemoglobin at 77% CO saturation; ■, hemoglobin at 53% CO saturation; ▲, hemoglobin at 34% CO saturation. *Broken line*, absorbance of carbonylhemoglobin (*lower line*) and absorbance of deoxyhemoglobin quenched in similar conditions (*upper line*). The absorbance of deoxyhemoglobin oxidized at −20 °C was equal, within experimental error (±2%), to the absorbance of methemoglobin prepared in aqueous medium and similarly quenched at subzero temperature.

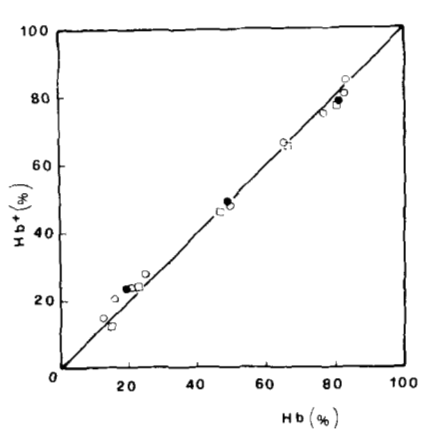

FIG. 2. **Methemoglobin fraction calculated from absorbance values at 630 nm, measured as shown in Fig. 1, plotted against the deoxyhemoglobin fraction of the equilibrated, partially CO-saturated solutions measured before quenching at subzero temperatures in hydro-organic buffer containing ferricyanide.** If the CO-liganded hemes are not oxidized at subzero temperature or during the quenching procedure, a 1:1 relationship should be obtained, as indicated by the *solid line*. □, ferricyanide in a 10-fold molar excess, −20 °C; ○, ferricyanide in a 30-fold molar excess, −20 °C; ●, ferricyanide in a 30-fold molar excess, −30 °C.

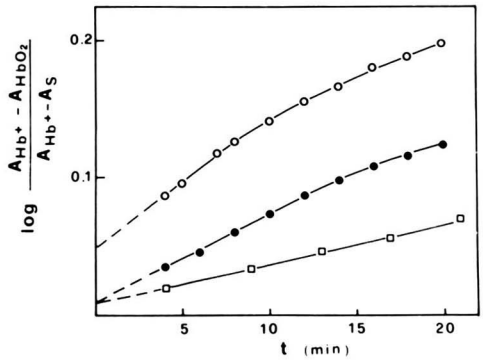

FIG. 3. **Kinetics of oxyhemoglobin oxidation by ferricyanide (30-fold molar excess) at subzero temperatures in hydro-organic phosphate buffer, pH 8.5.** Absorbance of methemoglobin (A_{Hb^+}), oxyhemoglobin (A_{HbO_2}), and partially oxidized oxyhemoglobin solutions (A_S) measured at 630 nm. □, oxyhemoglobin quenched at −20 °C in hydro-organic buffer containing oxygen in equilibrium with air; extrapolation to zero time showed that 4% hemoglobin was oxidized. ○, oxyhemoglobin quenched at −20 °C in deoxygenated buffer; extrapolation to zero time indicated 16% oxidation. ●, oxyhemoglobin quenched at −30 °C in deoxygenated buffer.

