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THE KINETICS OF THE REACTION OF CARBON MONOXIDE WITH FULLY OXYGENATED HAEMOGLOBIN IN SOLUTION AND ERYTHROCYTES

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SUMMARY

1. Spectrophotometric measurements, using a rapid mixing and stopped flow technique, have been made of the rate at which CO displaces O_2 from its combination with haemoglobin.

2. In haemoglobin solutions, buffered at pH 7·2 and 9·1, the reaction proceeds by a unimolecular dissociation as proposed by Gibson & Roughton (1955). In a Ringer–Locke solution, equilibrated with a P_{CO_2} of 3 cmHg and at pH 7·4, the reaction of HbO_2 with CO is a two-stage process, with a transition from one form of Hb_4O_6 to another.

3. An investigation of the reaction between CO and HbO_2 in erythrocytes, suspended in Ringer–Locke solution, indicates that the rate is determined by the chemical reaction and this also is a two-stage process.

4. The transition is probably associated with the reaction of CO_2 with Hb_4O_6, following the dissociation from fully saturated oxyhaemoglobin of an oxygen molecule. It alters the relative velocity constants of the reactions of O_2 and CO with Hb_4O_6 by 100:1.

5. The implications of these proposals of the equilibria of haemoglobin with CO and O_2 are discussed. The difference between the sigmoid equilibria curves at high HbO_2 and HbCO values can be explained as due to the different reaction pathways.

INTRODUCTION

The reaction of CO with fully oxygenated haemoglobin has both theoretical and practical interest. An analysis of this reaction, with haemoglobin in buffered solutions, has been given by Gibson & Roughton (1955). They demonstrated that the dissociation in these circumstances was unimolecular with a rate constant, r, given by

$$r = \frac{k_4}{4(1 + k_4'[O_2]/l_4'[CO])}, \quad (1)$$

where k_4' and l_4' are the rate constants of the combination of O_2 and CO respectively with haemoglobin, and k_4 the velocity constant of the reaction $Hb_4O_8 \rightarrow Hb_4O_6 + O_2$. When the reciprocal of r was plotted against $[O_2]/[CO]$ a straight line was obtained, which intercepts the vertical axis at $4/k_4$. This reaction mechanism was utilized by Roughton, Forster & Cander (1957) and later Holland (1969), in considering the corresponding situation of CO reacting with fully oxygenated haemoglobin in erythrocytes. These results were used to estimate θ, the rate of uptake of CO by whole blood (ml./(min × mmHg × ml.)), and applied to calculate the diffusing capacity of the pulmonary membrane, D_m. It was implicit in these calculations that the differences between the rates with haemoglobin in solution and those with intact cells were due to the latter situation involving diffusion and chemical reaction with the internal haemoglobin, and diffusion through the cell membrane. There was no direct evidence, however, to prove that the chemical reaction mechanism proposed by Gibson & Roughton (1955) was directly applicable within red blood cells.

The present study began with a comparison of this reaction in cells, at high CO concentrations, with the rate of deoxygenation after rapidly mixing and diluting cells in an isotonic solution containing $Na_2S_2O_4$. These were shown (Sirs, 1966, 1967) to be identical, over the initial 40 % of the reaction, suggesting that diffusion through the cell membrane is not a limiting factor and effective mixing occurs within the cell. The CO concentration was varied over a limited range, while keeping the ratio $[O_2]/[CO]$ small, with no change of r. This indicated that the limiting factor was simply the chemical rate constant k_4, and, at the high concentrations of CO used, the reaction mechanism of Gibson & Roughton (1955) appeared valid. It was estimated (Sirs, 1967) that the partial pressure of O_2 within the cells could not be greater than 0·4 cmHg above that in the external and well mixed environment. A further theoretical analysis of this situation has since suggested that if the cell interior was not efficiently mixed or the cell membrane had a finite resistance, the resultant increased O_2 concentration within the cell would significantly lower the rate constant r at low CO concentrations. In particular the reciprocal of r plotted against the ratio $[O_2]/[CO]$ would give different curves for each concentration of CO, which would not intercept the vertical axis at the same point. The present investigation of this possibility supports the proposal that the rate limiting factor with intact cells is simply the chemical rate of dissociation, but that this is more complicated than the reaction mechanism proposed by Gibson & Roughton (1955).

METHODS

Spectrophotometric measurements of the rate of displacement of O_2, from its combination with haemoglobin, by CO_2, were made with the stopped flow method of Sirs & Roughton (1963). A modification of this procedure, by which the oxyhaemoglobin is diluted at the same time as it is rapidly mixed with solutions containing carbon monoxide was also utilized (Sirs, 1966). The dilution factor was varied, by altering the respective bores of the reactant syringes, over a range 1:20, 1:5 to 1:1. This permitted a much wider range of $[O_2]/[CO]$ values, in the mixed solution, to be studied. In addition by diluting the oxyhaemoglobin 1:20 in concentrated CO solutions the rate observed is very close to the limit set by the rate constant, k_4, for the first O_2 molecule to dissociate.

