REACTION OF OXYHEMOGLOBIN WITH CARBON MONOXIDE

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ABSTRACT The reaction of oxyhemoglobin and carbon monoxide was studied kinetically at pH 7.8 in a variety of suspending media. The dielectric constant of the suspending media, as well as the viscosity (and hence the Fick diffusion coefficients), was varied with the use of glycine, glycerol, and sucrose. The results showed that the reaction was unaltered by the various additions to the media, provided that the pO_2 and the concentration of carbon monoxide were held constant. Since the concentration of oxygen varies from medium to medium at constant pO_2 while the pCO varies at constant concentration of carbon monoxide, the differences in the reactions with oxygen and carbon monoxide were emphasized. The lack of variation of the rate constants with changes in dielectric constant can be interpreted as indicating that electrostatic effects are unimportant in this reaction.

INTRODUCTION

In another paper (1) we have submitted evidence that the average rates of the reactions of reduced hemoglobin (Hb) with oxygen (O_2) and with carbon monoxide (CO) are diffusion-independent in certain viscous media and are not altered by the dielectric constant. Roughton (2), Roughton and Millikan (3), Gibson (4, 5), and Gibson and Roughton (6) have shown that the reactions of oxyhemoglobin with carbon monoxide involve the rate constants for the fourth dissociation and association, taking place sequentially as:

$$Hb_4(O_2)_4 \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} Hb_4(O_2)_3 + O_2$$

$$Hb_4(O_2)_3 + CO \underset{l_4}{\overset{l_4'}{\rightleftharpoons}} Hb_4(O_2)_3 \cdot CO$$

$$Hb_4(O_2)_3CO \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} Hb_4(O_2)_2CO + O_2$$

$$Hb_4(O_2)_2CO + CO \underset{l_4}{\overset{l_4'}{\rightleftharpoons}} Hb_4(O_2)_2(CO)_2$$

$$Hb_4(O_2)_2(CO)_2 \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} Hb_4O_2(CO)_2 + O_2$$

$$Hb_4O_2(CO)_2 + CO \underset{l_4}{\overset{l_4'}{\rightleftharpoons}} Hb_4O_2(CO)_3$$

$$Hb_4(CO)_3O_2 \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} Hb_4(CO)_3 + O_2$$

$$Hb_4(CO)_3 + CO \underset{l_4}{\overset{l_4'}{\rightleftharpoons}} Hb_4(CO)_4$$

In each case the rates k_4', k_4, l_4', and l_4 are applicable since the heme groups are supposedly unable to distinguish whether the other three have reacted with an oxygen or a carbon monoxide molecule.

We studied the reaction of oxyhemoglobin (HbO_2) with carbon monoxide in media of varying viscosity and dielectric constant. One may represent the measured average rate constant as the apparent rate, m', for the over-all reaction

$$HbO_2 + CO \overset{m'}{\rightleftharpoons} HbCO + O_2, \tag{1}$$

in which m' is defined by

$$m' = \frac{1}{(HbO_2)\cdot(CO)} \cdot \frac{d(HbCO)}{dt} \tag{1a}$$

and t represents time. Since these reactions were almost all conducted at oxygen concentrations which far exceeded the hemoglobin concentrations, an integrated form of (1a) can readily be developed. According to this scheme, m' is related to the true rate constants by the formula

$$m' = l_4' \cdot \frac{k_4}{k_4'(O_2)}. \tag{2}$$

Our original data (7) on the variation of m' with different media were improperly interpreted. A more extensive series of experiments have now confirmed that the data were valid even though the earlier interpretation was misleading.

As long as only water-like media are used, equation (2) cannot be distinguished from the equation

$$m' = Gl_4' \frac{k_4}{k_4' \cdot pO_2}, \tag{3}$$

in which the reciprocal of the solubility G is defined by

$$G = pO_2/(O_2). \tag{4}$$

If the medium is altered, as was done in the experiments described in this paper, it is not obvious whether the expression $l_4' k_4/k_4'$ should remain constant or $Gl_4' k_4/k_4'$ or neither. Our data support the constancy of the expression $Gl_4' k_4/k_4'$ in spite of large changes in G from one medium to the next.