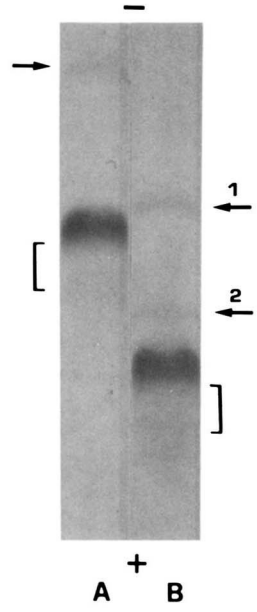

FIG. 4. **Isoelectric focusing separations at −25 °C (15 h) of samples of deoxyhemoglobin and carbonylhemoglobin quenched at the same subzero temperature in hydro-organic buffer containing ferricyanide in a 10-fold molar excess.** *A*, deoxyhemoglobin quenched at pH 8.5. The *arrow* indicates the band corresponding to the hybrid of HbA_0 and HbA_2 in met form. The *bracket* comprises HbA_1 and other minor hemoglobins in met form including products of incomplete deoxyhemoglobin oxidation. *B*, carbonylhemoglobin quenched as in *A*. *Arrow 1* indicates the band corresponding to the hybrid of HbA_0 and HbA_2 in CO form. *Arrow 2* indicates a product of partial oxidation of carbonylhemoglobin by ferricyanide. The *bracket* comprises HbA_1 and other minor hemoglobins in the CO form.

time (3 to 5 min) required for the spectrophotometric observation of the quenched solution. In fact, the absorbance at 630 nm of such a solution equaled that obtained by quenching an equivalent amount of methemoglobin in the same solvent. A solution of carbonylhemoglobin similarly quenched was stable for periods of 1 h or longer. Comparison with the solution of carbonylhemoglobin quenched in the absence of ferricyanide indicated that some oxidation to methemoglobin during the quenching procedure occurred (about 0.5%).

Fig. 2 shows the percentage of hemoglobin oxidized to methemoglobin in rapid quenching experiments, similar to those reported in Fig. 1, *versus* the percentage of deoxyhemoglobin contained in the original sample. The continuous line is the identity line. When the concentration of ferricyanide was increased from a 10- to a 30-fold molar excess over hemoglobin, extrapolation of absorbance values to zero time showed that 3 to 4% carbonylhemoglobin was oxidized.

Fig. 3 shows similar experiments using oxyhemoglobin solutions quenched into a hydro-organic buffer containing a 30-fold molar excess of ferricyanide.

Isoelectric Focusing of Deoxy- and Carbonylhemoglobin Solutions Quenched into a Hydro-organic Solvent Containing Ferricyanide at Subzero Temperatures—Deoxy- or carbonylhemoglobin aqueous solutions were quenched into a hydro-organic solvent, at −25 °C, containing ferricyanide in a 10-fold molar excess over hemoglobin, at pH 8.5. The solutions were then focused at −25 °C as previously described. Photographs relating to such experiments are shown in Fig. 4. Fig. 4*A* shows the pattern obtained for deoxyhemoglobin and Fig. 4*B* that for carbonylhemoglobin.

Isoelectric Focusing of Partially Oxidized Carbonylhemoglobin Solutions Quenched into a Hydro-organic Solvent at −25 °C—An oxyhemoglobin solution was oxidized for 30 min at 0 °C with half-equivalent ferricyanide. The solution