Human and sheep blood was collected by venepuncture, heparin at a concentration of 6 i.u./ml. of blood being used to prevent coagulation. The blood was either, used immediately or stored at 4° C. All measurements were completed within 24 hr of collecting the blood sample. The type of haemoglobin was identified electrophoretically. The results discussed in this paper were obtained on human Hb A and sheep Hb A haemoglobins. With experiments on cell suspensions the blood was diluted in an isotonic solution consisting of 130 mM-NaCl, 23·9 mM-$NaHCO_3$, 5·7 mM-KCl and 2·2 mM-$CaCl_2$. This was equilibrated with a P_{CO_2} of 3 cmHg, to adjust to pH 7·4 at room temperature, and a given P_{O_2}. The suspension was mixed and diluted with the same solution, without erythrocytes, equilibrated with 3 cm P_{CO_2} and known partial pressures of CO.

The haemoglobin solutions were prepared by centrifuging and washing cells in isotonic saline, followed by lysis with distilled water. The ghosts and debris were removed by centrifuging at 3000 *g* for 30 min. Observations were made in borate buffer solutions at pH 9·1 and 7·2, under similar conditions to those used by Gibson & Roughton (1955). The pH 9·1 buffer was made by mixing 100 ml. 0·2 M-boric acid with 45 ml 0·2 M-sodium hydroxide. The borate buffer at pH 7·2 was obtained by dissolving 1·24 g boric acid in 1 l 0·1 N sodium hydroxide solution and mixing this with 920 ml. 0·1 N hydrochloric acid. After the results obtained with suspensions of erythrocytes indicated some unusual features, the measurements in solution were extended to include studies on haemoglobin diluted in the isotonic solution given above, equilibrated with a P_{CO_2} of 3 cmHg and at pH 7·4. In these experiments the haemoglobin solution was equilibrated with various partial pressures of oxygen and then rapidly mixed and diluted in a solution of the same composition, without haemoglobin, containing carbon monoxide.

The rate of change of HbO_2 to HbCO was observed with a split-beam spectrophotometric detector (Sirs & Roughton, 1963), using two Farrand interference filters. At least six measurements were made using wave-lengths of 560 and 582 mμ, then six more at 520 and 460 μm, each pair of filters being chosen so that the absorption changes occur in opposition. This increases the sensitivity of the detector and minimizes any non-linearity of the change of deflexion with HbO_2, as determined by calibration mixtures. The temperature of all reactants was maintained to $\pm 0{\cdot}5°$ C in a thermostatically controlled bath, and water was pumped from this reservoir through the stopped-flow apparatus to maintain it at the same temperature. All the measurements reported in this paper were at or just above room temperature. The pH was checked with a standard glass electrode and pH meter. The equilibration of solutions with CO_2, CO and O_2 was carefully monitored, and solution concentrations measured using a Van Slyke technique. Small samples were taken directly from the cell suspensions and the outflow of the stopped flow apparatus and centrifuged to ensure the absence of haemolysis. Microscopic examination was systematically made to ensure there was no significant abnormality or crenation of the erythrocytes.

RESULTS

Haemoglobin in buffered solutions. These studies were undertaken to be completely sure that the technique was valid and the results obtained in the following sections were not due to an artifact, such as the effect of light in dissociating HbCO. The oxyhaemoglobin solutions were rapidly mixed and diluted with solutions containing known concentrations of CO. From the observed reaction curves of the change of HbO_2 with time, a plot of log (HbO_2) with time was obtained. This was linear in all cases and r was determined from the slope of the line. A plot of the reciprocal of r against $[O_2]/[CO]$ is given in Fig. 1, for sheep haemoglobin at pH 9·1

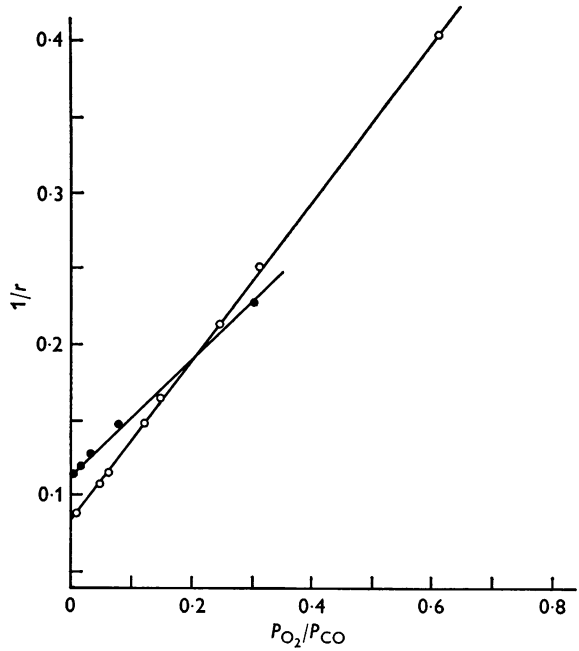


Fig. 1. A plot of the reciprocal of r against P_{O_2}/P_{CO} for haemogoblin in solution. ○, Human HbA in borate buffer at pH 7·2 and 24° C. ●, Sheep Hb in buffer solution at pH 9·1 and 18·5° C.