MATERIALS AND METHODS

For the experiments described in this paper, blood of a cow or of a dog was laked by a 10:1 dilution with deionized distilled water, filtered, and then further diluted with various

solutions, all of which were buffered to pH 7.8 with M/15 phosphate buffers. Further purification of the solutions of hemoglobin had been shown not to affect the results of similar experiments (1) and so was not carried out. With the samples of canine blood, complete absorption spectra were recorded in a Beckman DK-2 spectrophotometer from 700 to 380 mμ. These records, by comparison with the spectra of Lemberg and Legge (8), showed no measurable methemoglobin. The samples of hemoglobin were discarded no later than 48 hours after removal of the blood from the dog and were kept refrigerated except immediately before use.

The chemicals used for the buffer and for the suspending media were all cp grade. Data on the solubility of oxygen in solutions of sucrose and glycerol are published elsewhere (9, 10), as are data on the variation of dielectric constant in these media (9, 11). The dielectric constant, d, for solutions containing glycine was found from the formula (12)

$$d = d_0 + 21\ C,$$

in which d_0 is the dielectric constant in the absence of glycine and C is the concentration of glycine in moles per liter. The solubility of CO in buffered glycerol solutions was measured by means of the infrared gas analyzer.[1] The results are shown in Table I.

TABLE I

CO AND O_2 SOLUBILITY IN BUFFER SOLUTIONS AT 25°C

Glycerol w/w	Viscosity cp	Equil. conc.*‡, pCO = 1 atm. STP	Equil. conc.*§, pO_2 = 1 atm. STP
per cent		*mM*	*mM*
0	1.1	1.00	1.25
54.3	6.0	0.72	0.56
68.0	17.8	0.57	0.46
80.0	48.0	0.45	0.36
84.3	77.1	0.39	0.34

* Measured in 0.01 M phosphate buffer pH 7.4.

‡ Unpublished data of R. Forster, R. Coburn, and R. L. Berger.

§ Unpublished data of J. Jordan, R. L. Berger, and R. A. Javick.

The experiments with bovine hemoglobin reported herein made use of changes in optical density in a Beckman DU spectrophotometer modified in a fashion similar to that described previously (7, 13). Cuvettes were used at low viscosities and a stopped-flow system (1, 14) at all viscosities. For the experiments with canine hemoglobin, a Beckman DK-2 spectrophotometer with temperature-control apparatus was used. Special plastic stirrers made by the engineering section of the Mayo Clinic permitted rapid mixing of viscous solutions within the cuvettes. These are similar to the adder-mixer described by Boyer and Segal (15). One of these is shown in Fig. 1.

The solutions at a partial pressure of oxygen (pO_2) of 0.2 atmosphere were equilibrated with air, while the solutions at pO_2 = 1.0 atmosphere were prepared by bubbling

[1] The help of Dr. R. Forster and Dr. R. Coburn, Department of Physiology, Graduate School of Medicine, University of Pennsylvania, is gratefully acknowledged. They courteously contributed both equipment and time for these measurements.

