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Cooperativity in the Dissociation of Nitric Oxide from Hemoglobin*

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The dissociation of nitric oxide from hemoglobin, from isolated subunits of hemoglobin, and from myoglobin has been studied using dithionite to remove free nitric oxide. The reduction of nitric oxide by dithionite has a rate of 1.4×10^3 M^{-1} s^{-1} at 20° in 0.05 M phosphate, pH 7.0, which is small compared with the rate of recombination of hemoglobin with nitric oxide (25×10^6 M^{-1} s^{-1} (Cassoly, R., and Gibson, Q. H. (1975) *J. Mol. Biol.* **91,** 301–313)). The rate of NO combination with chains and myoglobin was found to be 24×10^6 M^{-1} s^{-1} and 17×10^6 M^{-1} s^{-1}, respectively. Hence, the observed progress curve of the dissociation of nitric oxide is dependent upon the dithionite concentration and the total heme concentration. Addition of excess carbon monoxide to the dissociation mixture reduces the free heme yielding a single exponential process for chains and for myoglobin which is dithionite and heme concentration independent over a wide range of concentrations. The rates of dissociation of nitric oxide from α chains, from β chains, and from myoglobin are 4.6×10^{-5} s^{-1}, 2.2×10^{-5} s^{-1}, and 1.2×10^{-4} s^{-1}, respectively, both in the presence and in the absence of carbon monoxide at 20° in 0.05 M phosphate, pH 7.0. Analogous heme and dithionite concentration dependence is found for the dissociation of nitric oxide from tetrameric hemoglobin. The reaction is cooperative, the intrinsic rate constants for the dissociation of the 1st and 4th molecules of NO differing about 100-fold.

With hemoglobin, replacement of NO by CO at neutral pH is biphasic in phosphate buffers. The rate of the slow phase is 1×10^{-5} s^{-1} and is independent of pH. The amplitude of the fast phase increases with lowering of pH. By analogy with the treatment of the HbCO + NO reaction given by Salhany *et al.* (Salhany, J. M., Ogawa, S., and Shulman, R. G. (1975) *Biochemistry* **14,** 2180–2190), the fast phase is attributed to the dissociation of NO from T state molecules and the slow phase to dissociation from R state molecules. Analysis of the data gives a pH-independent value of 0.01 for the allosteric constant c ($c = K_R/K_T$ where K_R and K_T are the dissociation constants for NO from the R and T states, respectively) and pH-dependent values of L (2.5×10^7 at pH 7 in 0.05 M phosphate buffer). The value of c is considerably greater than that for O_2 and CO.

Studies of the difference spectrum induced in the Soret region by inositol hexaphosphate are also reported. This spectrum does not arise directly from the change of conformation between R and T states.

The results show that if the equilibrium binding curve for NO could be determined experimentally, it would show cooperativity with Hill's n at 50% saturation of about 1.6.

Over 60 years ago Douglas *et al.* (1) were led by the similarity of the equilibrium binding curves of oxygen and carbon monoxide to propose that the dissociation curve of the one gaseous ligand could be converted to that of the other by multiplying the partial pressures by a common factor (Haldane's second law). Roughton (2) has recently demonstrated that for oxygen and carbon monoxide Haldane's second law can only be regarded as an approximation, for he found that the factor for interconverting the two equilibrium curves varied from 170 at the bottom of the curve to 270 at the top.

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Even if Haldane's law were exactly followed the kinetic origins of cooperativity are different for the two ligands since the ratio of the intrinsic rate constants of the 4th and the 1st ligand molecules in an Adair scheme (3) is widely different. The association constants have been compared by Gibson and Roughton (4) who suggested that the ratio of the 1st and 4th intrinsic combination velocity constants was 80 for carbon monoxide and 12 for oxygen. Combination of equilibrium (2, 5, 6) and kinetic data yields estimates for the ratio of the corresponding dissociation velocity constants of 0.125 for CO and 0.025 for oxygen. In other words, cooperativity for oxyhemoglobin is expressed more in the dissociation reactions than in the association reactions (7), while the opposite is true for carbon monoxide.

Much less is known of the NO reactions, but a recent study

of the combination of nitric oxide by Cassoly and Gibson (8) has suggested that the four association constants are identical. That is, the association reactions of nitric oxide do not contribute to the cooperativity of the overall reaction. If nitric oxide binding is cooperative as in the cases of carbon monoxide and oxygen, then any cooperativity must arise from the dissociation reaction.

Because of the high affinity of NO (equilibrium dissociation constant approximately 5×10^{-12} M (4, 9)) equilibrium studies have not so far been possible, and so it is not known if NO binding is cooperative. In this paper we report studies on the dissociation of NO which establish that the reaction is cooperative and which permit comparisons with other ligands.