and 18·5° C, and human haemoglobin at pH 7·2 and 24° C. These observations are in accord with the theory and experimental data of Gibson & Roughton (1955). The rate constant r is equivalent to $k_4/4$ when $[O_2]/[CO]$ is zero. From the plots the value of k_4 is 47·6 sec^{-1} and the ratio k'_4/l'_4 is 4·68 for the human haemoglobin. The corresponding value for sheep haemoglobin of k_4 is 33·9 sec^{-1} and k'_4/l'_4 is 3·1.

Haemoglobin in Ringer–Locke solutions. When a similar study was made

with erythrocyte suspensions, as outlined below, the values of the rate constant r at different $[O_2]/[CO]$ values did not accord with eqn. (1). A further study was therefore undertaken of the rate of change of HbO_2 to HbCO in the isotonic Ringer–Locke solution specified under Methods. The solution was equilibrated with a P_{CO_2} of 3 cmHg, in addition to the other gases used, in order to adjust to pH 7·4. The rate of change of HbO_2 with time again followed a simple first-order reaction. A plot of log (HbO_2) with time was linear, as previously reported by Sirs (1967), with a slope equal to the rate constant r. However, when the reciprocal of r was plotted

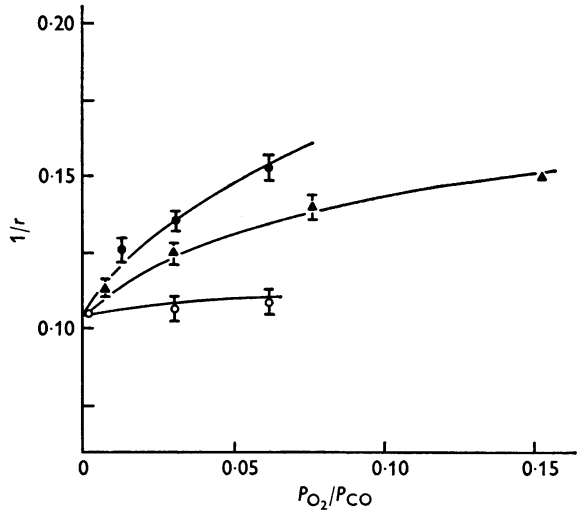


Fig. 2. The reciprocal of r plotted against P_{O_2}/P_{CO} for human haemoglobin in modified Ringer–Locke solution equilibrated with 3 cmHg P_{CO_2}, at pH 7·4 and 21° C. To change P_{O_2}/P_{CO} to $[O_2]/[CO]$ multiply former by 1·33, to allow for differences in solubility. The range of variation of the calculated values of $1/r$ are shown as bars about each point. ●, CO concentration 0·245 mM; ▲, CO, 0·49 mM; ○, CO, 1·22 mM. The curves have been drawn, using eqn. (8), with $4/k_4 = 0{\cdot}103$, $k'_3/l'_3 = 11$, $p/l'_4 = 0{\cdot}5 \times 10^{-3}$, $k'_4/l'_4 = 0{\cdot}1$.

against the ratio of $[O_2]/[CO]$, the results no longer fell on a single straight line. The experimental data for human haemoglobin in solution, at a temperature of 21° C., are shown in Fig 2; and for sheep haemoglobin at 23° C in Fig. 3. There is a family of curves which depend on the carbon monoxide concentration. These results are not compatible with the reaction mechanism proposed by Gibson & Roughton (1955).

Measurement of r in erythrocyte suspensions. Erythrocyte suspensions, similarly equilibrated with known partial pressures of O_2, have been rapidly mixed and diluted in isotonic solutions equilibrated with a range of partial pressures of carbon monoxide. The rate of HbO_2 change with

time follows a simple first-order reaction, with a rate constant r. In an initial series of experiments, undertaken with both sheep and human erythrocytes, the observations were restricted to a narrow range of $[O_2]/[CO]$ values, from zero to 0·05. The purpose of these experiments was to estimate whether the P_{O_2} within the cell was higher than the P_{O_2} in the surrounding media. If this were the case, the plot of $1/r$ against $[O_2]/[CO]$ would intercept the vertical axis at different points, depending on the carbon

