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THE EGRESS OF OXYGEN FROM HUMAN HbO_2 IN SOLUTION AND IN THE ERYTHROCYTE

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SUMMARY

1. Spectrophotometric measurements, using the rapid-mixing constant-flow and stopped-flow techniques, have been made of the rate of egress of oxygen from human HbO_2 in solution and in erythrocytes.

2. By 1:20 dilution in a medium containing a high concentration of carbon monoxide k_4, the rate constant of the reaction $Hb_4O_8 \rightarrow Hb_4O_6 + O_2$ has been determined. At pH 7·4 and 20° C, k_4 in solution was 42·0 sec^{-1} and within the intact cell 41·4 sec^{-1}. The respective activation energies were 19·3 and 18 kcal.

3. The rate of dissociation of HbO_2 to Hb, after mixing an erythrocyte suspension with $Na_2S_2O_4$, was identical, over at least 50 % of the reaction, with the change of HbO_2 to HbCO above.

4. An analysis of the data indicates that the cell membrane has little or no resistance to the passage of oxygen and the internal contents are effectively mixed.

INTRODUCTION

Recent research has suggested that the respiratory function of the erythrocyte is influenced by the flexibility of its membrane (Sirs, 1964, 1966*a*). In its inflexible form, which can most simply be brought about by the complete removal of oxygen, the rate of uptake of gaseous ligands (O_2, CO and NO) is determined by a process of diffusion through the erythrocyte membrane, followed by diffusion through and chemical reaction with the internal haemoglobin. The earlier work of Roughton (1959, 1963) falls mainly in this category. A significant exception to this concept was, however, reported by Legge & Roughton (1950). The normal erythrocyte, in the presence of oxygen and after the minimum of preparatory treatment, can be flexed and distorted by very small forces. In these circumstances, experiments on the rate of egress of oxygen from HbO_2 in sheep erythrocytes have suggested that the rate of exchange is determined simply by the chemical reaction rates (Sirs, 1966*b*, *c*). In the past there have been some striking examples of the imprudence of drawing general

conclusions from research on the blood of a single species. The present results therefore represent an extension of these data to human cells, with the added practical advantage of future application to abnormal haemoglobins and studies of their respiratory function in a number of diseases.

Previous measurements of the rate of egress from the red blood cell (Roughton, 1932; Lawson, Holland & Forster, 1965) have utilized the rapid-mixing technique with $Na_2S_2O_4$, to reduce the extracellular O_2 concentration to zero. Difficulties in the use of $Na_2S_2O_4$ have been reported by Sirs (1966*d*) with sheep cells, so a more rigorous calibration procedure has been used in the present experiments. The more reliable dilution technique (Sirs, 1966*b*), with and without CO in the isotonic diluting medium, has also been used to ascertain the rate constant, k_4, for the reaction $Hb_4O_8 \rightarrow Hb_4O_6 + O_2$ within the cell.

METHODS

The rate of egress of oxygen has been measured by spectrophotometric observation of the change of HbO_2 with time, using the rapid-mixing apparatus of Sirs & Roughton (1963). For the dilution studies the relatively simple modification of Sirs (1966*b*) was utilized.

The human blood was obtained by venepuncture, heparin being used to prevent coagulation. The blood was immediately stored at 4° C and measurements normally made within 24 hr of collecting the blood sample. All samples were electrophoretically identified as of type HbA. Immediately before an experiment the blood was diluted in an isotonic solution consisting of (g/l.): NaCl, 7·6; $NaHCO_3$, 2; KCl, 0·42; $CaCl_2$, 0·24. The suspension was equilibrated with a given P_{O_2} and a P_{CO_2} of 3 cm Hg to adjust to pH 7·4 at room temperature. A similar solution, without red blood cells, and equilibrated with 3 cm P_{CO_2} and oxygen-free nitrogen was used as the medium with which the cells were diluted in the rapid-reaction apparatus. The oxygen pressure applied to the cell suspension was adjusted so that initially more than 95% HbO_2 was present, but after 1:20 dilution in the stopped-flow apparatus the P_{O_2} was only sufficient to maintain less than 5% saturation. The oxygen concentration was reduced to zero in some experiments by mixing the cells with a solution containing (g/l.): $Na_2S_2O_4$, 4; NaCl, 5·36; $NaHCO_3$, 2; KCl, 0·42; $CaCl_2$ 0·24. In other experiments the diluting medium was equilibrated with 3 cm P_{CO_2} and 110 cm P_{CO} so that, after mixing, conversion of HbO_2 to HbCO occurred.