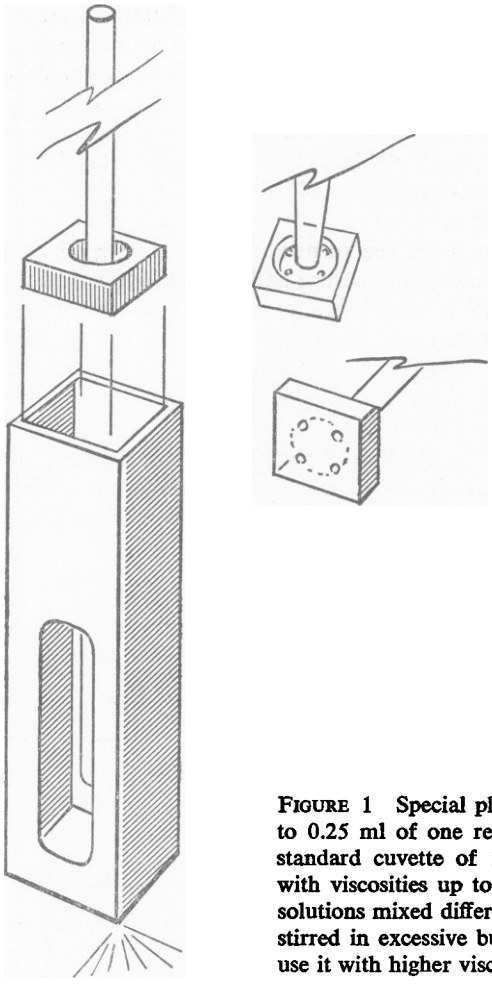

FIGURE 1 Special plastic mixer suitable for adding 0.04 to 0.25 ml of one reactant to another reactant within a standard cuvette of 1 cm^3. Rapid mixing was obtained with viscosities up to 10 centipoise even though the two solutions mixed differed greatly in their viscosities. Mixer stirred in excessive bubbles when attempts were made to use it with higher viscosities.

oxygen for 1 hour. Some minor inconsistencies may have been introduced by using the solutions at $pO_2 = 1.0$ atmosphere in open cuvettes, but the loss of oxygen during the brief experiments appeared very slight.

EXPERIMENTAL RESULTS

For all of the experiments solutions of carbon monoxide were prepared by bubbling carbon monoxide gas through pH 7.8, M/15 phosphate buffer. For the experiments with bovine blood, saturated solutions of carbon monoxide, with $pCO = 1.0$ atmosphere, were diluted with the buffered sucrose medium to give the desired concentration of carbon monoxide. (For the experiments using stopped-flow technics, this was twice the final concentration of carbon monoxide.)

For the experiments with canine blood, less concentrated solutions of carbon

monoxide were used, the pCO ranging between 0.2 and 0.5 atmosphere. Small aliquots of this solution were stirred into solutions of oxyhemoglobin in various suspending media at such a concentration that the final per cent of carbonyl hemoglobin (HbCO) was about 95 per cent of the total concentration of hemoglobin when $pO_2 = 0.2$ atmosphere. A few checks on other concentrations of carbon monoxide verified the validity of equation (1).

The bovine hemoglobin was used for a large number of repetitive determinations of m' at a restricted group of sucrose concentrations. The results of these determinations are presented in Table II; they are compared with the Fick diffusion constant

TABLE II

MEASUREMENT OF m′ IN pH 7.8 BUFFERED SOLUTIONS OF BOVINE OXYHEMOGLOBIN

Concentration sucrose w/w	Viscosity cp	Fick(10) diffusion constant	m'*	(O_2) pO_2 = 0.2 atm. STP	Measured in
per cent		$cm^2/sec.$	M^{-1} *sec.*$^{-1}$	*mM*	
0	1	2.1×10^{-5}	10.6×10^8	0.25	Cuvette
0	1	2.1	10.8	0.25	Flow system
50	15	1.0	10.0	0.09	Cuvette
50	15	1.0	10.8	0.09	Flow system
58	30	0.66	11.8	0.08	Flow system
62	60	0.45	10.3	0.07	Flow system

* For these experiments the initial HbO_2 concentration was 21 μM. The initial CO concentration was 3.1 μM. The variations in m' are almost within the spread of repetitive measurements, namely, $\pm$ 0.8 $\times$ 10^8 M^{-1} sec.$^{-1}$. The depletion of HbO_2 during the reaction was used to compute the value of m' from the half-time of the exponential curves.

for oxygen, which, it is assumed, should be similar to that for carbon monoxide, since the percentage decrease in solubility for CO in glycerol-water solutions is somewhat similar, as Table I shows.

Although the assumption of the similarity of the diffusion constants for oxygen and carbon monoxide is undoubtedly inexact, evidence has been presented (16) that the diffusion constants for hydrogen peroxide and oxygen vary in a similar although not identical fashion. In any case, Table II shows no evidence of any similarity between the variation of the Fick diffusion constant, which decreases about tenfold, and the values of m', which remain constant within experimental error.

Also listed in Table II are the concentrations of oxygen at a pO_2 of 0.2 atmosphere. According to the theory presented in the Introduction, m' might vary inversely with the concentration of oxygen. The data, however, leave little doubt that m' is actually constant and does not vary with the concentration of oxygen as the medium is changed.