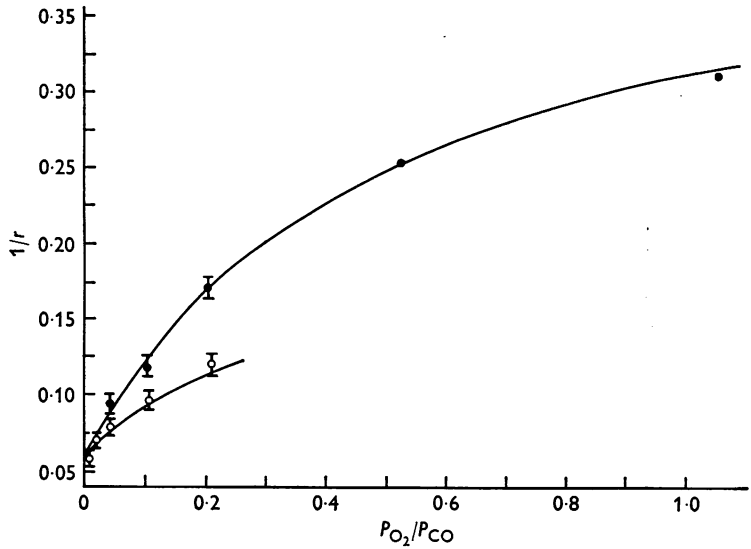


Fig. 3. A plot of the reciprocal of r against P_{O_2}/P_{CO} for sheep haemoglobin in Ringer–Locke solution equilibrated with a P_{CO_2} of 3 cmHg, at pH 7·4 and 23 ± 0·5° C. ○, CO concentration 1·1 mM, ●, CO, 0·245 mM. The curves have been drawn using the values, $4/k_4 = 0{\cdot}059$, $k'_8/l'_8 = 10{\cdot}5$, $p/l'_4 = 1{\cdot}65 \times 10^{-3}$ and $k'_4/l'_4 = 0{\cdot}1$.

monoxide concentration. No difference in the point of interception could be detected within the experimental errors of ± 7 %. It became apparent, however, that with $[O_2]/[CO]$ of the order 0·04–0·05 the values of $1/r$ were systematically higher at low CO concentrations than at high CO conditions.

In order to investigate this further a wider range of $[O_2]/[CO]$ was studied. With the limited quantity of blood available this restricted observations to two CO concentrations. The findings of a typical experiment are shown in Fig. 4. These results were obtained with the same blood sample as the results on haemoglobin in solution given in Fig. 3. The cell suspension experiments were made during the morning and those on Hb in solution during the afternoon of the same day.

The results were so different to what had previously been reported that

a number of experiments were undertaken to examine the possibility that the effects could be due to an experimental artifact. The purity of the gas supplies were checked with a mass spectrometer, but no significant impurities were found. The possibility that the incident light was significantly dissociating HbCO was eliminated by altering the light intensity and using different combinations of interference filters. The amount of reduced haemoglobin was shown to be negligible by studying the rate of dissociation at partial pressures of oxygen up to 100 cmHg. A careful check on the O_2 and CO concentrations was undertaken, but any small errors in these determinations could not possibly account for the large differences observed. The concentration of cells was also varied but no changes were found in the values of r at given $[O_2]/[CO]$ ratios. No evidence was obtained that these results were due to an artifact.

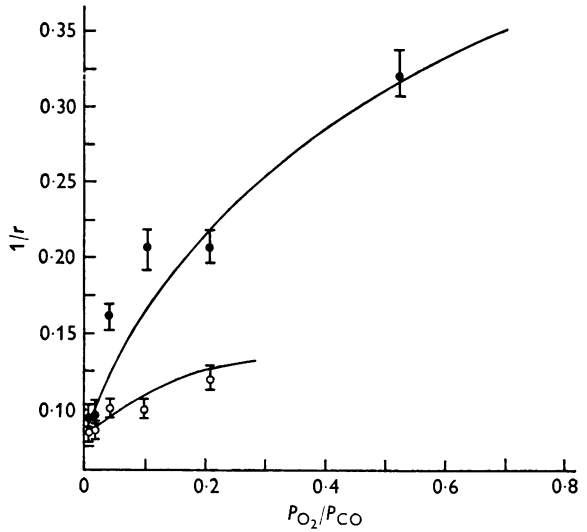


Fig. 4. A plot of the reciprocal of r against P_{O_2}/P_{CO} for sheep erythrocytes diluted in Ringer–Locke solution, equilibrated with a P_{CO_2} of 3 cmHg, at pH 7·4 and 23 ± 0·5° C. ○, CO concentration 1·23 mM, ●, CO, 0·246 mM. The curves were drawn using the values, $4/k_4 = 0{\cdot}079$, $k'_8/l'_8 = 12$, $p/l'_4 = 1{\cdot}2 \times 10^{-3}$ and $k'_4/l'_4 = 0{\cdot}1$.