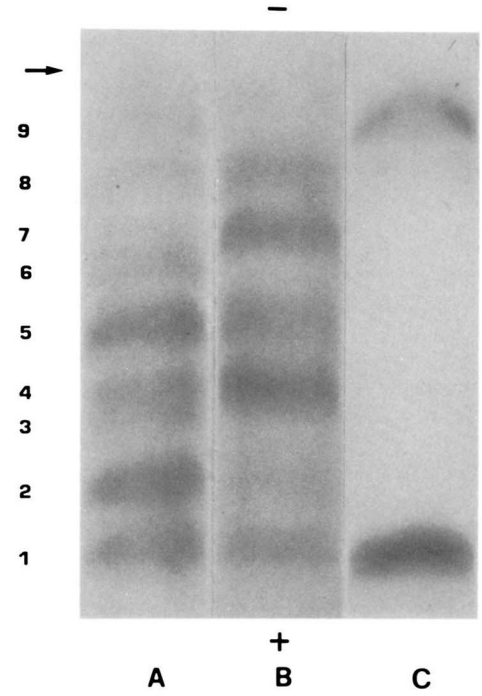

FIG. 5. **Isoelectric focusing separation at −25 °C of samples of partially oxidized carbonylhemoglobin.** *A*, sample obtained by 50% oxidation of oxyhemoglobin with ferricyanide at 0 °C for 30 min before saturation with CO. When this sample was focused at 4 °C, it showed the following composition in symmetrical species: 32% $\alpha_2^{CO}\beta_2^{CO}$, 24% $\alpha_2^+\beta_2^+$, 44% $\alpha_2^+\beta_2^{CO}$ plus $\alpha_2^{CO}\beta_2^+$, of which the latter was present in greater abundance. All nine theoretically predictable components are present and their identification is shown in Table I. The arrow indicates the band corresponding to the hybrid between HbA_0 and HbA_2 in carbonyl form. *B*, sample obtained by 50% oxidation of oxyhemoglobin at 20 °C for 2 h before saturation with CO. When this sample was focused at 4 °C, it showed the following composition: 28% $\alpha_2^{CO}\beta_2^{CO}$, 22% $\alpha_2^+\beta_2^+$, 50% $\alpha_2^+\beta_2^{CO}$ plus $\alpha_2^{CO}\beta_2^+$, of which the former was predominant. Components 3, 6, and 9 are present in too small a quantity to be detected. *C*, methemoglobin and carbonylhemoglobin focused on the same gel tube to allow the correct alignment of the components separated in *A* and *B*.

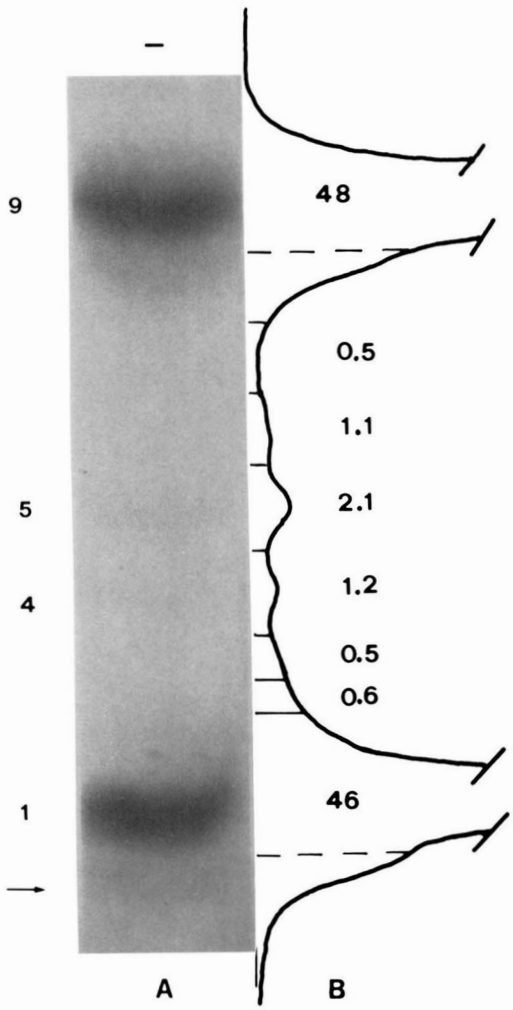

FIG. 6. **Isoelectric focusing separation (−25 °C) of the components of a sample of hemoglobin solution obtained by quenching and mixing in hydro-organic solvent at −25 °C, pH 8.5, containing ferricyanide in a 5-fold molar excess, equal amounts of solution of deoxyhemoglobin and carbonylhemoglobin.** *A*, photograph of a gel tube taken after 24 h of focusing. The separation between methemoglobin (component 9) and carbonylhemoglobin (component 1) is about 12 mm. The only two partially oxidized carbonylhemoglobin species distinctly visible in the slightly colored background are components 4 and 5 (see Table I for identification). *B*, densitometric scan at 465 nm of a color slide of the gel tube shown in *A*. It should be noted that the area of the peak corresponding to methemoglobin is increased compared with that of carbonylhemoglobin; this is due to the presence of a hybrid between HbA_0 and HbA_2 in the CO forms (2 to 3% of total hemoglobin), which focuses together with methemoglobin. HbA_{1C} in CO form is indicated by the *arrow*. In *B*, the *numbers* indicate the relative percentage of each single band.