MATERIALS AND METHODS

Stock solutions of human hemoglobin were prepared as previously described (7) and stripped of organic phosphates (10). The deoxygenated stock hemoglobin solution was stored at 4° and used within 1 week. Crystalline sperm whale myoglobin was purchased from Sigma Chemical Co. and used without further purification. Isolated hemoglobin subunits were the gift of Dr. M. J. McDonald and were prepared by the method of Bucci and Fronticelli (11) with the modifications of Geraci *et al.* (12) and McDonald and Noble (13). The purity of the isolated chains was confirmed by cellulose acetate electrophoresis and complete regeneration of the sulfhydryl groups were confirmed by the *p*-mercuribenzoate titration method of Boyer (14).

Buffers, prepared from reagent grade chemicals, were deoxygenated by bubbling with prepurified N_2. NO and CO gases were obtained from Matheson Gas Products. NO-saturated solutions were prepared by shaking deoxygenated buffer solutions in a tonometer containing 1 atm of nitric oxide gas at room temperature. CO-saturated solutions were prepared by bubbling appropriate buffers with CO gas at room temperature. Sodium dithionite (Manox brand, Hardman and Holden, Miles Platting, Manchester, England) was stored at 0° under a N_2 atmosphere. Sodium inositol hexaphosphate (Sigma Chemical Co., St. Louis, Mo.) was dissolved in water, titrated to pH 7.0 with HCl, and stored at 0° as a 50 mM stock solution.

Concentrations of hemoglobin, myoglobin, and chains were determined with a Zeiss PMQ II or Cary 15 spectrophotometer and calculated on a heme basis using the following extinction coefficients: $\epsilon^{576}_{HbO_2} = 15.2$ mM^{-1} cm^{-1}, $\epsilon^{418}_{HbNO} = 130$ mM^{-1} cm^{-1}, $\epsilon^{419}_{HbCO} = 191$ mM^{-1} cm^{-1}, $\epsilon^{581}_{MbO_2} = 14.6$ mM^{-1} cm^{-1}, $\epsilon^{422}_{MbNO} = 125$ mM^{-1} cm^{-1}, $\epsilon^{423}_{MbCO} = 187$ mM^{-1} cm^{-1}. All pH measurements were made with a Radiometer PHM22 pH meter.

Studies of the dissociation of nitric oxide were performed by diluting a stock nitrosylhemoglobin solution, which was freshly prepared by adding stock deoxyhemoglobin (approximately 3 mM) to a 2-fold molar excess of 2 mM NO solution, to the appropriate concentration in deoxygenated buffer solutions. The dissociation reaction was initiated by mixing 1 volume of a dithionite solution (0.3 to 6%) with 2 volumes of the dilute nitrosylhemoglobin solutions. Dissociation in the presence of carbon monoxide was initiated with a CO-saturated dithionite solution. Reducing the CO concentration to three-fifths had no effect on the result. The reaction mixtures were transferred to a closed cuvette which had been flushed with N_2 and the spectrum of the solution was periodically recorded on a Zeiss or Cary 15 spectrophotometer. Between recordings samples were incubated at 22° in a constant temperature bath. The pH was checked initially and at the end of the experiment. The data are reported as the optical density change which is the optical absorption at a given time during the reaction less the optical absorption at infinite time. The optical absorption at infinite time is calculated from the total heme concentration and the extinction coefficient at the wavelength of observation.

Stopped flow data were obtained using a Gibson-Durrum apparatus interfaced to a small computer as previously described (15).

Spectral differences between partially saturated nitrosylhemoglobin and the same saturation in the presence of 3 mM IHP[1] were recorded with a computer-controlled digital spectrophotometer constructed in the laboratory. The instrument recorded the absolute absorption spectrum of each solution which was introduced in turn into a cuvette remaining fixed in place throughout the experiment. The difference spectra were generated arithmetically from the raw data (80 points/spectrum) and were stored on magnetic tape. The wavelength reproducibility of the spectrophotometer as judged by the differences between successive spectra of oxyhemoglobin A was better than 0.1 Å. The limit appeared to be set by the effects of temperature changes in the room on the monochromator.

[1] The abbreviation used is: IHP, inositol hexaphosphate.

RESULTS

Dissociation of NO from Myoglobin and from Chains—As a preliminary, the reaction of NO with dithionite was examined, following the reaction spectrophotometrically at 380 nm. Dithionite solutions ranging from 1.4 to 115 mM (0.025 to 2%) were rapidly mixed in the stopped flow with 2 mM NO solutions. The observed reaction at each of the dithionite concentrations was first order with respect to nitric oxide, and a plot of the observed first order rates against the concentration of dithionite used gave a linear relation with a slope of 1.4×10^3 M^{-1} s^{-1} at 20°, 0.05 M potassium phosphate, pH 7.0. Dithionite has proven to be a powerful reductant for a number of biologically significant molecules for which a detailed mechanistic study has been presented by Lambeth and Palmer (16). According to their analysis, nitric oxide would appear to be reduced by $S_2O_4^{-2}$ rather than SO_2^-.

