THE REACTIONS OF HEMOGLOBIN IN VARIOUS MEDIA

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ABSTRACT The rate constants for the association of oxygen and of carbon monoxide with reduced hemoglobin and for the dissociation of oxygen from hemoglobin were studied in a variety of media. All three of these rates were essentially independent of diffusion rate and dielectric constant throughout a wide range. These results are very different from those found for other heme proteins whose rates are diffusion-controlled in very viscous solvents.

The interpretation of the average measured rates described herein in terms of the four rate constants discussed by Gibson and Roughton indicates that our measurements reflect most strongly the constants for the first reacting molecule in each case. For the purposes of these reactions, the reactants may be regarded as neutral molecules.

INTRODUCTION

The aim of the experiments reported herein was to investigate the roles of two physical characteristics of the medium—the diffusion rate of small molecules and the dielectric constant—in the reactions of hemoglobin (Hb) with oxygen (O_2) and carbon monoxide (CO). In contrast to the reactions of other heme proteins, namely myoglobin (1), catalase (2), and peroxidases (3), the reactions of hemoglobin described herein were found to be essentially independent of the diffusion rate of the small molecules and the dielectric constant throughout the ranges studied. The implications of these findings are discussed in the Comment section.

The major disadvantage of hemoglobin for the studies described in this paper is that there are four heme groups per molecule; all of them may interact, giving rise to a number of different rate constants (4). The simplest way of avoiding this complexity is to measure an average rate of reaction of reduced hemoglobin with oxygen, for example. This was the method of Hartridge and Roughton (5) and of Roughton and Millikan (6–8) and was also adopted for the present study.

Gibson and Roughton (9, 10) assumed that all four heme groups of the hemo-

globin molecule were equivalent but that they could interact; accordingly, they assumed that any one of four rate constants applied for the reactions of hemoglobin with oxygen, depending on whether three, two, one, or no oxygen molecules were combined with the same hemoglobin molecule. They carried out a series of reactions to resolve the first and fourth of these constants. Perutz' (11) crystallographic studies have shown that the four heme groups are not equivalent; rather, there are two pairs of equivalent heme groups per hemoglobin molecule.

In view of these complexities, the method utilizing an average appears to have been a more practical choice. Allowing hemoglobin to represent the average heme group, the reactions we studied may be written:

$$\mathrm{Hb} + \mathrm{O_2} \overset{k'}{\rightleftharpoons} \mathrm{Hb \cdot O_2} \tag{1}$$

$$\mathrm{Hb} + \mathrm{CO} \overset{l'}{\rightleftharpoons} \mathrm{Hb \cdot CO} \tag{2}$$

$$\mathrm{Hb \cdot O_2} \overset{k}{\rightarrow} \mathrm{Hb} + \mathrm{O_2}, \tag{3}$$

where k', l', and k are the average reaction rate constants.

THEORY

Reactions 1 and 2 depend on a small molecule and a larger one coming together to react. The small molecule moves more rapidly; one may think of it as diffusing to an active site on the large one, which remains fixed in place. The small molecule moves through the medium in a series of discrete jumps, remaining at each quasi-equilibrium position for a finite length of time. Each time that the small molecule moves to a position adjoining the active site is called an encounter. During the short time that the small molecule remains at this site, it may collide with the large molecule many times by vibrating and rotating about its quasi-equilibrium position.

Two extreme cases occur. First, if the duration of each encounter is very long, a sufficient number of collisions occur to assure that a reaction takes place. In this case the number of encounters per second will be identical with the number of molecular reactions per second. Since the encounter rate is controlled primarily by the diffusion rate, this type of reaction is called a diffusion-controlled reaction.

In the second extreme, each encounter is so short that comparatively few collisions occur. Under these conditions most encounters end as the small molecule jumps away from the active site. If the diffusion rate is halved, the encounter rate will also be halved, but the length of each encounter will be doubled. Since the number of collisions per second is proportional to the product of these two rates, it would remain approximately constant. In such a case, the reaction is diffusion-independent.

