

# The Binding of Carbon Monoxide by Human Hemoglobin

PROOF OF VALIDITY OF THE SPECTROPHOTOMETRIC METHOD AND DIRECT DETERMINATION OF THE EQUILIBRIUM\*

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## SUMMARY

Stoichiometric titrations of human hemoglobin with carbon monoxide show that the interaction of hemoglobin with ligand is accompanied by proportional changes in both the Soret and visible absorption bands. This linearity has been observed in neutral solutions, both in the presence and absence of 2 M NaCl, and in alkaline solutions at pH 9.2. It is concluded that the liganded intermediates taking part in the equilibrium do not differ significantly in spectra from the equivalent hypothetical mixtures of reduced and fully liganded hemoglobin.

The CO equilibrium determined in dilute hemoglobin solutions ( $4.5 \times 10^{-3}$  M to  $2.34 \times 10^{-7}$  M) is characterized by a relatively high Hill constant (2.3) and a free ligand concentration of  $4.3$  to  $5.6 \times 10^{-8}$  M at 50% saturation. The results are of special interest in light of the sedimentation studies and kinetic experiments which indicate that a strongly ligand-linked equilibrium between dimeric and monomeric (or single chain) hemoglobin predominates in this concentration range.

Kinetic and equilibrium studies of the interaction of hemoglobin with ligands have been simplified by spectrophotometric procedures relating the changes in absorption spectrum to the degree of saturation of hemoglobin with ligand (1-3). The validity of the inherent assumption that the spectral changes are directly proportional to fractional saturation has been frequently discussed. Although indirect evidence supports this assumption (4-6), Rifkind and Lumry have recently suggested that non-linear spectral changes are responsible for cases of apparent deviation from the Adair equation (7).

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Proportionality between spectral change and fractional saturation can be readily shown by stoichiometric titration. The following relationship between total ligand concentration ( $X_0$ ) and fractional saturation ( $Y$ ) resulting upon the addition of ligand to a solution of fixed protein concentration ( $P_0$ ) is derived from the empirical Hill equation.  $K$  is the equilibrium concentration of free ligand at 50% saturation and  $n$  is the Hill constant.

$$\frac{X_0}{P_0} = Y + \frac{K}{P_0} \sqrt[n]{\frac{Y}{1-Y}}$$

Whenever  $P_0 \gg K$ , the addition of 0 to 1 mole of ligand per eq of protein gives complete binding of the ligand within the limits of experimental error and  $X_0/P_0 = Y$ . Thus linearity in the stoichiometric plot of change in absorbance against  $X_0/P_0$  is sufficient to establish proportionality.

The CO dissociation curves of whole blood (8, 9) and measurements of the partition constant between CO and O<sub>2</sub> (10) indicate that the affinity of hemoglobin for CO is more than 200 times greater than that for O<sub>2</sub>. Therefore, direct demonstration of proportionality between spectral change and fractional saturation is feasible for the binding of CO by hemoglobin since stoichiometric addition takes place at protein concentrations suitable for spectrophotometry.

There have been, however, no direct determinations of the CO equilibrium for pure hemoglobin. Evaluation of this equilibrium by the spectrophotometric method is possible only at very low protein concentrations similar in magnitude to the binding constants (11). The ligand-binding equilibria in such dilute solutions are of special interest since sedimentation and gel filtration studies (12, 13) show a marked dissociation of ligand-bound hemoglobin into dimers or monomers. In addition, kinetic experiments (14, 15) indicate that there is a strongly ligand-linked equilibrium between dimeric and monomeric (or single chain) hemoglobin.

We show that proportional changes in both the visible and Soret absorption bands accompany the stoichiometric binding of 1 mole of CO per eq of heme residue in human hemoglobin. Correlation of the spectral changes with fractional saturation is

used to evaluate the CO equilibrium in dilute hemoglobin solutions.

#### EXPERIMENTAL PROCEDURE

**Materials**—Human hemoglobin prepared by the toluene method (16) was used within a few days of its preparation. Concentrations, in heme equivalents, were calculated from the absorption spectrum of deoxyhemoglobin with extinction coefficients of  $1.33 \times 10^5 \text{ cm}^2$  per mmole at  $430 \text{ m}\mu$  and  $1.25 \times 10^4 \text{ cm}^2$  per mmole at  $555 \text{ m}\mu$ .

Solutions of CO prepared by equilibration of water with 1 atmosphere of CO at  $20^\circ$  were standardized by stoichiometric titration of either of the isolated  $\alpha$  and  $\beta$  chains (17, 18). The concentrations of CO were always well within 10% of the predicted value of  $10^{-3} \text{ M}$ .