The dissociation of NO from isolated subunits of hemoglobin or from myoglobin in the presence of dithionite gives results such as those of Fig. 1, *A* and *B*. The reaction has a decelerating time course which is dependent upon the concentration of dithionite (Fig. 1*A*) and which is heme concentration-dependent (Fig. 1*B*). Analogous results were obtained for β chains and for myoglobin.

The dissociation process could be reduced to a single exponential which was substantially independent of heme and dithionite concentrations (Fig. 2) by the addition of 0.34 mM CO to the reaction mixture. The added CO bound to the free heme sites as judged by spectral changes. The rate of dissociation from α and from β chains was 4.6×10^{-5} s^{-1} and 2.2×10^{-5} s^{-1}, respectively, comparable to the rates determined by Antonini *et al.* (17), while the dissociation rate from myoglobin was 1.2×10^{-4} s^{-1}.

Dissociation of NO from Hemoglobin in Absence of CO—At pH 9.1 the progress of the dissociation in the absence of CO is similar to that described for chains and myoglobin (Fig. 3), *i.e.* the apparent progress of the reaction is dependent on the concentration of dithionite (Fig. 3*A*) and on the total heme concentration (Fig. 3*B*). While the results shown in Fig. 3 were obtained at 430 nm, the dissociation at pH 9.1 was relatively wavelength-independent in the Soret band. At pH 7.0 the dissociation of nitric oxide in the absence of CO could be followed at 442 or 406 nm without spectral complications as discussed below. The time course of the dissociation process at 442 nm using 60 μM hemoglobin and 1% dithionite is shown in Fig. 4. The progress of the dissociation reaction at neutral pH is more rapid than that at pH 9.1 with comparable dithionite and total heme concentrations.

Spectral Studies of Nitrosylhemoglobin—While the dissociation process in the absence of CO at pH 9.1 for hemoglobin, like chains and myoglobin, is relatively wavelength-independent, the wavelength dependence of the reaction at neutral pH is more complicated (Fig. 5). This spectral complication can be resolved by taking into account the IHP-induced difference spectrum reported by Salhany *et al.* (18) for nitrosylhemoglobin.

The nature of this difference spectrum was investigated

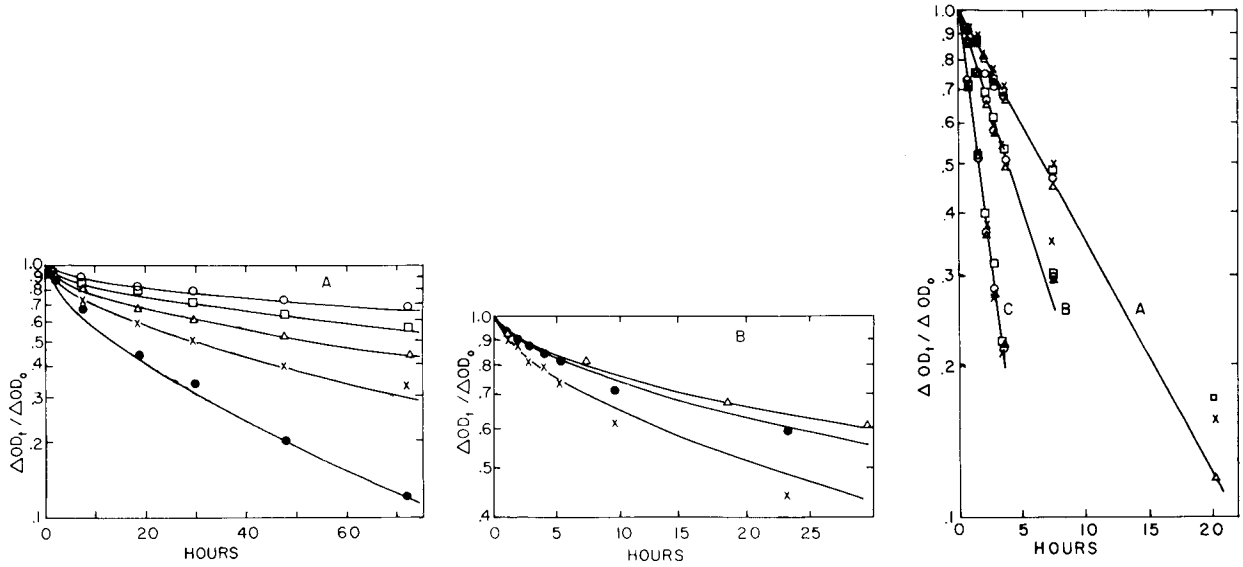


FIG. 1 (*left and center*). Effect of heme and dithionite concentrations on the dissociation of nitric oxide from α chains in 0.05 M potassium phosphate buffer, pH 7.0, at 22°. The progress of the dissociation was followed at 430 nm. In *A*, the concentration of total heme was 55 μM for each of the curves with varying concentrations of dithionite. O, 0.1% dithionite; □, 0.2% dithionite; Δ, 0.4% dithionite; ×, 0.8% dithionite; ●, 2.0% dithionite. In *B*, the concentration of dithionite was 0.4% for each of the curves with varying total heme concentrations. Δ, 51.5 μM heme; ●, 41.9 μM heme; ×, 21.0 μM heme. The *solid lines* are calculated as described under "Discussion."