A number of alternate explanations were tested. These included: (*a*) that the

original data were wrong; (*b*) that the reaction rate m' was highly dependent on the dielectric constant of the suspending medium; and (*c*) that the original reaction scheme presented in the Introduction was wrong. These explanations were all shown to be false by the results of a series of experiments on canine hemoglobin summarized in Table III. The initial concentration of carbon monoxide was controlled by adding measured amounts of a standardized solution of carbon monoxide in buffered water. For most of the experiments included in the data in Table III, the initial concentration of carbon monoxide was about 30 μM, and the initial concentration of oxyhemoglobin was 3 μM. A control series of experiments with initial concentrations of carbon monoxide of 1 to 100 μM gave similar results for m'.

In the first column is listed the composition of the suspending medium, in the second its dielectric constant, and in the third and fourth columns, the Fick diffusion constant for oxygen and the concentration of oxygen in a solution, with $pO_2 = 0.2$ atmosphere. The fifth, sixth, and seventh columns give the values of m' determined at pO_2 of 0.2 and 1.0 atmosphere.

TABLE III

MEASUREMENTS OF m' IN BUFFERED SOLUTIONS OF CANINE OXYHEMOGLOBIN*

Concentrations and viscosities	Dielectric constant (9)	Fick(10) diffusion constant	(O_2) pO_2 = 0.2‡	(CO) pCO = 1.0‡	m'§ M^{-1} sec.$^{-1}$($\times 10^{-8}$) Dog *A* pO_2 = 0.2‡	Dog *B* pO_2 = 0.2‡	Dog *B* pO_2 = 1.0‡
		cm^2/sec.	mM	mM			
Buffer	78	2.1×10^{-5}	0.25	1.00	16	10.9	2.0
0.5 M glycine	89	2.0	0.25||	1.00||	15		
1.0 M glycine	100	2.0	0.25||	1.00||	19	12.0	2.5
50 per cent glycerol 6 cp	69	1.2	0.11	0.72	18	12.7	2.5
50 per cent glycerol + 0.5 M glycine	80||	1.2||	0.11||	0.72||	16		
40 per cent sucrose 6 cp	68	1.3	0.11	0.72||	19	12.7	2.9
40 per cent sucrose + 0.5 M glycine	79||	1.3||	0.11||	0.72||	18		

* Measured at pH 7.8. Initial HbO_2 concentration was about 3 μM and initial CO concentration was about 30 μM. These values are based on Beckman DK-2 spectrophotometer records.

‡ In atm. STP.

§ At constant pO_2, for either dog's hemoglobin, all variations of m' were within the range of maximal spread on repetitive measurements. Blood from dog *B* was used to estimate a value for the constant M in water defined by:

$$M = \frac{(HbCO)}{(HbO_2)} \cdot \frac{(O_2)}{(CO)}.$$

This value of 260 ± 50 is within experimental error of Roughton's (2) value for sheep hemoglobin of 215.

|| Estimated value.

A comparison of the last two columns leaves no doubt that m' is inversely proportional to pO_2, thereby supporting the reaction scheme shown in the Introduction. The data with and without glycine can leave little room for doubt that m' is unaltered by major changes in the bulk dielectric constant of the medium. Likewise, the data for widely varying concentrations of oxygen strongly support the original data that m' varies inversely with pO_2 but not with (O_2).

Typical records from the DK-2 spectrophotometer for buffer and for 1M solutions of glycine at pO_2 of 0.2 and 1.0 atmosphere are shown in Fig. 2. It can be noted that the change in optical density resulting from the addition of the same