Von Smoluchowski (12) showed that for reactions between similar molecules under conditions of diffusion control, the encounter rate is given by:

$$k = 4\pi D N r \, 10^{-3} f, \tag{4}$$

where D is the diffusion constant in cgs units, N is Avogadro's number, r is the sum of the radii of the reacting groups, and f is a constant depending on geometric effects as well as on electrostatic ones. For two spherically symmetric, uncharged molecules, f is 1. On the other hand, for a small hemispherical site on the side of a large molecule, f is ½. In general, for a small molecule such as oxygen that lacks spherical symmetry reacting with a heme group not exposed to the outer surface of the molecule, f may be expected to be less than ¼. Then equation (4) may be approximated by:

$$k \doteq DNr\,10^{-3}. \tag{5}$$

The constant f can be increased or decreased by electrostatic effects. When this occurs, the reaction rate is altered by variations in the dielectric constant of the medium.

A numerical substitution in equation (4) (13) shows that when a molecule whose active site is on the surface reacts with a spherically symmetric molecule whose diffusion rate is typical of oxygen in water, the diffusion-controlled rate constant k'_d should be given by:

$$k'_d \doteq 10^8\ \text{M}^{-1}\ \text{sec}^{-1}.$$

This value is too large for hemoglobin reactions. If one notes that in crystalline hemoglobin the heme groups are not on the surface, it seems reasonable to reduce the value of f by a factor of about 10. If one also reduces D by a factor of 10, as was done in the experiments reported herein, then diffusion control could occur for bimolecular reactions with rate constants of the order of magnitude of 10^6 M^{-1} sec.^{-1}. Results of this nature were obtained with myoglobin (1), catalase (2), and peroxidase (3).

In the range of viscosities in which the reactions are independent of the diffusion rate of O_2 or CO, the reaction rates should be a function of the individual molecules themselves rather than of the suspending medium. It should be possible to apply absolute rate theory to these reactions to compute an entropy of activation (14). If dielectric effects occur, they can be included in the entropy of activation (ΔS^*).

MATERIALS AND METHODS

Media. The reactions were studied in media which, in addition to water and buffers, contained varying amounts of glycerol, sucrose, and methylcellulose; all solutions were made from chemically pure substances. The glycerol-water solutions varied from 1 to 90 centipoise, the sucrose-water solutions from 1 to 200 centipoise, and methylcellulose-water solutions were used at 23 and 69 centipoise. The dielectric constant of the glycerol and sucrose solutions varied from 75 for water at 25°C to about 40 (15, 16). The dielectric constants of methylcellulose were more difficult to measure because of the high dissipative losses. Some measurements (15) indicated the dielectric constant to be 160. However, considering the shape and large size of the methylcellulose molecule, it

seems likely that the effective dielectric constant was that of water in the immediate neighborhood of the heme groups.

The variations of the diffusion constant for oxygen in the glycerol and sucrose media have been reported elsewhere (17). In this study it varied from a maximum of more than 3×10^{-5} cm²/sec. to a minimum of 0.25×10^{-5} cm²/sec. (Fig. 1). Similar