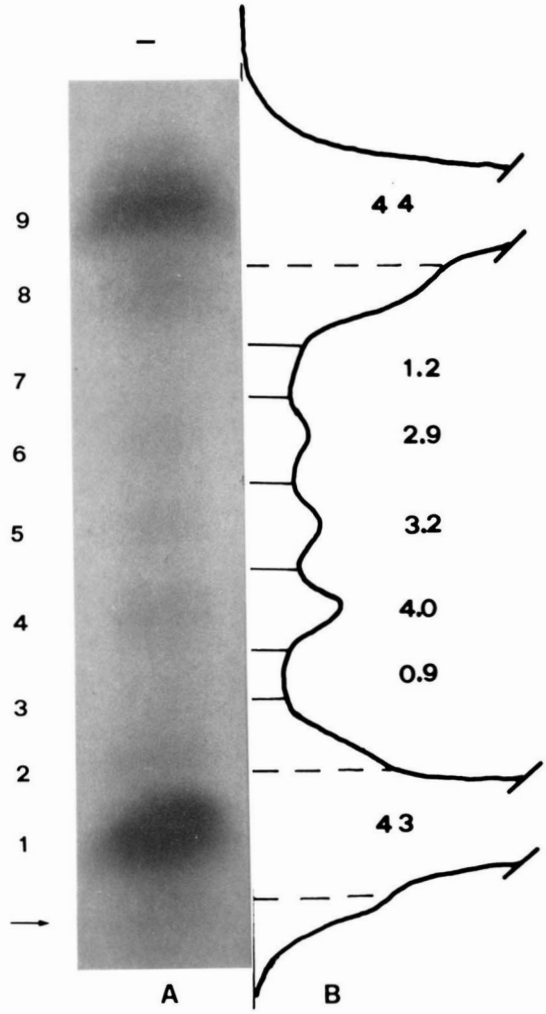

FIG. 7. **Isoelectric focusing separation (−25 °C) of the components of a solution of hemoglobin 50% saturated with CO at 20 °C and quenched in a hydro-organic solvent, as described in the legend to Fig. 6.** *A*, photograph of gel tube taken after 24 h of focusing. All partially oxidized carbonylhemoglobin components identified in Table I are isolated. HbA_{1C} in CO form is indicated by the *arrow*. *B*, densitometric scan of a color slide of the gel tube shown in *A*. Only the bands corresponding to components 4, 5, and 6 give well defined peaks. Other partially oxidized components contribute to raising the base-line of the scan compared with that of Fig. 6*B* or render asymmetric the anodic side of the methemoglobin peak (component 8) and the cathodic side of the carbonylhemoglobin peak (component 2). In *B*, the *numbers* indicate the relative percentage of each single band.

was then saturated with CO to displace all bound oxygen and then quenched into a hydro-organic solvent at −25 °C. Fig. 5*A* shows the pattern obtained by focusing such a solution. Fig. 5*B* shows a similar pattern obtained when the oxyhemoglobin solution was oxidized at 20 °C for 2 h before saturation with CO and then quenched at low temperature. Fig. 5*C* shows the focusing pattern of carbonyl- and methemoglobin separately quenched into the hydro-organic buffer at −25 °C.

Isoelectric Focusing of Hemoglobin Solutions in Equilibrium with CO at 50% Saturation—Equal amounts of deoxyhemoglobin and of carbonylhemoglobin were separately quenched at −25 °C into a hydro-organic solvent containing ferricyanide in a 5-fold molar excess over hemoglobin and then mixed together at −25 °C. The focusing pattern of this mixture separated at −25 °C is shown in Fig. 6. When a solution of deoxyhemoglobin in equilibrium with CO at 50% saturation was similarly quenched and then focused at −25 °C, the pattern of Fig. 7 was obtained.