The haemoglobin solutions were prepared by centrifuging and washing the cells in isotonic saline, followed by lysis with distilled water. The ghosts and debris were removed by centrifuging at 3000 *g* for 30 min. All oxygen was removed from the solution and replaced by oxygen-free nitrogen. The reduced haemoglobin was then stored at 4° C until used. For observations at pH 7·4 the Hb solution was diluted in the modified Ringer–Locke solution given above and equilibrated with O_2 and CO_2 in a similar manner. Some experiments were made at pH 8·2 simply by removing the CO_2 partial pressure.

Temperatures were varied between 4 and 38° C with a thermostatic control previously described (Sirs & Roughton, 1963). The temperature of the reacting mixture was measured with a thermometer placed in the outflow of the constant-flow apparatus and by a thermocouple inserted in the observation tube of the stopped-flow system. The pH was measured with a standard glass electrode and pH meter. Small samples were taken directly from the cell suspensions and from the outflow, and centrifuged to ensure the absence of haemolysis. Microscopic examination was also made to ensure no significant abnormality or crenation of the red blood cells was present.

RESULTS

Measurements of 'r' in solution. The applicability of the technique to human haemoglobin was tested by studying the reaction of

$$CO + HbO_2 \rightarrow HbCO + O_2$$

in solution. Gibson & Roughton (1955) have shown that, providing the sum of the oxygen and carbon monoxide concentrations is high, this follows a simple first-order dissociation, the rate constant of which is 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), which reduces to $k_4/4$ when $(O_2)/(CO)$ is

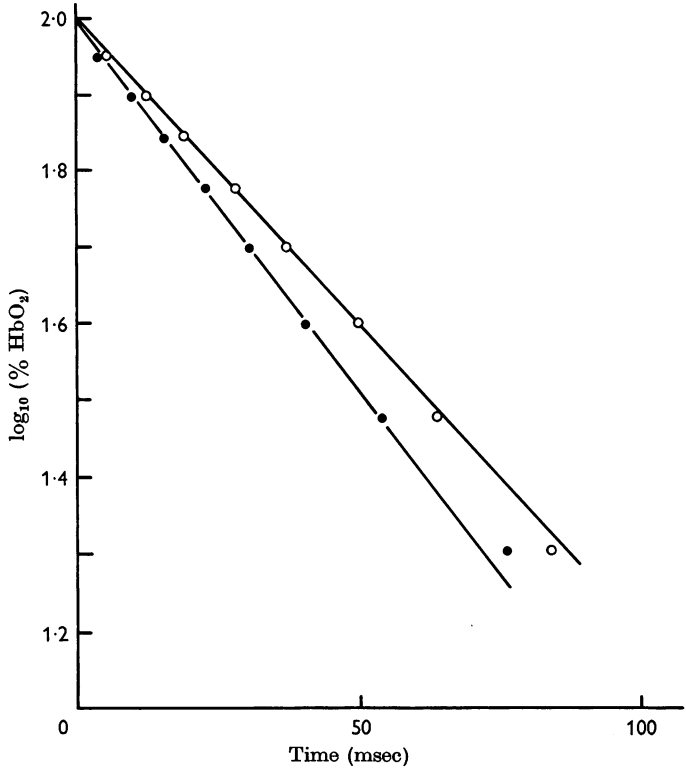

Fig. 1. A plot of $\log_{10}$ (% HbO_2) with time, obtained with the stopped-flow method diluting cells and Hb solution 1:20 in Ringer–Locke equilibrated with 110 cm P_{CO} at pH 7·4. ●, HbO_2 in solution equilibrated with 11 cm P_{O_2}, temperature 26·8° C, r = 22·4 sec^{-1}. ○, HbO_2 in erythrocytes equilibrated with 6 cm P_{O_2}, temperature 25° C, r = 17·4 sec^{-1}.

small. The haemoglobin solution, equivalent to a 1:10 dilution of whole blood, was equilibrated with 11 cm Hg P_{O_2} and 3 cm Hg P_{OC_2}, to maintain pH 7·4. This was diluted in the rapid reaction apparatus with a Ringer–Locke solution equilibrated with 100 cm Hg P_{CO} and 3 cm P_{CO_2} at pH 7·4. Thus, after mixing, the P_{O_2} is reduced to 0·52 cm Hg while the P_{CO_2} only falls to 95·5 cm, so that the ratio $(O_2)/(CO)$ is small and the rate determined by $k_4/4$ to within 2%. The change of HbO_2 to HbCO was observed