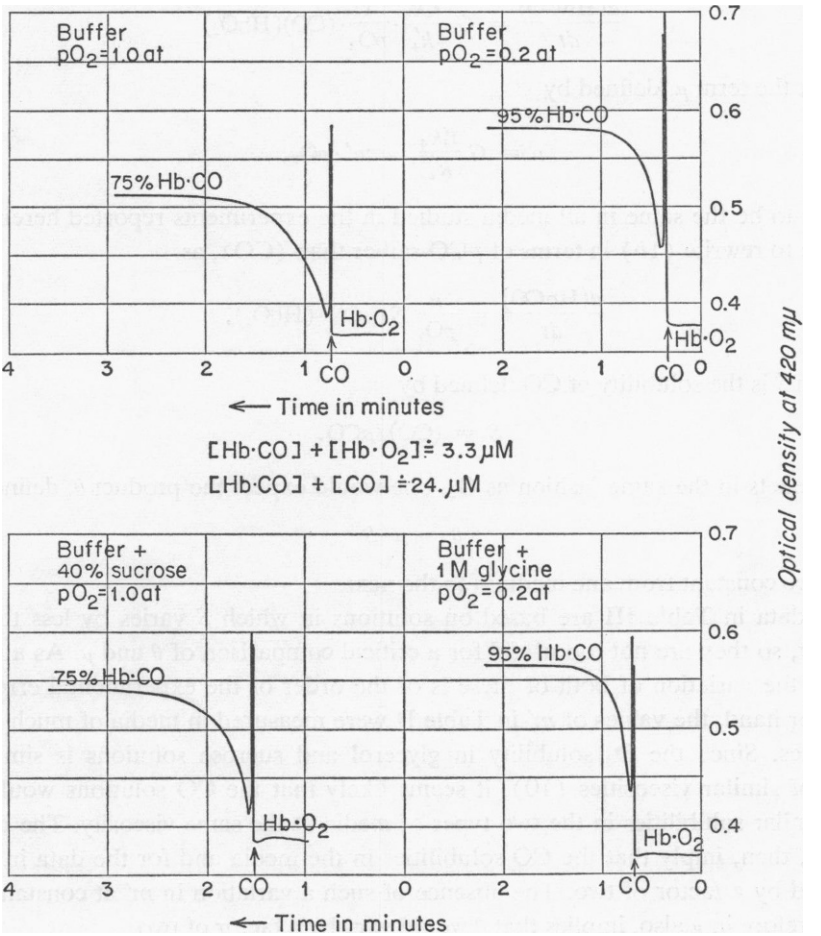

FIGURE 2 Reactions of $Hb \cdot O_2$ with CO. Curves illustrate effect of pO_2 on rate and end point of reaction. Curves also indicate that neither 1 M glycine nor 40 per cent sucrose alters the reaction, provided similar pO_2 values are compared. In all curves 0.04 ml of a buffer solution saturated with CO was added at a stage indicated by arrow.

amount of carbon monoxide is less at a pO_2 of 1 atmosphere than at a pO_2 of 0.2 atmosphere. This is also in accord with the theory presented in the Introduction. It is sometimes concluded from this theory (8) that the ratios of oxyhemoglobin to carbonylhemoglobin should be proportional to the ratio of pO_2 to pCO in the final solution. Thus, if a pO_2 of 0.2 atmosphere leaves 5 per cent of the hemoglobin as oxyhemoglobin, then a pO_2 of 1 atmosphere should leave approximately 25 per cent. The data in Fig. 2 support this type of relationship.

To recapitulate, the discussion so far has indicated that to compare media of different compositions the rate equation (1*a*) may be rewritten

$$\frac{d(\mathrm{HbCO})}{dt} = G \cdot \frac{l_4' k_4}{k_4'} \cdot \frac{1}{pO_2} \cdot (\mathrm{CO})(\mathrm{HbO_2}) \tag{1b}$$

and that the term μ, defined by

$$\mu = G \frac{l_4' k_4}{k_4'} = m' \cdot pO_2,$$

appears to be the same in all media studied in the experiments reported herein. It is possible to rewrite (1*b*) in terms of pCO rather than (CO), as

$$\frac{d(\mathrm{HbCO})}{dt} = \frac{\mu}{pO_2} S \cdot p\mathrm{CO} \cdot (\mathrm{HbO_2}), \tag{1c}$$

in which S is the solubility of CO defined by

$$S = (\mathrm{CO})/p\mathrm{CO}.$$

If CO reacts in the same fashion as O_2, one would expect the product θ, defined by:

$$\theta = \mu \cdot S$$

to also be constant from one medium to the next.

The data in Table III are based on solutions in which S varies by less than 30 per cent, so they are not too useful for a critical comparison of θ and μ. As a matter of fact, the variation of both of these is of the order of the experimental error. On the other hand, the values of m' in Table II were measured in media of much higher viscosities. Since the O_2 solubility in glycerol and sucrose solutions is similar in media of similar viscosities (10), it seems likely that the CO solutions would also have similar solubilities in the two types of media at the same viscosity. The data of Table I, then, imply that the CO solubilities in the media and for the data in Table II varied by a factor of two. The absence of such a variation in m' at constant pO_2, and therefore in μ also, implies that θ would vary by a factor of two.