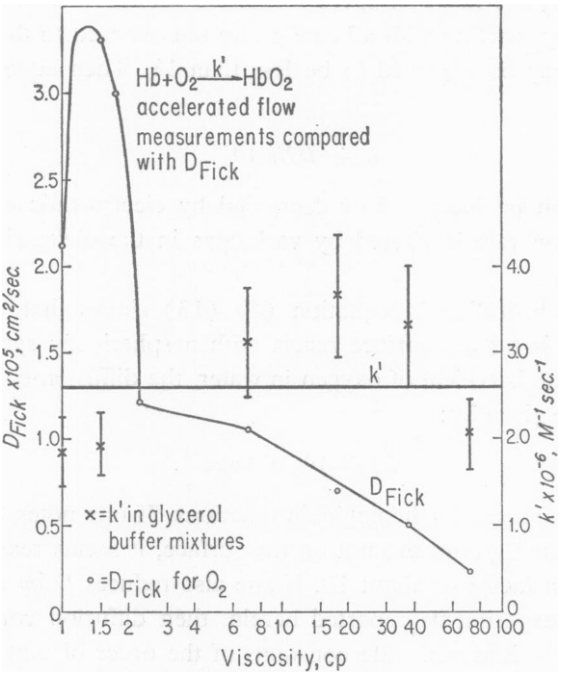

FIGURE 1 Variation with viscosity of the suspending medium of the Fick diffusion constant for oxygen D_{Fick} and the average reaction rate constant k' for the combination of reduced hemoglobin and oxygen. Glycerol at varying concentrations was used to control the viscosity.

measurements in the methycellulose solutions introduced technical difficulties that were never overcome.

For experiments at pH 7.2, a 0.01 M phosphate buffer was used in all media. For those at pH 9.3, a 0.01 M ammonium buffer was employed.

Reactants. The hemoglobin used in most of these experiments was obtained from the blood of a ram owned by the Pennsylvania Experimental Station of the United States Department of Agriculture. However, in some experiments blood from other sheep as well as from rats was tested. The differences in results appeared small when compared to our limits of error.

Differences in the degree of purification also had little effect on the measured rate constants. The crudest method used was to lake the blood in distilled water. The red blood cell ghosts were sometimes centrifuged down. At other times, the cells were washed in isotonic saline before laking. Although it is quite possible that the debris from broken cells could plug the flow system, the various degrees of purification did not appear to affect the reactions.

To permit observation of the constant k, the hemoglobin solutions were mixed with sodium hydrosulfite solutions; the latter solutions were used within a few hours after they were made up. The ammonium sulfide used for the slow reduction of hemoglobin was formed by bubbling hydrogen sulfide through an ammonium hydroxide solution. Spectrophotometric checks were performed to be certain all measured reactions were completed before any appreciable methemoglobin or sulfhemoglobin was formed. The carbon monoxide used to observe the rate constant l' was Matheson cp grade, passed through a vanadium pentoxide chain (15). It was dissolved to saturation in a buffer solution made up with water. The latter was then diluted with more buffer and, as needed, with glycerol, sucrose, or methylcellulose to give a final solution having the desired concentration of buffer, viscosity, dielectric constant, and concentration of carbon monoxide.

In some of the experiments in which the reactions of hemoglobin with oxygen were measured, the oxygen was removed from the solution by bubbling with nitrogen, and then a measured amount of air-saturated water was added to give the desired concentration of oxygen. More repeatable results were obtained by using the oxygen in air-saturated solutions. Such concentrations in the glycerol solutions were obtained from the Bunsen coefficients given by Seidell (18).

Measuring Apparatus. All the reactions were measured in terms of changes in optical density. Optical densities were also used to determine the concentrations of hemoglobin. Detailed spectra for the various hemoglobin compounds are given by Lemberg and Legge (4).

The reactions observed all had a fairly short half-time. Accordingly, a flow system was used to measure them. The heart of the flow system was a specially designed mixing chamber in which it was possible to mix rapidly solutions with viscosities as high as 200 centipoise. This chamber is illustrated in Fig. 2. The reaction of hemoglobin with oxygen was observed by means of the accelerated-flow method, while the others were measured with a stopped-flow technic (19).

In order to increase the half-times of the bimolecular reactions, low concentrations of hemoglobin were used. This step was of additional importance in that it reduced the differences between the two solutions being mixed. With most mixing chambers it was impossible to use the flow system if even a 5 per cent difference existed between the viscosities of the two solutions combined in the mixing chamber.