DISCUSSION

Procedure for Stabilizing the Intermediate Compounds between Hemoglobin and CO

The key problem of any attempt to isolate intermediates in the reaction between hemoglobin and its heme ligands is to develop a separation method that will not alter their relative concentrations. Because of the inherent complexity of this problem, it seems pertinent here to discuss in detail the various steps of the procedure followed in this study.

Stabilization of CO in Its Binding Site—This was accomplished by quenching the hemoglobin solution into a hydro-

organic solution containing ferricyanide, at −25 °C, using the injection technique previously described (3). Separate quenching experiments to be reported elsewhere have shown that under identical conditions of solvent composition and temperature the dead time of quenching the hybridization reaction between HbA_0 and HbC and the hydrolysis of 2,4-dinitrophenyl acetate ranges between 60 and 100 ms. Fig. 2 shows that when a carbonmonoxy-, partially saturated hemoglobin solution is quenched at −25 °C as described, the amount of oxidized hemes found after quenching is equivalent to the amount of deoxy hemes contained in the same sample before quenching. The experimental error in the determination of deoxy- or methemoglobin in this experiment was, at most, ±1.5%.

Fig. 1 shows that after the quenching has occurred the concentration of CO-bound hemes is stable for a long time. Since deoxy hemes are quantitatively oxidized to their met form (Fig. 2), it follows that the total amount of CO-bound hemes before and after quenching is not altered by the quenching procedure. Any alteration in the original pattern of combination between CO and hemoglobin sites would thus require that, during the dead time of quenching, a rapid dissociation of CO from its original site of combination occurs, followed by rapid combination with another site. However, if according to Sharma *et al.* (7), the fastest CO dissociation step from hemoglobin is assumed to have a rate constant of 0.09 s^{-1} ($t_{1/2}$ = 7.3 s), it then follows that during the dead time of the quenching process into the hydro-organic solvent (100 ms) only about 1% of CO bound to any intermediate species would have time to dissociate from hemoglobin. Such conditions are drastically changed in the case of oxygen, as shown in Fig. 3, since the rate of dissociation of O_2 from hemoglobin is several-fold higher than that of CO. The data reported in Fig. 3 clearly show that the oxidation of oxyhemoglobin proceeds at a substantial rate at −20 °C and in the presence of a back pressure of oxygen.

Dimer-Dimer, Heme-Heme, and Electron Exchange Reactions—During the dead time of quenching, or after the partially CO-saturated hemoglobin solutions have been quenched at −25 °C and the deoxy hemes transformed into their met form, exchange reactions between dimers and hemes or electron transfer reactions can alter with time the original pattern of distribution of oxidized or CO-bound hemes between various hemoglobin molecules. Experiments (to be reported elsewhere) have shown that the tetramer-dimer dissociation constant of tetrameric liganded HbA_0 and HbC equals 0.35 s^{-1} at 20 °C. If we assume that the dead time of the quenching procedure is 100 ms, no more than 1.7% hybridization between various intermediate compounds of hemoglobin and CO, which may be present in a partially CO-saturated solution in equilibrium, should thus occur during this time. Obviously, the hybridization reaction during the dead time can only involve rapidly dissociating species such as fully CO-saturated hemoglobin and methemoglobin but not deoxyhemoglobin, for which the rate of tetramer dissociation into dimers is known to be several orders of magnitude slower.

Fig. 6 shows that the amount of hybrid species of the ($\alpha^{CO}\beta^{CO}\alpha^{+}\beta^{+}$) type originating from fully CO-saturated hemoglobin and methemoglobin after the quenching process and during the separation procedure is very small, *i.e.* about 2%. The experiment also shows, confirming our previous observations (3), that no significant heme or electron exchange reactions between methemoglobin and carbonylhemoglobin are likely to occur during the time required for the separation experiments at −25 °C.