The interpretation of the above observations is that CO and O_2 react with $Hb_4(O_2)_3$ in different fashions, the CO being constant for equal concentrations in various media, the O_2 for equal partial pressures. In all media the ratio of carbonylhemoglobin to oxyhemoglobin will be given by the equation

$$\frac{(HbO_2)}{(HbCO)} = M_{CO} \frac{pO_2}{(CO)},$$

in which M_{CO} is a constant that is independent of the medium. Note that M_{CO} is different from the constant M usually used:

$$\frac{(HbO_2)}{(HbCO)} = M \frac{(O_2)}{(CO)}.$$

While M_{CO} remains constant from one medium to the next, M does not.

COMMENT

Dependence on pO_2 rather than on (O_2). The result of this study—that the apparent rate constant m' for the reaction of oxyhemoglobin and carbon monoxide varies inversely with the value of pO_2 rather than of (O_2), or the concentration of oxygen—was surprising to us. Both in the average reaction of oxygen with reduced hemoglobin and in the average reaction of carbon monoxide with reduced hemoglobin, the concentrations rather than the partial pressures were the significant variables as the medium was varied. The concentration also appears to be the significant parameter of the carbon monoxide in its reaction with oxyhemoglobin.

In the comparison of standard states in different media, the free energy associated with small gaseous molecules will be constant if the partial pressure is constant. The data presented in the previous paper (1) show that under these conditions the average rate constant for the association reaction

$$Hb + O_2 \xrightarrow{k'} Hb \cdot O_2 \tag{5}$$

varies. On the other hand, with a unit concentration of O_2 in all the standard states, k' remained constant. The reverse reaction

$$HbO_2 \xrightarrow{k} Hb + O_2 \tag{6}$$

should not depend upon pO_2 concentration. Thus it appears that the equilibrium constant

$$K = \frac{k}{k'} \tag{7}$$

should remain constant as the medium is varied, provided that unit concentrations are used for the standard states. The equilibrium constant, K, may be related to the change in partial molal free energies, $\Delta\tilde{F}_0$, by the expression:

$$K = e^{-\Delta\tilde{F}_0/RT}. \tag{8}$$

A constant K implies a constant $\Delta\tilde{F}_0$. The latter is related to the partial molal free energies in the standard state by the expression

$$\Delta\tilde{F}_0 = \tilde{F}_{0,HbO_2} - \tilde{F}_{0,Hb} - \tilde{F}_{0,O_2}. \tag{9}$$

The oxygen partial molal free energy in the standard state referred to unit concentrations varies from one medium to the next since the O_2 solubility at constant pO_2 varies. As $\Delta\tilde{F}_0$ does not seem to vary from one medium to the next, $\tilde{F}_{0,Hb} - \tilde{F}_{0,HbO_2}$ apparently varies in such a way as to compensate for the variations in $\tilde{F}_{0,O_2}$ from one medium to the next.

An alternate hypothesis is that equations (4) to (9) are really incomplete. In particular, a modified form of (4) and (5), such as

$$Hb \cdot (H_2O)_b + O_2 \underset{k}{\overset{k'}{\rightleftharpoons}} HbO_2 + bH_2O \tag{10}$$

in which b is a number which could not be specified from our data, would allow us to conclude that the variations in $\tilde{F}_{0,Hb} - \tilde{F}_{0,HbO_2}$ were not in any simple way tied to the variations in $\tilde{F}_{0,O_2}$. Keilin and Hartree (17), and Haurowitz (18) have presented evidence supporting equation (10).

The dependence of the rate of reaction of CO with HbO_2 on the value of pO_2 could be explained on the above model and reconciled with the k, k' data, provided that we assume that the value of b is different for the fourth oxygen than for the others. A possible scheme for this is illustrated in Fig. 3. Another way of expressing the same ideas is presented in Fig. 4, which shows hypothetical sketches of free energy variations during the several O_2 and CO reactions with Hb and H_2O. Although the scheme presented in equation (10) and expanded in Fig. 3 and 4 would