Theoretical analysis

One of the original purposes of this investigation was to examine the extent to which diffusion processes may influence the rate of exchange of O_2 by erythrocytes. The similarity of the curves of the reciprocal of r with $[O_2]/[CO]$, for both cells and haemoglobin in Ringer–Locke solution, would

suggest that the main rate determining factor is the chemical reaction rate. There are differences between these two sets of data, however, the value of r being a little higher in solution than cells under comparable conditions, and it is necessary to consider if this could in any way be due to diffusion. For the reaction of CO with HbO_2 when diffusion through the cellular haemoglobin is significant, it has been shown by Sirs (1966) that the equation governing the rate of O_2 egress would be given by

$$\frac{d[O_2]}{dt} = D\frac{d^2[O_2]}{dx^2} - r[HbO_2],$$

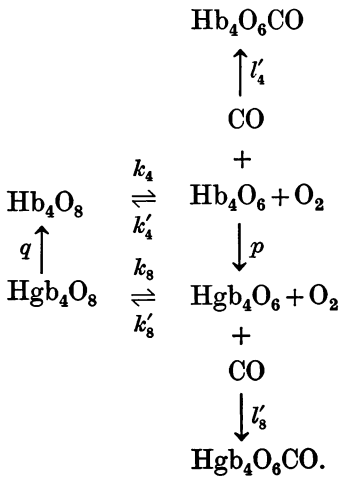
where D is the diffusion coefficient, t time and x the distance within the cell. By putting in the appropriate boundary conditions, the solution of this equation is

$$\frac{d[HbO_2]}{dt} = -r[HbO_2].$$

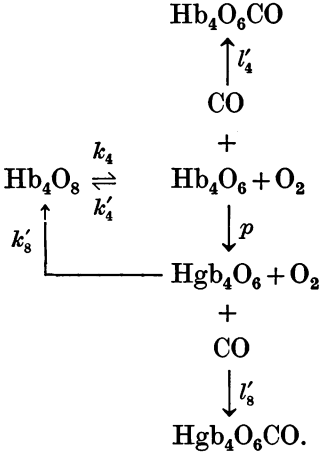
The rate is thus a unimolecular process with an exponential decay. This is in agreement with the experimental observation that the plot of log $[HbO_2]$ with time is linear. With cells, however, though the rate constant r depends on the $[O_2]/[CO]$ ratio, the $[O_2]$ value is that inside the cell. Depending on the degree to which diffusion is involved, this would be higher than in the external media surrounding the cells. If, however, this were the case the values of r plotted against the known $[O_2]/[CO]$ value in the external media would intercept the vertical axis at different points, depending on the carbon monoxide concentration. At high CO concentrations a small difference of O_2 would be negligible and r would be equivalent to $k_4/4$ when $[O_2]/[CO]$ was zero. For low CO concentrations r would be significantly smaller. The initial experiments on cell suspensions were undertaken to examine if the intercept with the vertical axis, when r was plotted against $[O_2]/[CO]$, varied with the CO concentration. Within experimental error the extrapolated curves crossed the vertical axis at the same point, irrespective of [CO]. This suggests that the only rate limited factor in the exchange of O_2 by red blood cells is the chemical reaction rate.

The question remains, since the results with intact cells and haemoglobin in Ringer–Locke solution do not agree with Gibson & Roughton's proposals, what is the chemical reaction mechanism that accounts for this data? There are three main criteria that any proposed mechanism must satisfy. The rate of change of oxyhaemoglobin with time must be a first-order reaction; the rate constant r of the reaction will depend on the CO and O_2 concentrations in the manner shown in Figs. 2–4; and the equilibrium condition must be consistent with the established Haldane equa-

tion. A number of possible mechanisms were considered, but the only one consistent with the experimental observations appeared to be



The first dissociation is equivalent to that proposed by Gibson & Roughton (1955). There is, however, the additional step, that Hb_4O_6 can change at a rate constant p into Hgb_4O_6, a form of oxyhaemoglobin with three molecules still attached but with different reaction velocity constants. The nomenclature adopted is that used by Gibson & Roughton, the velocity constants with O_2 being indicated by k, and with CO by l. The number of unknown constants in this mechanism makes it difficult to estimate them sufficiently accurately to provide the necessary quantitative proof. An examination of this scheme and the experimental data indicated, however, that the rate constant q is likely to be considerably larger than k_8 and k'_8. The calculations are then simplified and the proposed mechanism becomes



Applying the law of Mass Action,

$$\frac{d[Hb_4O_8]}{dt} = -k_4[Hb_4O_8] + k'_4[Hb_4O_6]\,[O_2] + k'_8[Hgb_4O_6]\,[O_2]. \quad (2)$$

$$\frac{d[Hb_4O_6]}{dt} = -p[Hb_4O_6] + k_4[Hb_4O_8] - k'_4[Hb_4O_6]\,[O_2] - l'_4[Hb_4O_6]\,[CO], \quad (3)$$

and $$\frac{d[Hgb_4O_6]}{dt} = p[Hb_4O_6] - k'_8[Hgb_4O_6]\,[O_2] - l'_8[Hgb_4O_6]\,[CO]. \quad (4)$$