FIG. 2 (*right*). Dissociation from α chains, from β chains, and from myoglobin in the presence of CO. Dissociation was performed in 0.05 M potassium phosphate, pH 7.0, at 22°. The reaction was followed at 420 nm. The CO concentration was 0.34 mM. O, 2% dithionite, 50 μM heme; Δ, 1% dithionite, 50 μM heme; □, 1% dithionite, 25 μM heme; ×, 0.5% dithionite, 50 μM heme. *Curve A*, α chains; *Curve B*, β chains; *Curve C*, myoglobin.

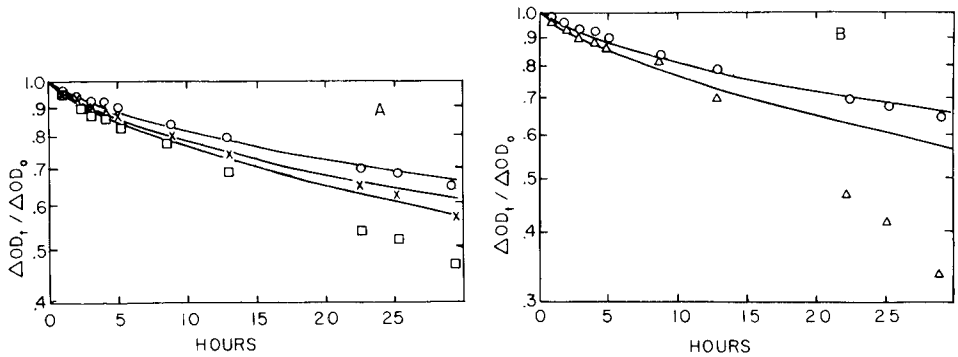


FIG. 3. Effects of dithionite and heme concentrations on the dissociation of nitric oxide from hemoglobin at pH 9.1. The reaction was initiated as described under "Materials and Methods." Dissociation from hemoglobin was performed in 0.05 M sodium borate, pH 9.1, at 22°. The progress of the dissociation was followed at 430 nm. In *A*, the concentration of total heme was 46 μM for each of the curves with varying concentrations of dithionite. O, 1.0% dithionite; ×, 1.5% dithionite; □, 2.0% dithionite. In *B*, the dithionite concentration was 1% for each of the curves with varying total heme concentrations. O, 46 μM; Δ, 23 μM.

through the generation of IHP-induced difference spectra using hemoglobin solutions which were partially saturated with nitric oxide. Addition of 3 mM IHP to the partially saturated hemoglobin solutions in 0.05 M phosphate, pH 7.0, at room temperature produced the difference spectra shown in Fig. 6. The maximal absorption is 27 $mM^{-1}\,cm^{-1}$ (approximately 20% of the extinction of nitrosylhemoglobin at 420 nm). Although the difference spectra appeared to be homogeneous, having an isosbestic point at 406 nm, normalization of the spectra at 420 nm and subtraction from the difference spectrum of the lowest percentage saturation solution gave results which indicated the existence of a second component of the IHP-induced difference spectrum (Fig. 7). While the data points at wavelengths less than 422 nm and greater than 442 nm are largely scattered, falling within the solid lines shown in Fig. 7, the data points between 422 nm and 442 nm form consistent patterns, having a maximum absorption at 432 nm. The maximum extinction for this spectrum is significantly less than that of the previous difference spectrum.

NO Dissociation from Hemoglobin in Presence of CO—In 0.05 M borate, pH 9.1 (Fig. 10) the replacement of NO by CO is independent of dithionite and total heme concentration and is described by a single exponential throughout the time course. The rate of dissociation is $1.4 \times 10^{-5}\,s^{-1}$. As the pH is lowered the dissociation is still found to be independent of dithionite and total heme concentrations; however, the progress of the reaction begins to deviate from a single exponential shown in Fig. 8, *B*, *C*, and *D*, for pH 7.7, 7.15, and 6.8, respectively, with the appearance of an initial rapid phase. The amplitude of the faster phase increases as the pH is decreased, but the slope of the slower exponential phase remains nearly unchanged with change in pH having a rate of approximately $9.5 \times 10^{-6}\,s^{-1}$.

These results were obtained by observation at 420 nm, however similar progress curves were obtained at 406 nm and 442 nm.