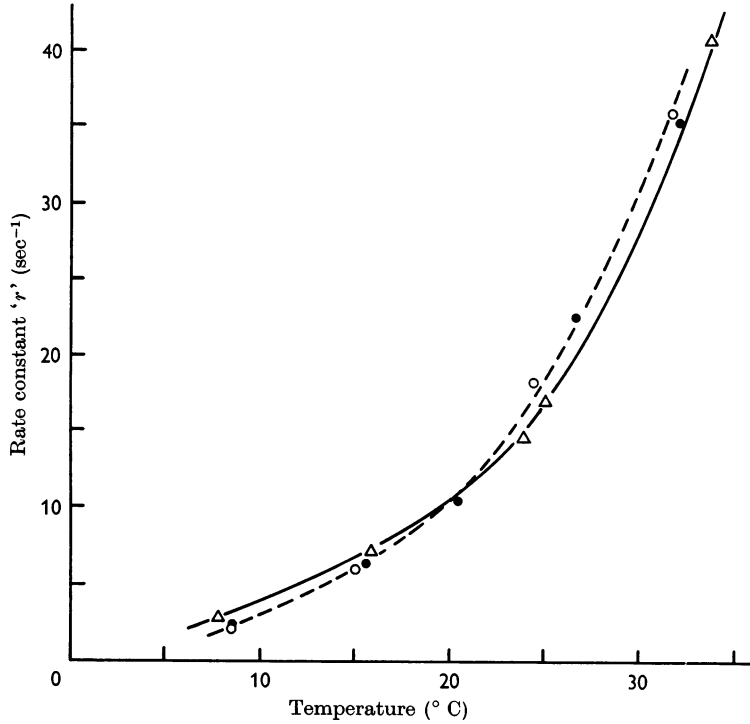

Fig. 2. The variation of the rate constant 'r' with temperature. Circles and interrupted line obtained in solution with blood samples from two male adults. Triangles and continuous line using an erythrocyte suspension obtained from the same donor as the open circles. All measurements were made at pH 7·4 and each point represents the mean of six curves at two different combinations of light filters.

spectrophotometrically, using light filters at two combinations of wavelengths, 560 and 582 mμ, then 520 and 460 mμ. No significant difference of the reaction curves was observed between these light filters. A plot of log HbO_2 with time was linear, as indicated in Fig. 1, in agreement with the Gibson & Roughton theory, and from the slope of the line 'r' can be calculated. Variation of the initial P_{O_2} from 4 to 12 cm, and of P_{CO} between 50 and 110 cm Hg, had no significant effect on the value of 'r'

within the experimental error of $\pm 5\%$. This confirms that the $(O_2)/(CO)$ term can be neglected and 'r' is equivalent to $k_4/4$. These rate constants, and the activation energy obtained from the variation of 'r' with temperature indicated in Fig. 2, are in close agreement with the data of Gibson, Kreuzer, Meda & Roughton (1955), obtained at pH 7·4 with the more laborious displacement method. The rates in solution were obtained on blood collected from only two individuals. No difference was found between them, though a large difference of the rates of egress under similar conditions was found in studies using intact cells. This is thought to be due to the inflexibility of the red blood cells of this donor and not to changes of $k_4/4$ within the cell.