The experimental conditions were chosen so that the combined O_2 and CO concentrations effectively maintained the haemoglobin in its saturated form throughout the reaction. The amounts of Hb_4O_6 and Hgb_4O_6 present at any time can be considered small or constant. It follows that both $d/dt[Hb_4O_6]$ and $d/dt[Hgb_4O_6]$ are zero. Eqns. (3) and (4) can then be rewritten as

$$[Hb_4O_6] = \frac{k_4[Hb_4O_8]}{p + k'_4[O_2] + l'_4[CO]} \quad (5)$$

and $$[Hgb_4O_6] = \frac{p[Hb_4O_6]}{k'_8[O_2] + l'_8[CO]}. \quad (6)$$

Substituting these into equation (2), gives

$$\frac{d[Hb_4O_8]}{dt} = -r[Hb_4O_8], \quad (7)$$

where $$\frac{1}{r} = \frac{4}{k_4}\left(1 + \frac{k'_8}{l'_8}\frac{[O_2]}{[CO]}\right)\left(\frac{1 + p/l'_4[CO] + k'_4[O_2]/l'_4\,[CO]}{1 + p/l'_4[CO] + k'_8[O_2]/l'_8\,[CO]}\right). \quad (8)$$

The factor of 4 is necessary to allow for the fact that four O_2 atoms are tied to each haemoglobin molecule and spectrophotometric observations do not distinguish between them.

The value of k_4 can be determined from a plot of $1/r$ against $[O_2]/[CO]$, at the intercept on the vertical axis when $[O_2]/[CO]$ is zero. Numerical values of the ratios, k'_8/l'_8, p/l'_4 and k'_4/l'_4 can be obtained by substituting into eqn. (8) the experimental values of $1/r$ and $[O_2]/[CO]$ at three different points. The three simultaneous equations can then be solved for each ratio. The curves drawn on Figs. 2–4 were determined in this way, with small adjustments to obtain a better over-all fit. The respective values of the constants are given in the legend of each Figure. The accuracy for k_4 is $\pm 7\,\%$, for k'_8/l'_8 and $p/l'_4 \pm 15\,\%$ and for k'_4/l'_4 only $\pm 100\,\%$. The reason for the latter inaccuracy is that $1/r$ is only sensitive to k'_4/l'_4 at high values of $[O_2]/[CO]$. Attention was concentrated initially at low values of $[O_2]/[CO]$ in order to establish the relevance of diffusion to this situation, as outlined

above, so the data at present available to improve this accuracy is limited. The agreement between experiment and theory is sufficiently close, however, to establish the validity of the proposed mechanism.

Equilibrium considerations

If haemoglobin is equilibrated with mixtures of O_2 and CO, under conditions such that the amount of free haemoglobin is negligible, the proportion of oxyhaemoglobin to carboxyhaemoglobin is given by the Haldane equation

$$[\mathrm{HbCO}]\,[\mathrm{O_2}] = M[\mathrm{HbO_2}]\,[\mathrm{CO}]. \tag{9}$$

On the basis of the present proposals this can be derived in the following manner. At equilibrium

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{Hgb_4O_6CO}] = l'_4[\mathrm{Hb_4O_6}]\,[\mathrm{CO}] + l'_8[\mathrm{Hgb_4O_6}]\,[\mathrm{CO}] - l_4[\mathrm{Hb_4O_6CO}] = 0, \tag{10}$$

where l_4 is the velocity constant for the dissociation of Hb_4O_6CO. The dissociation of Hgb_4O_6CO is neglected because with such a slow rate it would change to the Hb_4O_6CO form first. Equation (2) would also be zero. Combining these with eqn. (6) we get

$$\frac{k_4[\mathrm{Hb_4O_8}]}{l_4[\mathrm{Hb_4O_6CO}]} = \frac{[\mathrm{O_2}]}{[\mathrm{CO}]}\left\{\frac{(k'_4/p)(k'_8[\mathrm{O_2}]/l'_8 + [\mathrm{CO}]) + k'_8/l'_8}{(l'_4/p)(k'_8[\mathrm{O_2}]/l'_8 + [\mathrm{CO}]) + 1}\right\}. \tag{11}$$

With k'_8/l'_8 approximately equal to 10 and the CO concentration less than one-hundredth of that for O_2, this simplifies to

$$\frac{[\mathrm{Hb_4O_8}]}{[\mathrm{Hb_4O_6CO}]} = \frac{[\mathrm{O_2}]}{[\mathrm{CO}]}\left(\frac{l_4}{k_4}\right)\frac{(k'_4[\mathrm{O_2}]/p + 1)}{(l'_4[\mathrm{O_2}]/p + l'_8/k'_8)}. \tag{12}$$

If the O_2 concentration is constant, the % HbCO to % CO is a rectangular hyperbola (Haldane & Smith 1897; Roughton, 1964). The theory suggests that the value of M will decrease with lowered P_{O_2}. The magnitude of this can be calculated using the kinetic values derived previously. For a change of P_{O_2} by $\pm 50\,\%$ the variation of M would be approximately $\pm 20\,\%$. Thus as the absolute pressure is increased, even though the ratio of O_2 to CO concentrations is constant, the proportion of carboxyhaemoglobin is increased. This occurs because less Hb_4O_6 is then changed to Hgb_4O_6 and as a result the relative reaction constants change one hundredfold in favour of binding CO. This effect was not observed, however, by Rodney, O'Neal & Collison (1969), for reasons which will become apparent in the Discussion.