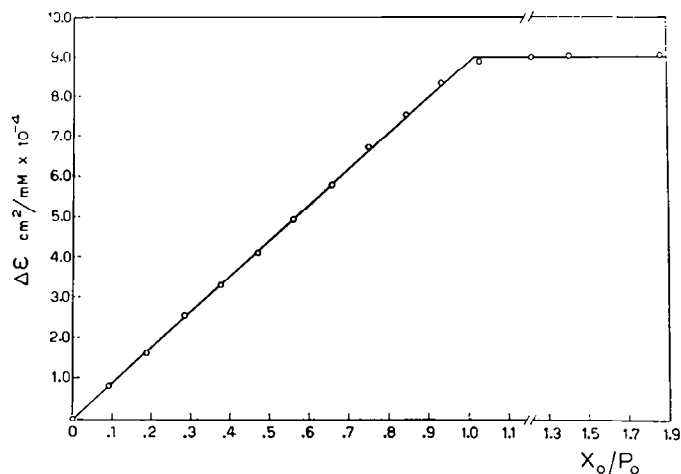


Fig. 1. Stoichiometric titration of human hemoglobin with CO. The changes in molar absorptivity ( $\Delta\epsilon$ ) were measured at  $420 \text{ m}\mu$  as a function of the ratio of total CO concentration to total hemoglobin concentration ( $X_0/P_0$ ). Conditions:  $5.0 \times 10^{-6} \text{ M}$  hemoglobin in  $0.10 \text{ M}$  phosphate, pH 7.0 ( $25^\circ$ ).

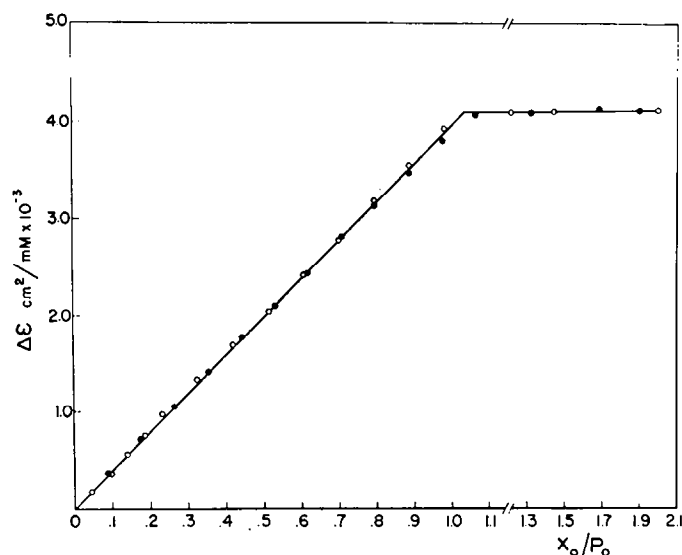


Fig. 3. Stoichiometric titration of human hemoglobin with CO. Changes in molar absorptivity were measured at  $538 \text{ m}\mu$ . Conditions:  $\circ$ ,  $6.9 \times 10^{-6} \text{ M}$  hemoglobin in  $0.05 \text{ M}$  disodium tetraborate, pH 9.18 ( $25^\circ$ );  $\bullet$ ,  $6.38 \times 10^{-6} \text{ M}$  hemoglobin in  $0.10 \text{ M}$  phosphate +  $2 \text{ M}$  NaCl, pH 7.0 ( $25^\circ$ ).

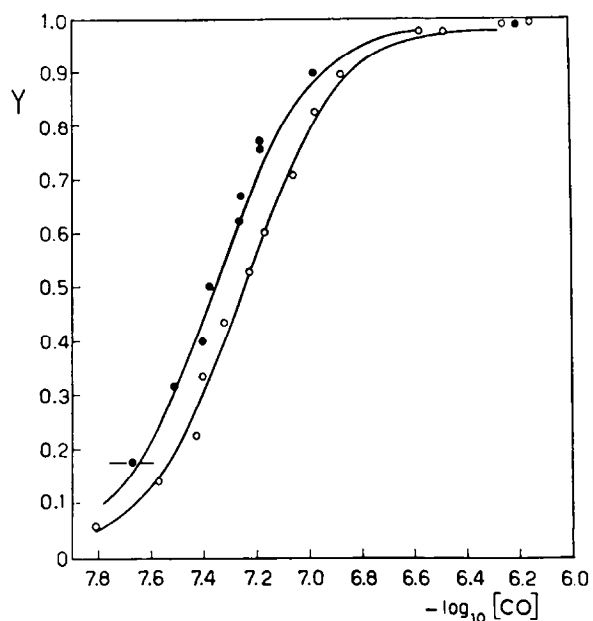


Fig. 4. The carbon monoxide equilibrium of human hemoglobin. Conditions:  $4.5 \times 10^{-8} \text{ M}$  hemoglobin ( $\bullet$ ) and  $2.34 \times 10^{-7} \text{ M}$  hemoglobin ( $\circ$ ) in  $0.10 \text{ M}$  phosphate, pH 7.0 ( $25^\circ$ ). CO is the equilibrium concentration of free CO in moles per liter and Y is the fractional saturation.

Buffers prepared in glass-distilled water from reagent grade chemicals were degassed immediately before use. All solutions contained 0.2 to 0.4 mg per ml of sodium dithionite.