We conclude from the available data and from the evidence reported in this paper that (i) during the quenching time CO is stabilized to within ±1.5% saturation in its binding site, (ii) at most, 2% hybridization between various intermediate compounds of hemoglobin with CO may occur during the quenching procedure, (iii) no more than 2% hybridization between the various tetrameric species occurs after quenching or before separation of the valence hybrids, and (iv) no oxidation exceeding 1 to 2% of CO-bound hemes by excess ferricyanide similarly occurs during or after the quenching procedure.

Relative Isoelectric Focusing Positions of the Valence Hybrids of Hemoglobin

If a partially CO-saturated hemoglobin solution is rapidly oxidized, a spectrum of valence hybrids results. Each oxidized heme in such a solution will, in the ideal case, correspond to the same heme in the deoxy state in the original solution. The problem is now to identify, according to their isoelectric points, all possible valence hybrids of hemoglobin present in the quenched solution.

We have previously shown (3) that all valence hybrids formed from rapid dimer-dimer hybridization between the four "stable" symmetrical species $\alpha_2^{CO}\beta_2^{CO}$, $\alpha_2^{+}\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^{+}$, and $\alpha_2^{+}\beta_2^{+}$ can be isolated in electrofocusing experiments at −25 °C. To determine the relative positions of the valence hybrids in the gels used in our separation experiments at −25 °C, it should be kept in mind that the difference in isoelectric point value between methemoglobin and carbonylhemoglobin under the experimental conditions of solvent composition and sub-zero temperature used is only 0.1 pH unit. This makes it difficult to assign a precise isoelectric point value to each hybrid species and to use its determination for their identification. It is simpler instead to identify each hybrid species by matching the pattern of isolated components with that obtained using a standard solution containing all possible hybrids.

The patterns reported in Fig. 5, *A* and *B*, are somewhat similar to the "transient" patterns shown by Park (8) in her pioneer experiments on the isolation of valence hybrid species of hemoglobin. Carbonylhemoglobin (component 1) and methemoglobin (component 9) are readily identified by comparing the patterns of Fig. 5, *A* and *B*, with the pattern shown in Fig. 5*C*. Another species, ($\alpha^{+}\beta^{+}\alpha^{CO}\beta^{CO}$), which focuses symmetrically with respect to $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{+}$, is identified as component 5. Hybridization experiments (3) between $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{CO}$ gave a hybrid species ($\alpha+\beta^{CO}\alpha^{CO}\beta^{CO}$) whose isoelectric point was higher than that of the hybrid species ($\alpha^{CO}\beta^{CO}\alpha^{CO}\beta^{+}$) resulting from $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^{+}$. Thus, the species nearest to $\alpha_2^{CO}\beta_2^{CO}$ in the gel is the hybrid with one oxidized heme on the β chain, *i.e.* $\alpha^{CO}\beta^{CO}\alpha^{CO}\beta^{+}$ (component 2). Application of similar concepts to the hybridization data previously reported (3) led us to identify component 8 of Fig. 5, *A* and *B*, as $\alpha^{+}\beta^{+}\alpha^{+}\beta^{CO}$. The parent symmetrical species of $\alpha^{CO}\beta^{CO}\alpha^{CO}\beta^{+}$ are $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^{+}$. The distance between $\alpha_2^{CO}\beta_2^{+}$ and $\alpha^{CO}\beta^{CO}\alpha^{CO}\beta^{+}$ is thus the same as the distance between the latter and $\alpha_2^{CO}\beta_2^{CO}$. This led us to identify component 3 as $\alpha_2^{CO}\beta_2^{+}$.

Using the same line of reasoning, all the valence hybrids of partial oxidation of HbCO shown in Fig. 5, *A* and *B*, were identified and are shown in Table I. Of all valence hybrids previously reported (Ref. 3, Fig. 6), only $\alpha_1^{+}\beta_1^{+}\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_1^{+}\beta_1^{CO}\alpha_2^{CO}\beta_2^{+}$ have not been separated. They probably appear on the focusing pattern as a single species.