DISCUSSION

During the course of the investigation of the dissociation of nitric oxide from hemoglobin in the absence of carbon monoxide at pH values near neutrality, it became apparent that spectral complications such as those shown in Fig. 5 could be attributed to the formation of an intermediate spectrum. The difference between the fully liganded spectrum and the intermediate spectrum is similar to the IHP-induced difference spectrum reported by Cassoly (19) and by Salhany *et al.* (18). We subsequently investigated the nature of the IHP-induced difference spectrum through the use of hemoglobin solutions which were partially saturated with nitric oxide as illustrated in Fig. 6. At all levels of saturation the IHP-induced difference spectrum had a large contribution at 420 nm, an isosbestic point at 406 nm, and a minimal extinction at wavelengths greater than 440 nm. Consequently, kinetic measurements were made at 406 nm and 442 nm. While the intermediate spectrum was prominent at neutral pH in the absence of carbon monoxide, it was absent in dissociation from isolated chains, from myoglobin, and from hemoglobin in the presence of CO, and was negligible in the dissociation from hemoglobin at pH 9.1.

The study of the dissociation of nitric oxide was performed by the addition of dithionite to a solution of nitrosylhemoglobin, giving results such as those shown in Fig. 1, *A* and *B*, for chains. The decelerating time course seen for this simple

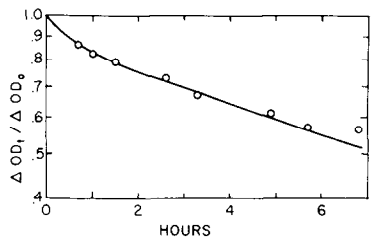


FIG. 4. Dissociation of nitric oxide from hemoglobin at pH 7.0 in the absence of CO. The reaction was initiated as described under "Materials and Methods." Dissociation from hemoglobin was followed in 0.05 M potassium phosphate, pH 7.0, at 22°. The total heme concentration was 60 μM and the dithionite concentration was 1%. The progress of the dissociation was followed at 442 nm. The curve was calculated as discussed under "Discussion."

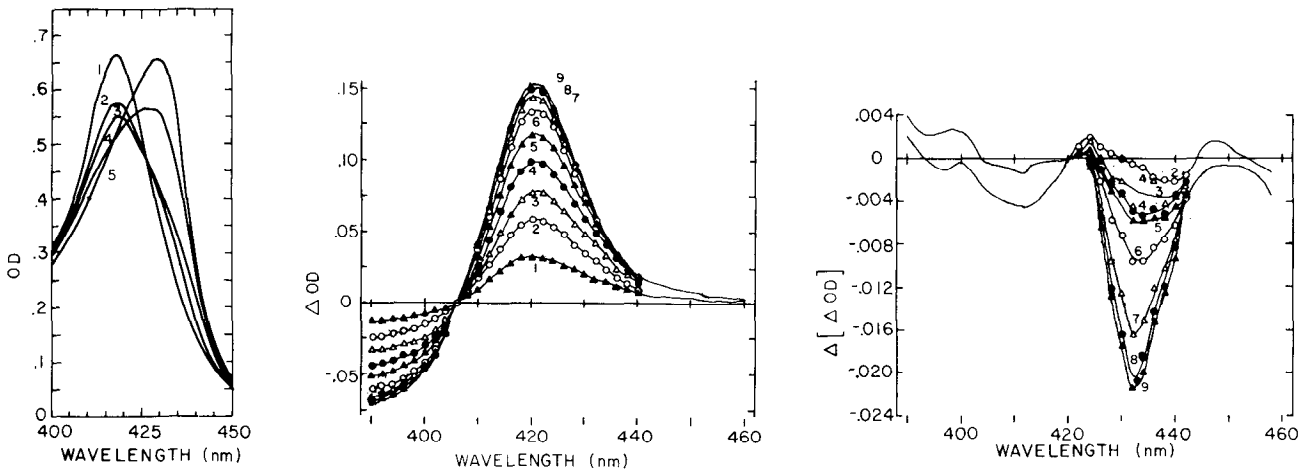


FIG. 5 (*left*). Soret spectrum of dissociation at pH 7.0 in the absence of CO. Conditions were the same as those described in Fig. 4 except that the heme concentration was 55 μM. The spectra were taken at the following time intervals after initiation of the reaction: *Curve 1*, 0 hour; *Curve 2*, 0.5 hour; *Curve 3*, 2.0 hours; *Curve 4*, 5.0 hours; *Curve 5*, 21.25 hours.

FIG. 6 (*center*). IHP difference spectra for partially saturated nitrosylhemoglobin solutions. IHP difference spectra were generated as described under "Materials and Methods." Partially saturated nitrosylhemoglobin solutions were prepared by diluting 1 ml of 3 mM deoxyhemoglobin into 49 ml of anaerobic 0.05 M potassium phosphate solution, pH 7.0, which contained less than saturating concentrations of nitric oxide. These partially saturated nitrosylhemoglobin solutions were divided into two 18.8-ml aliquots. To one aliquot 1.2 ml of anaerobic 50 mM IHP were added; to the other aliquot 1.2 ml of deoxy buffer were added. The final heme concentration was approximately 55 μM. The spectra were run at room temperature. Percentage saturation: *Curve 1*, 17%; *Curve 2*, 30%; *Curve 3*, 40%; *Curve 4*, 52%; *Curve 5*, 62%; *Curve 6*, 70%; *Curve 7*, 88%; *Curve 8*, 97%; *Curve 9*, 100%.