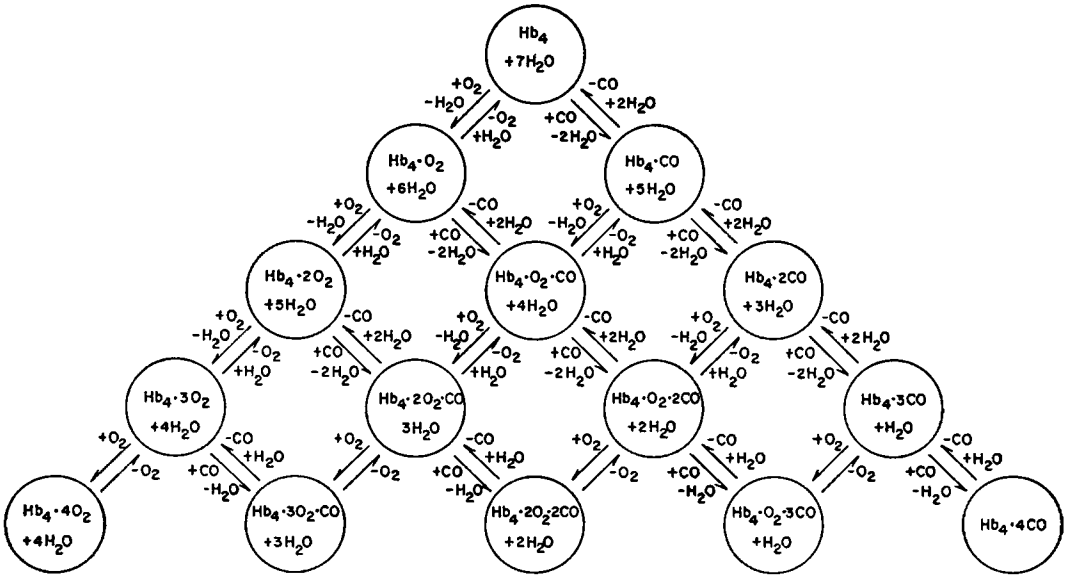

FIGURE 3 Reactions of hemoglobin with carbon monoxide and oxygen. Numbers of water molecules involved fit data in this paper, but any multiple of these numbers would do so also. Lowest row shows steps important in reaction of CO with $Hb \cdot O_2$.