**Procedure for Spectrophotometric Titration**—A quartz cuvette of suitable light path was filled with degassed buffer and closed with a soft plastic stopper bearing a needle for overflow of excess solution. A small glass chip was included for mixing. Solutions of dilute hemoglobin were introduced through the stopper with a syringe. Additions of CO solution were made with a micrometer syringe. After each increment of CO, the cuvette was incubated in the dark in a Colora constant temperature bath.

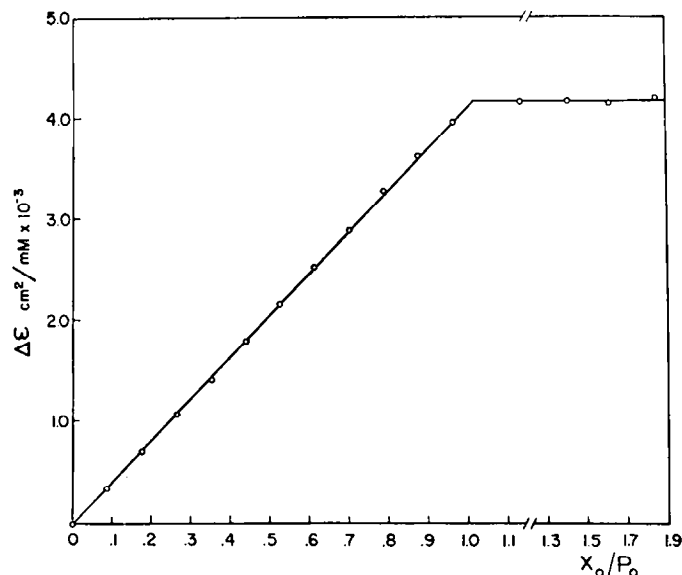


Fig. 2. Stoichiometric titration of human hemoglobin with CO. The changes in molar absorptivity were measured at  $538 \text{ m}\mu$ . Conditions:  $6.71 \times 10^{-6} \text{ M}$  hemoglobin in  $0.10 \text{ M}$  phosphate, pH 7.0 ( $25^\circ$ ).

All experiments were performed in a darkened room with the Cary model 14 spectrophotometer. The spectra were recorded either in the visible or Soret regions. Attainment of binding equilibrium was checked by readings at different time intervals. The constancy of the isosbestic points indicated that there was no loss of protein during the course of an experiment.

#### RESULTS AND CONCLUSIONS

Stoichiometric titrations of hemoglobin with CO, performed in 0.1 M phosphate buffer at pH 7 and 25°, are presented in Figs. 1 and 2. The changes in molar absorptivity plotted as a function of the ratio  $X_0/P_0$  clearly show the stoichiometric binding of 1 mole of CO per eq of heme group with proportional changes in both the Soret (420 m $\mu$ ) and visible (538 m $\mu$ ) absorption bands. The upper limits for deviation from linearity are 2 to 3% at fractional saturations of 0.2 to 0.9 and 5 to 7% at fractional saturations less than 0.2. Measurements at 430, 555, and 568 m $\mu$  confirm this linearity. The small deviations can be explained by experimental error and incomplete binding of CO. From the data presented in the next section, it is estimated that 97 to 98% of the CO was bound under these conditions. The spectra are characterized by well defined isosbestic points in both the Soret and visible regions. Titrations performed in neutral solutions containing 2 M NaCl and in alkaline solutions of pH 9.2 (Fig. 3) indicate that proportionality is maintained under these conditions.

Although more precise measurements might reveal significant small deviations from linearity, it is certain that there are no deviations large enough to cause serious error in equilibria evaluated by spectrophotometric procedures. The assumption that this proportionality also applies to the binding of O<sub>2</sub> is reasonable since the absorption spectra are very closely related. In addition, the changes in the magnetic properties of the heme iron are known to be parallel for the binding of CO and O<sub>2</sub> (19). Stoichiometric titrations of hemoglobin with O<sub>2</sub> at pH 7 would be difficult since protein concentrations greater than 10<sup>-3</sup> M are required.

Carbon monoxide equilibria, determined by the spectrophotometric method in 0.1 M phosphate buffer at pH 7 and 25°, are presented in Fig. 4. The equilibrium observed at a hemoglobin concentration of  $2.34 \times 10^{-7}$  M is described by a Hill constant of 2.3 and a value for  $\bar{K}$  of  $5.6 \pm 1.3 \times 10^{-8}$  M. The uncertainty in  $\bar{K}$  was calculated from the probability of binding and an estimated error in  $Y$  of 4% at 50% saturation (11). The results

obtained at a hemoglobin concentration of  $4.5 \times 10^{-8}$  M correspond to a Hill constant of 2.3 and a value for  $\bar{K}$  of  $4.4 \pm 0.8 \times 10^{-8}$  M. The error in  $Y$  at 50% saturation was estimated to be 6%. Although some dependence on protein concentration cannot be ruled out, these results are not significantly different. The approximate value for  $\bar{K}$  predicted from oxygen equilibria (20) and measurements of the partition constant between CO and O<sub>2</sub> (10) is  $4 \times 10^{-8}$  M at a hemoglobin concentration of 10<sup>-7</sup> M. The Hill constant is remarkably high for a system believed to consist largely of deoxy dimers and liganded monomers (21, 22).

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