FIG. 7 (*right*). Inhomogeneity in the IHP-induced difference spectra. The spectra in Fig. 6 were normalized to be equal at 420 nm. The differences between the lowest level of saturation and all subsequent partially saturated hemoglobin solutions are plotted as a function of wavelength in the Soret band.

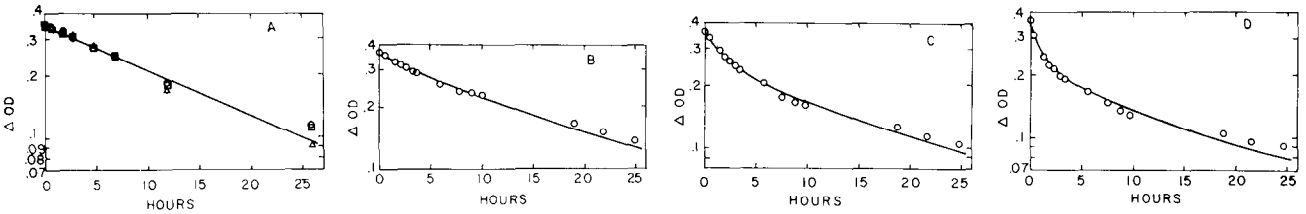


FIG. 8. Dissociation from hemoglobin in the presence of 0.34 mM CO. The reaction was initiated as described under "Materials and Methods." The reaction was performed at 22°. *A*, the conditions were 0.05 M sodium borate, pH 9.1. O, 0.2% dithionite, 56 μM heme; □, 1.0% dithionite, 28 μM heme; Δ, 0.2% dithionite, 56 μM heme; ×, 1.0 dithionite, 28 μM heme. *B*, the conditions were 0.05 M potassium phosphate, pH 7.7, 55 μM heme, and 0.2% dithionite. *C*, the conditions were 0.05 M potassium phosphate, pH 7.15, 55 μM heme, and 0.2% dithionite. *D*, the conditions were 0.05 M potassium phosphate, pH 6.8, 55 μM heme, and 0.2% dithionite. The solid lines in *B*, *C*, and *D* were calculated as discussed under "Discussion."

system cannot be explained by the depletion of reducing agent since the reaction between dithionite and nitric oxide is first order in dithionite and since the initial concentration of dithionite is at least 100 times greater than the initial NO concentration. However, the results may be explained by a competition between dithionite and unliganded hemes for free NO. The association rate of nitric oxide with isolated α chains is the same as that of tetrameric hemoglobin (second order rate constant of 24×10^6 M^{-1} s^{-1}); thus, a concentration of free heme sites of 5 μM and a 1% dithionite concentration would yield comparable probabilities of free nitric oxide reacting with dithionite or recombining with heme. The dissociation process should then be described by the following scheme:

$$\mathrm{HbNO} \underset{j'}{\overset{j}{\rightleftharpoons}} \mathrm{Hb} + \mathrm{NO}$$

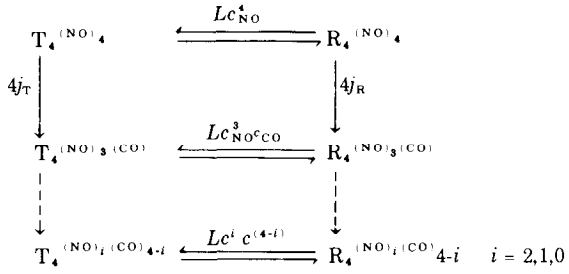
$$DT + \mathrm{NO} \xrightarrow{k} \text{(products)}$$

SCHEME 1

where DT is the concentration of dithionite and the nature of the products of the dithionite reaction is uncertain. This mechanism predicts a dependence of the observed dissociation rate on the concentration of dithionite and on the total heme concentration, and was used to calculate the curves in Fig. 1, *A* and *B*. The rates of dissociation of nitric oxide from α chains, from β chains, and from myoglobin in the presence and in the absence of carbon monoxide are in excellent agreement with expectation. The independence of heme and dithionite concentrations for the dissociation of NO in the presence of CO offers evidence that dithionite reacts only with unbound nitric oxide. The rate of dissociation from tetrameric hemoglobin at pH 9.1 in the presence of carbon monoxide is 1.4×10^{-5} s^{-1}. This rate also describes at least the initial portion of the dissociation curve in the absence of CO (illustrated by the curves in Fig. 3, *A* and *B*, calculated with Scheme 1). When the pH is lowered to pH 7.0, the estimated rate of dissociation in the absence of carbon monoxide (j) based on Scheme 1 is 1.55×10^{-4} s^{-1}.