As a confirmation of the identification procedure, it should be noted that in Fig. 5, *A* and *B*, the asymmetrical hybrids present in a greater amount are those which result from the hybridization of the predominent symmetrical species. This explains why two components present in Fig. 5*A* are apparently missing in Fig. 5*B*. They are components 3 and 6. The

TABLE I

Identification of the hemoglobin components of Fig. 5

Component No.	Species or hybrid	Parent species of hybrid
1	$(\alpha_2^{CO}\beta_2^{CO})$	
2	$(\alpha^{CO}\beta^{+})(\alpha^{CO}\beta^{CO})$	$(\alpha_2^{CO}\beta_2^{CO})$; $(\alpha_2^{CO}\beta_2^{+})$
3	$(\alpha_2^{CO}\beta_2^{+})$	
4	$(\alpha^{+}\beta^{CO})(\alpha^{CO}\beta^{CO})$	$(\alpha_2^{CO}\beta_2^{CO})$; $(\alpha_2^{+}\beta_2^{CO})$
5	$(\alpha^{+}\beta^{+})(\alpha^{CO}\beta^{CO})$	$(\alpha_2^{CO}\beta_2^{CO})$; $(\alpha_2^{+}\beta_2^{+})$
6	$(\alpha^{+}\beta^{+})(\alpha^{CO}\beta^{+})$	$(\alpha_2^{CO}\beta_2^{+})$; $(\alpha_2^{+}\beta_2^{+})$
7	$(\alpha_2^{+}\beta_2^{CO})$	
8	$(\alpha^{+}\beta^{+})(\alpha^{+}\beta^{CO})$	$(\alpha_2^{+}\beta_2^{CO})$; $(\alpha_2^{+}\beta_2^{+})$
9	$(\alpha_2^{+}\beta_2^{+})$	

former is the symmetrical species $(\alpha_2^{CO}\beta_2^{+})$, which is present in a very small amount in the aqueous sample, and the latter is its product of hybridization with methemoglobin. The concentrations of these two components at equilibrium are probably too small to be detected.

Effect of Minor Components on the Focusing Patterns of Valence Hybrids

Minor components other than HbA_0 hemoglobin and partially CO-bound or methemoglobins, which may be present in the original sample, deserve comment since their presence can interfere with the identification and quantitation of the partially CO-liganded intermediates. There are three kinds of these minor contaminants: (i) minor hemoglobins present in the blood of normal subjects, such as HbA_2 and HbA_{1C}, (ii) the by-products of the quenching reaction, and (iii) met- or carbonmonoxyhemoglobin present in the hemoglobin preparation.

In Fig. 4*A*, starting from the cathodic side and proceeding toward the anode, such contaminants are identified as the hybrid of HbA_0 and HbA_2 in its met form (indicated by an *arrow*). Completely oxidized HbA_2 focuses in a more cathodic position and cannot be seen in the separation shown. It is interesting to note that the concentration of $(\alpha^{+}\beta^{+})^{A_2}(\alpha^{+}\beta^{+})^{A_0}$ was found to be about twice the original concentration of HbA_2, since most of this minor hemoglobin is in hybrid form. The identification of these hemoglobins was obtained by separate experiments using purified hemoglobin A_2. HbA_{1C}, in met form, focuses slightly below met-HbA_0 on the anodic side, as usually observed in focusing experiments. It was not established whether this minor hemoglobin is isolated as a hybrid with HbA_0. Other subfractions of HbA_1 probably account for the slight smear of colored protein observed on the anodic side. Such components are present in less than 1% concentration, and their position in the gel is indicated by the *bracket* in Fig. 4*A*. This zone also includes products of incomplete deoxyhemoglobin oxidation resulting from the presence of small amounts (<2%) of carbonylhemoglobin and oxyhemoglobin in the deoxyhemoglobin preparation. These products correspond to components 8 and 6 of Fig. 5*A*.