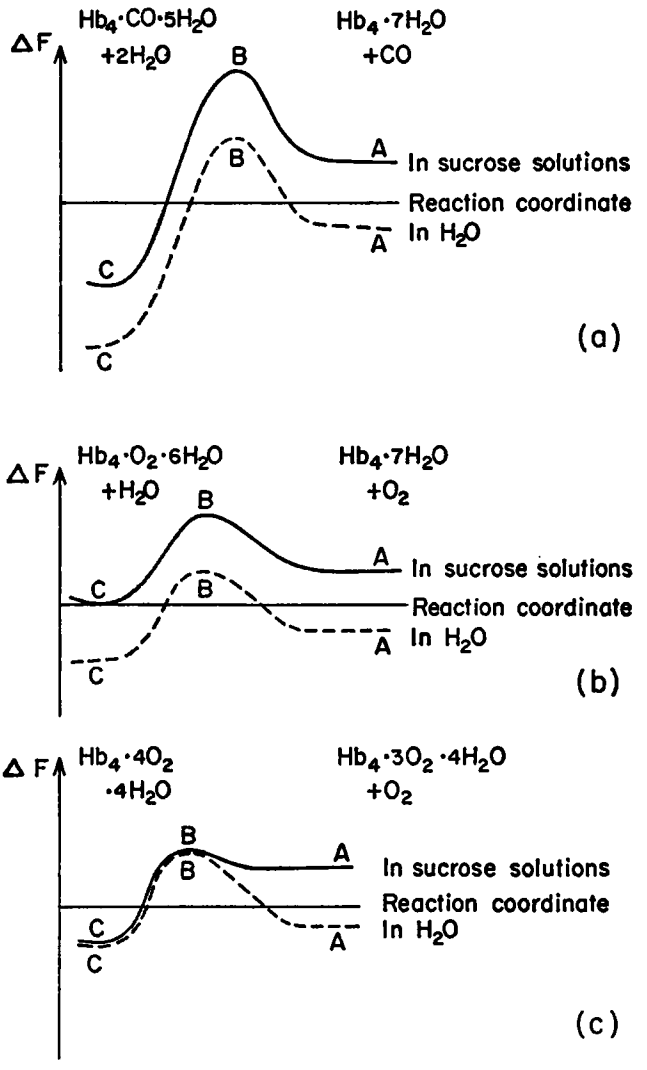

FIGURE 4 Sketches of free energy changes in reactions of hemoglobin with carbon monoxide and oxygen. Association rates are exponential functions of free energy change from *A* to *B*. Dissociation rates are exponential functions of free energy change from *C* to *B*. Equilibrium constants are exponential functions of free energy change from *A* to *C*. (*a*) Curves that determine l_1', l_1, and L_1. Other curves for additional CO molecules are all assumed to be similar. Hence none of these rates or equilibria are altered by CO solubility in media containing glycerol or sucrose. (*b*) Similar curves that determine k_1', k_1, and K_1. Curves for second and perhaps third O_2 are assumed similar in form. Hence none of these rates or equilibria are altered by changes of O_2 solubility due to sucrose or glycerol in reaction medium. (*c*) Curves for determination of k_4', k_4, and K_4, showing dependence of k_4' and K_4 on solubility of O_2.

explain our data, it is clearly possible that all the mechanisms we have considered are wrong (19). No similar need to include H_2O in the reaction has been noted in certain other studies of heme proteins in viscous media (11, 16, 20–22).

Dielectric Data. The lack of variation of the reaction rate m' with changes in the dielectric constant can be interpreted as indicating that, for the purposes of this reaction, the hemoglobin, oxygen, and carbon monoxide molecules can be regarded as neutral molecules whose dipole moments, if they are finite, either are not oriented in any fixed fashion relative to one another or are at right angles to each other and to the line joining the two dipole centers. This is contrary to the conclusions of Griffith (23). It is well known that pH can alter some of these rate constants (3); the dielectric data suggest that these changes are a result of conformational changes of the hemoglobin rather than of direct participation of charged groups or dipoles in controlling these reactions.

Diffusion Effects. No indication has been found that the rate constant m' is diffusion-limited even in extremely viscous media. Since it has been shown (1) that the rate constant k_4 is probably not diffusion-limited, equation (2) implies that any changes in l_4' due to diffusion effects are matched by equal percentage changes in k_4'.

The data in this paper can be expressed in a somewhat different manner. When considering different water-like media, our data indicate that Roughton's equilibrium constant, K_4, varies with the solubility of oxygen. On the other hand, the corresponding equilibrium constant for the first oxygen molecule to associate, K_1, might be independent of the medium, in spite of major changes in the solubility of oxygen (1). This makes it unlikely that the saturation curve for oxyhemoglobin *in vitro* and in the cell would have the same shape. Rather, it appears that oxygen should be at a lower concentration in a concentrated solution of hemoglobin within the red blood cell at the same pO_2. Thus the part of the saturation curve at low pO_2 should be different in the red blood cell. This is in accord with a recently published article (24).

In addition, the initial rate of combination of oxygen with hemoglobin should be lower in the cell because of the lower solubility of oxygen. This lower rate has been observed by Gibson and associates (25), who, following the interpretation of Nicolson and Roughton (26), ascribed this difference to a limitation due to diffusion through the membrane of the red blood cell. On the other hand, our data suggest that the rate of replacement of carbon monoxide by oxygen in carbonylhemoglobin should be nearly the same in the red blood cell and in solution. This was found by Roughton (27–32) to be the case. The interpretation of Nicolson and Roughton (26) indicated that no differences would be expected from diffusion limitation for the replacement of carbon monoxide by oxygen in carbonylhemoglobin, so that this is not a critical test.

This work is based in part on experiments in the Biophysics Laboratories of the Pennsylvania State University, University Park, and in part on experiments at the Mayo Clinic. Some of the

data were included in Dr. Berger's thesis for the degree of Master of Science in Physics, Pennsylvania State University, 1953, and some material used is from Dr. Berger's thesis for the degree of Ph.D. in Physics, Pennsylvania State University, 1956. Material from this paper was presented at the International Biophysics Congress, Stockholm, Sweden, 1961. Dr. Ackerman gratefully acknowledges the technical assistance of Nevin Nolder.

Received for publication, June 12, 1962.

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