As the pH is lowered in replacement experiments (Fig. 8), the rate of the slow phase does not deviate significantly from the pH 9.1 rate and at pH 7 the NO replacement rate (9.5×10^{-6} s^{-1}) is approximately 16 times smaller than the value calculated for j in the absence of CO using Scheme 1. Fitting these data to an Adair scheme (3) assuming a statistical relation between the rates for the last 3 molecules of nitric oxide which dissociate and assuming the association reactions to be noncooperative (8), the rate of dissociation of the first nitric oxide molecule is at least 80-fold slower than that for the last NO molecule to dissociate.

The cooperative dissociation of nitric oxide from hemoglobin is consistent with the two-state allosteric model of Monod, Wyman, and Changeux (20). Salhany *et al.* (21) have suggested that fully liganded nitrosylhemoglobin at pH 7.0 contains a significant population of the low affinity (T) quaternary structure under suitable solution conditions, while flash photolysis experiments (22) indicate that fully liganded carbon monoxyhemoglobin is essentially all in the high affinity (R) quaternary structure. In accord with this evidence replacement of nitric oxide by carbon monoxide (Fig. 8) at near-neutral pH would lead to a conformational transition from a mixture of R and T states for nitrosylhemoglobin to all R quaternary structure as the carbon monoxy derivative. Since the relative proportion of fully liganded T quaternary structure present is dependent upon the value of Lc^4 (20) (L = T/R, $c = K_R/K_T$), the value of c for NO (c_{NO}) must be greater than c for CO (c_{CO}) in order to describe the differential population of quaternary structures for fully liganded nitrosyl and carbon monoxyhemoglobin. Fits to the NO replacement experiments (illustrated by the calculated lines in Fig. 8, *B*, *C*, and *D*) to a scheme based on the two-state model (Scheme 2) were obtained by fixing the value of c_{CO} at 0.003 (23), fixing the value of the dissociation rate from the R state



SCHEME 2

(j_R) at approximately 9.5×10^{-6} s^{-1} as estimated from the slow phase of the NO replacement curves, fixing the value of c_{NO} at 0.01 based on the value of j_R and on the value of j_T (approximately 1×10^{-3} s^{-1}) estimated with the use of α- and β-manganese hybrids of hemoglobin (24),[2] and varying the value of L at different pH values. While c_{NO} was found to be pH-independent, the value of L required to fit the replacement reaction was very pH-dependent as is shown in Fig. 9. The scheme provides an alternative explanation for the results of Gibson and Roughton (25) who reported that the rate of replacement of NO by CO was strongly dependent on pH. The change in rate which they observed would thus be due to a change in the allosteric parameter, L, with pH, the rate of dissociation of NO from R and T states being pH-independent.

A linear relation between log L and pH is predicted assuming tight, exclusive binding of protons to the T state. Our results (Fig. 9) yield a slope of -1.7. This is less than the number of 2.4 to 2.8 protons per tetramer which are released upon ligand binding to deoxyhemoglobin (26, 27). The 1.7 protons would be derived exclusively from the quaternary change in conformation, and protons would serve to stabilize the T state. The preferential binding of protons as allosteric effectors would require that release of protons should lag behind the binding of ligands especially in kinetic experiments. However, equilibrium and kinetic evidence (27, 28), shows that the release of protons is linear with ligand binding, and this has been interpreted by correlating proton release with changes in tertiary structure. This paradox may be resolved if there is a relatively greater release of protons during the early stages of binding of ligand, which might arise from unequal effects of ligand binding to α and β chains as was found by Olson and Gibson (28) on binding of *n*-butyl isocyanide to hemoglobin. It is interesting that a recent reinvestigation of the release of protons during equilibrium binding of oxygen has shown saturation to lead proton release at high pH (29), contrary to the earlier reports of Antonini *et al.* (27).

If the values of 2.5×10^7 for L and 0.01 for c are accepted, the IHP-induced difference spectrum in Fig. 6 cannot be explained

[2] M. J. McDonald, E. G. Moore, and Q. H. Gibson, unpublished results.

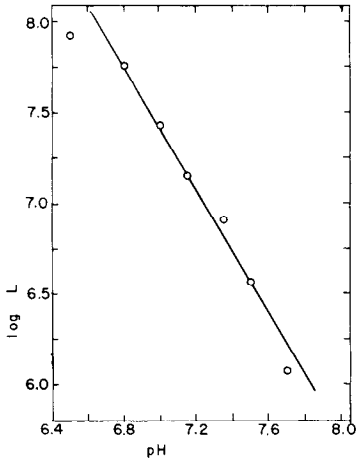


FIG. 9. Relationship between log L and pH. The fitted values of L from the CO replacement experiments shown in Fig. 8 are plotted logarithmically as a function of pH.