The flow system was placed in a sensitive spectrophotometer. Two different instruments were used during these measurements. One was basically a Beckman DU model, modified as described by Chance (20); the other was a split-beam type that utilized a Bausch & Lomb monochromator. In both cases the limiting element proved to be the performance of the flow system rather than the recording spectrophotometer.

RESULTS

$Hb + O_2$. The rate constant for this reaction was shown by Millikan and Roughton (6–8) to be independent of pH. Accordingly, this constant was measured at pH 9.0 and 25°C. Two sets of experiments were performed: in the first set the oxygen of the medium was controlled in order to slow the reaction to such an extent that it could be observed as a stopped-flow reaction; in the second set the solutions containing oxygen were equilibrated with air. Both sets of experiments supported

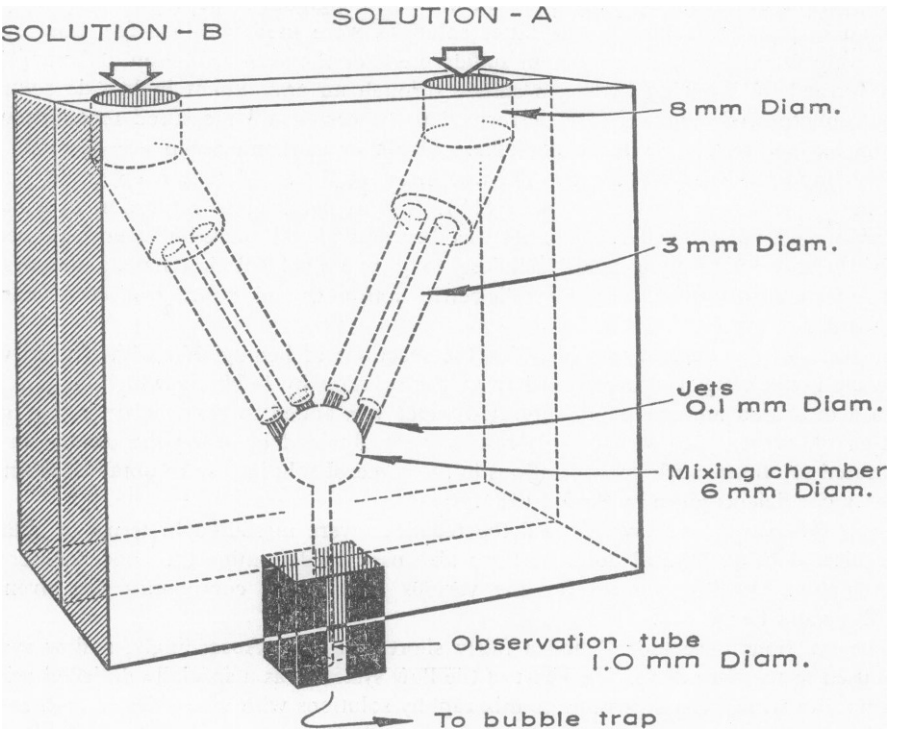

FIGURE 2 This mixing chamber permits stopped-flow as well as accelerated-flow measurements in a modified Beckman DU spectrophotometer. The two solutions are forced from syringes into the clear plastic block. The two flow tubes divide and redivide to form 16 jets. The small size of these jets assures complete mixing, even at comparatively low flow velocities. Accelerated-flow measurements at various heights along the observation tube showed that mixing occurs near the bottom of the spherical chamber, although small variations in the jets caused major changes in the mixing pattern within this chamber.

the constancy of k' when the dielectric constant of the medium and the diffusion rate of O_2 were varied. Fig. 1 shows the data in glycerol-water media. Similar results were obtained with the use of sucrose-water and methylcellulose-water solutions.