DISCUSSION

The results and analysis given in the previous sections would appear to confirm that the rates of uptake and egress of O_2 by haemoglobin within erythrocytes, are, in normal circumstances, simply determined by the chemical reaction rates. Indeed it is surprising considering the relative change in chemical activity, that the rates are so similar for haemoglobin in dilute solution and within the cell. There would not appear to be any gross differences in the structure of haemoglobin in solution and within the erythrocyte. In particular, the results imply that the velocity constant k_4 is only slightly affected by pH, CO_2 and 2,3-diphosphoglycerate (DPG).

The change in haemoglobin from one form, Hb_4O_6, to the other, Hgb_4O_6, would appear to be linked to the reaction of CO_2. The difference of pH between the haemoglobin in buffered solutions and the modified Ringer–Locke solution is too small to be significant, and the reaction of haemoglobin with hydrogen ions, being ionic, is relatively instantaneous. The only factor that is different in these experiments with haemoglobin in solution is the presence of CO_2. The mechanism is probably

$$\begin{array}{ccc} & \overset{k_4}{\rightleftharpoons} & \\ Hb_4O_8 & \underset{k_4'}{} & Hb_4O_6 + O_2 \\ + & & + \\ CO_2 & & CO_2 \\ \uparrow u & & \downarrow v \\ CO_2Hb_4O_8 & \overset{k_8}{\rightleftharpoons} \underset{k_8'}{} & CO_2Hb_4O_6 + O_2. \end{array}$$

Thus the notation Hgb_4O_6 used previously is equivalent to $CO_2Hb_4O_6$. This would appear to confirm the direct effect of CO_2 (Rossi-Bernardi & Roughton, 1967) on the reactions of haemoglobin with oxygen. The magnitude of the velocity constant v can be estimated from p derived previously, using

$$\frac{p}{l_4'} = \frac{v[CO_2]}{l_4'}.$$

A partial pressure of 3 cmHg CO_2 was used in these experiments, and the respective values of v/l_4' are 1·16 for sheep haemoglobin in solution and 0·84 for the same haemoglobin inside the erythrocyte. Since l_4' is some ten times faster than k_4', this means that the reaction of Hb_4O_6 with CO_2 would occur faster than recombination with O_2, under physiological conditions at the tissues. It is possible that the fall of v within the cell is associated with the binding of phosphate ions to haemoglobin, but the amount of

DPG in sheep cells is small. The preliminary data on human haemoglobin indicate a larger change.

The relevance of the alteration of the CO and O_2 velocity constants after reaction with CO_2 to structural alterations of the haemoglobin molecule is uncertain. There is no reason to believe the differences can in any way be associated with the collision frequency of O_2 or CO_2 molecules. The reaction of a small gas molecule with a large protein molecule would not directly involve a significant change of entropy. It could be possible, however, that the reaction and conformational changes of haemoglobin are simultaneous, and the entropy of activation may be altered in this way. The reaction of carbon monoxide with fully oxygenated haemoglobin is, however, a simple exponential rate process, until at least 50% of the reaction has occurred, so there must be no difference between Hb_4O_8 and Hb_4O_6CO in respect of dissociation and further chemical reaction. This would imply that the conformational change is the same whether CO or O_2 reacts with haemoglobin, and the relative change of one hundredfold in the velocity constants cannot be attributed directly to this. It could be, however, that the speed of the conformation change may be different for these ligands. In buffered solutions, without CO_2, there is no comparable effect of two reaction states, and this would suggest that following dissociation and reaction with hydrogen ions, any conformational changes must also be effectively instantaneous. The only remaining possibility is that the activation energy for CO and O_2 with the haem must vary. This is consistent with the movement of the iron atom and displacement of the haem proposed by Perutz (1970). There is no data on the effect of temperature on these constants available to resolve this possibility. There is a similarity in these results to the reactive form of haemoglobin observed by Gibson (1959) using flash photolysis. His experiments indicated, however, that the reactive form of haemoglobin appeared more evident at pH 9·1 than at pH 7·1. The conditions under which the reactive form can be observed are also very dependent on temperature and pH.

The results have a direct application to the use of CO in lung function tests. In order to calculate the diffusing capacity of the pulmonary membrane, D_M, from the over-all diffusing capacity of the lung, D_L, it is necessary to know the rate of uptake of gas by the red cells, θ. Estimates of θ in whole blood have been given by Roughton *et al.* (1957) and Holland (1969). Both of these sources have assumed that the kinetics of CO with HbO_2 within the cell obey the Gibson & Roughton (1955) equation given earlier and that the process is one of diffusion through the cell membrane followed by diffusion through and chemical reaction with the internal haemoglobin. Neither of these assumptions is justified. Data are not as yet available over a similar range of P_{O_2}/P_{CO} values, as given in this

paper, at 37° C, from which more realistic values of θ could be estimated.