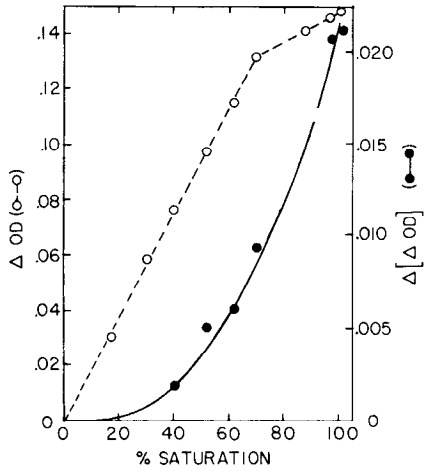


FIG. 10. Change in the difference spectra as a function of percentage saturation. The amplitude of the primary IHP-induced difference spectrum (ΔA) in Fig. 6 represented at 420 nm and the amplitude of the secondary IHP-induced difference spectrum ($\Delta[\Delta A]$) represented at 432 nm are plotted as a function of percentage saturation. The solid line is calculated as described under "Discussion."

as the R and T conformational spectrum. The change at 420 nm on addition of IHP taken from Fig. 6 plotted as a function of percentage saturation gives the dashed line in Fig. 10. The secondary IHP-induced spectrum (Fig. 7), however, gives changes which lag saturation in a way calculable from the two-state allosteric model. The solid line in Fig. 10 is the proportion of R state calculated with these values of the allosteric parameters determined from other experiments. The secondary IHP-induced difference spectrum in Fig. 7 is qualitatively similar to the conformational difference spectrum found for carbon monoxyhemoglobin in flash photolysis studies (22). The spectrum in Fig. 7 was obtained by indirect means, and should not be overemphasized, although it appears to show that such a spectrum exists. The primary IHP-induced difference spectrum (Fig. 6) would appear to be related to the liganded T quaternary state. Analogous spectral changes have recently been found for human oxyhemoglobin A (31, 32) and carp carbon monoxyhemoglobin (31).[3] The IHP-induced change for nitrosylhemoglobin is qualitatively different from those of other liganded derivatives in the Soret band for it appears as a decrease in amplitude in the spectrum of nitrosylhemoglobin instead of a shift in the position of the absorption band (31). However, it seems unlikely that any of these large primary IHP-induced spectral shifts corresponds to the difference spectrum for conformation change of the liganded form from R to T.

The treatment given above, which is quite analogous to that used by Salhany *et al.* (21) in considering the replacement of CO by NO, is successful in representing the experimental results. Some difficulties and paradoxes remain, however, which suggest that the simple two-state model should not be taken too literally. First, the values of L required are substantially higher than earlier estimates. The value at pH 7 exceeds the estimate of Edelstein (30) by 5-fold, and that of Hopfield *et al.* (23) by 15-fold, but Edelstein's method, like that used here, involves the fourth power of a ratio so that the discrepancy is less serious than it might at first appear. Second, and very interestingly, the definition of R and T states on the basis of functional behavior with a ligand seems to depend on the nature of the ligand. As already pointed out by Cassoly and Gibson (8) $Hb_4(NO)_3$ binds NO at a rate appropriate to the T state and CO at a rate suitable to an R state. The experiments in Fig. 8 establish that at pH 7, 0.05 M phosphate, NO dissociates from $Hb_4(NO)_4$ as though the T state was significantly populated. Since, by definition, the T state is $1/c$ times more favored in $Hb_4(NO)_3$ than in $Hb_4(NO)_4$, the paradox of the R-like rate of CO binding to $Hb_4(NO)_3$ is heightened. The R-like rate of CO binding to $Hb_4(NO)_3$ has also been observed in experiments by flash photolysis (33). These results may mean that the reaction mechanism of ligand binding is complex with several rate-determining steps which are different for CO and NO, and which change from R to T, in functional terms, at different points in liganding. If correct, stereochemical factors would enter into the determination of rates indirectly, rather than by simple space-filling considerations, as already suggested by the work of Lakowicz and Weber (34) and Alpert and Lindquist (35) which shows that protein structures in solution offer little obstruction to the diffusion of oxygen, and by analogy, NO and CO also.

Apart altogether from the model used to represent the results, it is clear that the rate of dissociation of NO from partially liganded hemoglobin is substantially (perhaps 100 times) faster than that from the R state $Hb_4(NO)_4$. It follows that the hemoglobin-NO equilibrium, if it was experimentally accessible, would show cooperativity with an n value in Hill's equation of 1.6 at pH 7. The value of n would be less at pH 6 and greater at pH 9 with a well marked Bohr effect. However, since the equilibrium dissociation constant is of the order of 10^{-11} to 10^{-12} M, these statements may enjoy immunity from the test of experiment for some time.

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[3] M. J. McDonald, C. A. Sawicki, and Q. H. Gibson, manuscript in preparation.

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