In order to arrive at a numerical value for k' it is necessary to solve the equation:

$$\frac{d(\mathrm{Hb})}{dt} = -k'(\mathrm{Hb})(\mathrm{O_2}) + k(\mathrm{HbO_2}),$$

If $t_{\frac{1}{2}}$ is the time for one-half of the final oxidation at constant (O_2) to occur, then:

$$k' = \frac{ln2 - kt_{\frac{1}{2}}}{t_{\frac{1}{2}}(\mathrm{O_2})}.$$

This formula was used to compute k'. The value of k is significant in determining the value of k'. It will be shown later that k is independent of the diffusion rate of O_2 and the dielectric constant; it was set equal to 15 sec.$^{-1}$ for computing k'. This gave k' an average value of 2.6×10^6 M^{-1} sec.$^{-1}$, which is within the range reported for the value in water by Gibson and associates (21).

The plot of k' *versus* viscosity shown in Fig. 1 might be interpreted as indicating a slight rise in k' as the viscosity is increased from 1 to 25 cp, followed by a fall in k' at higher viscosities. Since the bulk dielectric constant decreases as the viscosity rises from 1 to 25 cp, the rise in k' (if it exists) could be due to a small dielectric effect. Likewise the apparent fall in k' at higher viscosities could reflect the change in the diffusion constant of O_2. However the experimental errors in the measurements reported here are so great that further experiments would be required to substantiate these small trends.

HbO_2 + Hydrosulfite. An excess of hydrosulfite was used, and therefore the oxygen was largely removed after mixing; this was tested by varying the concentration of hydrosulfite and working in a range in which this concentration was unimportant. (At very low hydrosulfite concentrations the rate of reduction of hemoglobin will be controlled by the rate of removal of oxygen by the hydrosulfite. At very high concentrations, the hydrosulfite denatures the hemoglobin. About 2 gm/liter proved a convenient range in which to work.) The rate constant k was found to be about 15 sec.$^{-1}$, at both pH 7.2 and pH 9.

As in the reaction previously cited, no changes greater than the experimental error ($\pm$ 20 per cent) were observed in k, even though the viscosity was increased to 150 to 200 centipoise with glycerol and sucrose. Therefore, this rate constant also appears to be independent of the diffusion rate of O_2 and of the dielectric constant of the medium (within the experimental error).

Hb + CO. For this reaction, hemoglobin was reduced with hydrosulfite. It was then mixed in the flow system with solutions containing about 0.06 mM carbon monoxide to give a final carbon monoxide concentration after mixing of 0.03 mM. The concentration of hemoglobin was about 0.01 mM. Since the back reaction (dissociation) was so slow, it could be neglected. Thus the constant l' could be easily measured; it was related to the half-time approximately by:

$$l' \doteq \frac{ln2}{t_{\frac{1}{2}}(CO)}.$$

Values of l' were found to be about 1.4×10^5 M^{-1} sec.$^{-1}$ in all media used. This is summarized graphically in Fig. 3.

COMMENT

These studies have indicated that the reaction rates k, k', and l' are all diffusion-independent and also independent of the dielectric constant at pH 9. The problem

of the interpretation of these constants in terms of the four successive reactions of a hemoglobin molecule is quite complex. Gibson and his coworkers (9, 10, 21) have shown that the reaction rates k'_1 and k'_4 for the associations of the first and last oxygen atoms with a hemoglobin molecule are quite different in their pH dependence, their temperature dependence, and their absolute value.

Since k'_1 is smaller than k'_4, any increase in k'_1 would have been reflected in our experiments as an increase in k'. On the other hand, it is quite conceivable that appreciable increases in k'_4 would not have been detected by our experiments. This is indicated by Gibson and Roughton's pH studies (10), which show that an increase of fourfold in k'_4 as the pH is increased has a negligible effect on the observed value of k'.

The data reported in this paper for the association rate k' of oxygen with reduced hemoglobin indicate that k'_1 is probably not diffusion-limited at pH 9 and 27°C. One can also conclude that k'_1 is probably unaltered by a decrease in the dielectric constant. Similar arguments to those for k'_1 can be applied to l'_1 and also to k_4.