It is now possible to discuss further the variation of the equilibrium constant, M, with pH, P_{CO_2} and pressure. The data of Rodney *et al.* (1969) would suggest that neither pH nor pressure has any effect on M. They discount earlier data of Roughton (1954), Allen & Root (1957) and Joels & Pugh (1958), which indicated that M changed with pH. All of these data are, however, consistent once it is appreciated that CO_2 is an important factor. Without CO_2, the Gibson & Roughton kinetic mechanism applies and there would be no effect of pH or absolute pressure for a given $[O_2]/[CO]$ ratio. Only one experiment of the results given by Rodney *et al.* was made with CO_2 present. Under *in vivo* conditions, in the presence of CO_2, the data and proposals presented in this paper should apply, and these are consistent with the variations of M noted by Allen & Root with varying P_{CO_2} values. There are no similar data available of the effect of absolute pressure in the presence of CO_2. However the present results suggest that a very small amount of CO in the air cylinders of subaqua divers could present a serious hazard as more CO will combine with haemoglobin the deeper they go.

The precise data of Joels & Pugh (1958), and Roughton (1965), also clearly demonstrate the change of M when equilibria curves of oxyhaemoglobin are compared at high saturation. A possible explanation of this phenomena may be given on the basis of the present analysis. At low P_{CO} and P_{O_2} values, the reaction rate with haemoglobin is relatively slow so that CO_2 will combine first. As the pressure of CO required to fully saturate haemoglobin is low, this will be the case throughout the HbCO dissociation curve. The pathway for CO reacting with haemoglobin would be via the form Hgb, considered earlier. With O_2, a much higher concentration is necessary for saturation. Under these circumstances more of the oxygen will react via the Hb pathway, with a different velocity constant. At high saturations of haemoglobin the reactions of CO and O_2 thus proceed by different pathways.

REFERENCES

ALLEN, T. A. & ROOT, W. S. (1957). Partition of carbon monoxide and oxygen between air and whole blood of rats, dogs and men as affected by plasma pH. *J. appl. Physiol.* **10**, 186–190.

GIBSON, Q. H. (1959). The kinetics of reactions between haemoglobin and gases. *Prog. Biophys. biophys. Chem.* **9**, 1–53.

GIBSON, Q. H. & ROUGHTON, F. J. W. (1955). The kinetics of dissociation of the first oxygen molecule from fully saturated oxyhaemoglobin in sheep blood solutions. *Proc. R. Soc.* B **143**, 310–334.

HALDANE, J. & LORRAIN SMITH, J. (1897). The absorption of oxygen by the lungs. *J. Physiol.* **22**, 231–258.

HOLLAND, R. A. B. (1969). Rate at which CO replaces O_2 from O_2Hb in red cells of different species. *Resp. Physiol.* **7**, 43–63.

JOELS, N. & PUGH, L. G. C. E. (1958). The carbon monoxide dissociation curve of human blood. *J. Physiol.* **142**, 63–77.

PERUTZ, M. F. (1970). Stereochemistry of cooperative effects in haemoglobin. *Nature, Lond.* **228**, 726–739.

RODNEY, F. L., O'NEAL, J. D. & COLLISON, H. A. (1969). Oxygen and carbon monoxide equilibria of human adult haemoglobin at atmospheric and elevated pressure. *Blood* **33**, 57–65.

ROSSI-BERNARDI, L. & ROUGHTON, F. J. W. (1967). The specific influence of carbon dioxide and carbamate compounds on the buffer power and Bohr effects in human haemoglobin solutions. *J. Physiol.* **189**, 1–29.

ROUGHTON, F. J. W. (1954). The equilibrium between carbon monoxide and sheep haemoglobin at very high percentage saturations. *J. Physiol.* **126**, 359–383.

ROUGHTON, F. J. W. (1964). Transport of oxygen and carbon dioxide. In *Handbook of Physiology*, section 3: Respiration, vol. I, ed. FENN, W. O. & RAHN, H., pp. 767–825. Washington, D.C.: American Physiological Society.

ROUGHTON, F. J. W. (1965). The relative affinities of human blood under physiological conditions for oxygen and carbon monoxide. *J. Physiol.* **179**, 65*P*.

ROUGHTON, F. J. W., FORSTER, R. E. & CANDER, L. (1957). Rate at which carbon monoxide replaces oxygen from combination with human haemoglobin in solution and in the red cell. *J. appl. Physiol.* **11**, 269–276.

SIRS, J. A. (1966). Egress of oxygen from sheep erythrocytes. *Biochim. biophys. Acta* **112**, 538–549.

SIRS, J. A. (1967). The egress of oxygen from human HbO_2 in solution and in the erythrocyte. *J. Physiol.* **189**, 461–473.

SIRS, J. A. & ROUGHTON, F. J. W. (1963). Stopped-flow measurements of CO and O_2 uptake by haemoglobin in sheep erythrocytes. *J. appl. Physiol.* **18**, 158–165.