The pattern of Fig. 4*B* refers to carbonylhemoglobin. *Arrow 1* identifies the position of the hybrid between HbA_0 and HbA_2 bound with CO. *Arrow 2* indicates a product corresponding to component 4 in Fig. 5*A* which is derived from the partially oxidized species present in the hemoglobin preparation. To a minor extent, the same component is a product of partial oxidation of carbonylhemoglobin by ferricyanide. The intensity of this band increases with increasing ferricyanide concentration, but remains constant on exposure of carbonylhemoglobin to the oxidant at low temperature. This indicates that some oxidation (<1%) of carbonylhemoglobin occurs during the quenching procedure. The *bracket* identifies the position of HbA_{1C} and other minor hemoglobins in carbonyl form.

Isoelectric Focusing Pattern of Partially Saturated CO-Hemoglobin Solutions

Fig. 6*A* shows the pattern obtained when equal amounts of deoxy- and carbonylhemoglobin are separately quenched at −25 °C into a hydro-organic solvent containing ferricyanide (5-fold molar excess over hemoglobin), mixed together, and then separated as previously described. A corresponding densitometric scan is also shown. The areas under the peaks of carbonylhemoglobin and methemoglobin are approximately the same. Each of them is not symmetrical since the minor hemoglobin HbA_{1C} (perhaps in hybrid form) focuses at the anodic side of the methemoglobin and of the carbonylhemoglobin bands. The asymmetricity of the two peaks is similar, indicating an equal contribution of HbA_{1C} in the two forms. In the slightly colored background between the major peaks, the scan indicates only two other distinct peaks, which correspond to components 4 and 5. The latter component is the hybrid $(\alpha^{+}\beta^{+})(\alpha^{CO}\beta^{CO})$, which is formed from $\alpha_2^{+}\beta_2^{+}$ and $\alpha_2^{CO}\beta_2^{CO}$ during the separation process.

The pattern of Fig. 6 can be considered as a "blank" with respect to the experiment reported in Fig. 7, since it should show (i) all minor contaminants such as HbA_{1C} and HbA_2, which were present in the hemoglobin preparation, (ii) products of partial oxidation of deoxy- or carbonylhemoglobin, and (iii) hybrids formed during the separation procedure. As a blank experiment *it does not include* any hybridization or CO exchange that may occur during the dead time of quenching a partially CO-saturated hemoglobin solution.

Fig. 7 shows the pattern obtained when a 50% CO-bound hemoglobin solution was quenched at −25 °C into a hydro-organic buffer containing ferricyanide in a 5-fold molar excess. The amounts of fully CO-saturated and methemoglobin (corresponding to deoxyhemoglobin in the original solution before quenching) are approximately the same and together add up to more than 80% of the total of all components. The peak of methemoglobin (about 44%) is more skewed on the anodic side than is the peak of carbonylhemoglobin (43%), indicating the presence of component 8 (Table I) in addition to HbA_{1C}. The peak of carbonylhemoglobin (about 43%) is also asymmetric on the cathodic side due to the presence of component 2. It should be stressed that components 2 and 8, which can be distinctly visualized, are not completely resolved by the scanning procedure due to the presence of the large bands of carbonyl- and methemoglobin. Between the peaks of carbonylhemoglobin and methemoglobin three distinct small peaks are identified as components 4 (4%), 5 (3%), and 6 (3%). Components 3 and 7 do not give distinct peaks, but contribute to the enhancement of the base-line of Fig. 7 as compared with Fig. 6. Thus, if no systematic errors have been made and if the assumptions made in this paper are correct, the peaks shown in Fig. 7, corrected for the presence of blank amounts of various components shown in Fig. 6, should give us the original pattern of distribution of the CO molecule in a hemoglobin solution at equilibrium. It should be clear that one blank effect has not been taken into account, *i.e.* that which occurs in the initial dead time of quenching (100 ms). If the data available in the literature and our deductions are correct and no other thus far unidentified rapid reactions are involved, the pattern of Fig. 7 should physically prove the existence of intermediates in nonvanishing amounts in the reaction between hemoglobin and CO.

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