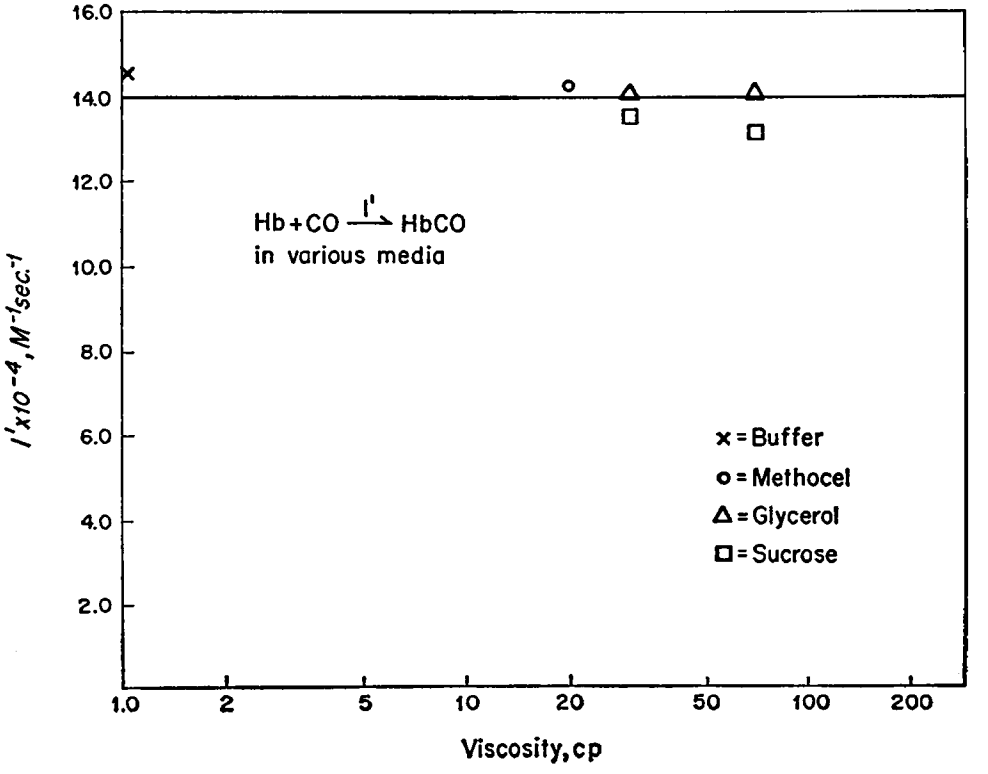

FIGURE 3 Variation with viscosity in various media of the average reaction rate constant l' for the combination of reduced hemoglobin and carbon monoxide.

The reactions studied in this paper form a sharp contrast to those of myoglobin, catalase, and peroxidase, which are diffusion-controlled under the conditions described in this paper; their rate constants are limited by diffusion to values lower than those observed for k'. One can explain this difference by assuming that the factor f in equation (4) is larger for hemoglobin than for the other heme reactions studied in this fashion.

The rate constants discussed in this paper have all been based on the concentrations of the reactants. If instead of oxygen concentration one uses pO_2, a new rate constant, k'_ν, can be found for the O_2 + Hb reaction, in which k'_ν is defined by:

$$k'_\nu = \frac{1}{(\mathrm{Hb})pO_2} \frac{d(\mathrm{HbO_2})}{dt}$$

The values of k'_ν, computed from the data used in constructing Fig. 1, vary at viscosities above 7 cp in a fashion similar to that of the diffusion constant for O_2. Thus k'_ν is the physiologically significant rate constant since tissue exchange depends upon O_2 tension.

This paper is based on experimental work performed in the Biophysics Laboratories, Physics Department, Pennsylvania State University, University Park. Some of the work was included in Dr. Berger's thesis for the degree of Doctor of Philosophy in Physics and Mr. Blair's thesis for the degree of Master of Science in Physics.

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