Measurement of 'r' in erythrocytes. Experiments with sheep erythrocytes (Sirs, 1966*b*) have indicated that equation (1) also applies when normal erythrocytes are similarly diluted in an isotonic medium. Providing P_{O_2} is low and P_{CO} high, after mixing, the rate constant 'r' would be a measure of $k_4/4$ within the cell. Any impedance to the transport of oxygen, such as a low diffusion constant through haemoglobin or resistance by the cell membrane, would, however, raise the oxygen concentration within the cell so that 'r' would then be correspondingly less than $k_4/4$. This possibility has been investigated by observing the dissociation of HbO_2 to HbCO within the cells after rapid mixing and simultaneous 1:20 dilution. The whole human blood was initially diluted 1:10 in the modified Ringer–Locke solution and equilibrated with P_{O_2} within the range 4–12 cm Hg. The P_{CO} applied to the isotonic diluting medium was varied from 50 to 110 cm Hg. No significant difference of 'r' was observed with normal discoidal erythrocytes after variation of the P_{O_2} or P_{CO}. In all these experiments the initial HbO_2 was more than 95% saturated, the pH 7·4 and temperature 20–22° C. The concentration of oxygen within the cell cannot therefore be large, for some difference would then have been found, and 'r' must be a valid measure of $k_4/4$. Further confirmation of this interpretation, and that the process is effectively a first-order dissociation, is obtained by plotting log HbO_2 with time, as shown in Fig. 1. This is again linear over a wide range with a slope of 'r'. Altering the light filters from 520 and 460 mμ to 560 and 582 mμ had no effect on the recorded reaction curve. The same rate was obtained with an initial cell suspension of 1:5 whole blood in Ringer–Locke solution and 1:15 initial dilution. The variation of 'r' with temperature, obtained with 4 to 11 cm Hg P_{O_2} and the diluting medium equilibrated with 110 cm P_{CO}, is shown in Fig. 2. Blood samples from twelve adults, including both sexes, have been studied. The value of the rate constant 'r' obtained with ten of these agreed within $\pm 7\%$. With one individual the rate constant was 20% higher, and the difference was confirmed with two further samples

taken from this donor during the following 6 months. The twelfth sample gave a low value of r. Further investigation revealed that 'r' was very dependent in this case on the P_{CO} used. Whereas at 110 cm P_{CO} the rate was 15% below the others, at 50 cm P_{CO} it had fallen by 30%. The comparable rate in solution, as indicated above, showed no similar abnormality. No other clinical symptoms were apparent and the individual concerned led a normal life. Further investigations are in progress.

The rate of egress from cells after mixing with $Na_2S_2O_4$. Previous observations, with sheep erythrocytes, of the rate of conversion of HbO_2 to Hb after mixing an erythrocyte suspension with a solution containing $Na_2S_2O_4$ indicated that errors could arise with $Na_2S_2O_4$ due to differences of optical homogeneity. The control procedures adopted in the earlier experiments of Sirs (1966*d*) have therefore been used.

With the constant flow technique, a rather involved procedure was followed to provide a check on the added effect of dithionite and enable a calibration of % HbO_2 against deflexion to be obtained. All the solutions contained red blood cells equivalent to 1 ml. of whole blood in 100 ml. of suspension. Four suspensions were: used (*a*) reduced cells, 0% HbO_2; (*b*) cells equilibrated with 5 cm air, x% HbO_2; (*c*) cells equilibrated with 8 cm P_{O_2}, 100% HbO_2; (*d*) blood which had been initially reduced and then diluted in an isotonic solution containing $Na_2S_2O_4$ (4 g/l.). All solutions were equilibrated with 3 cm P_{CO_2} and maintained at pH 7·4. The exact percentage saturation of the suspension equilibrated with 5 cm air was not determined, as its function was simply to provide a constant reference between 0 and 100% HbO_2 in order to check the sensitivity and linearity of calibration at different points along the observation tube. The following solutions and mixtures were flowed in turn through the mixing-chamber and down a 2 mm constant-bore observation tube; (1) x% HbO_2 cells; (2) reduced Hb cells, no $Na_2S_2O_4$; (3) reacting mixture, reduced cells + HbO_2 cells equilibrated with 4 cm P_{O_2}, no $Na_2S_2O_4$, with uptake of O_2; (4) HbO_2 cells; (5) HbO_2 cells + reduced cells in a medium containing $Na_2S_2O_4$ (4 g/l.), producing egress of O_2; (6) reduced cells in the dithionite medium; (7) repeat of (5); (8) repeat of (5) with sudden stopping of the outlet to indicate the reacted end-point. The optical density of each of these flowing solutions was determined with a split-beam spectrophotometric detector (Sirs & Roughton, 1963) and displayed on a pen recorder. When a filter of 432 mμ was placed in one light path, of the split-beam detector, and 480 mμ in the other, the recorded deflexions (2), (6) and (8) did not agree. The reduced cells in dithionite appeared to be more reduced than those in Ringer–Locke solution (*a*). The stopped flow end-point (8) fell between these two. When light filters of longer wave-length than 480 mμ were used, such as 560 and 542 mμ, this difference did not

appear. A complete series of points, obtained at different distances down the observation tube from the mixing-point, is shown in Fig. 3. The value of '*r*', calculated in terms of the initial rate of change of HbO_2 relative to the 100 % deflexion, is 14 sec^{-1} at 23° C.

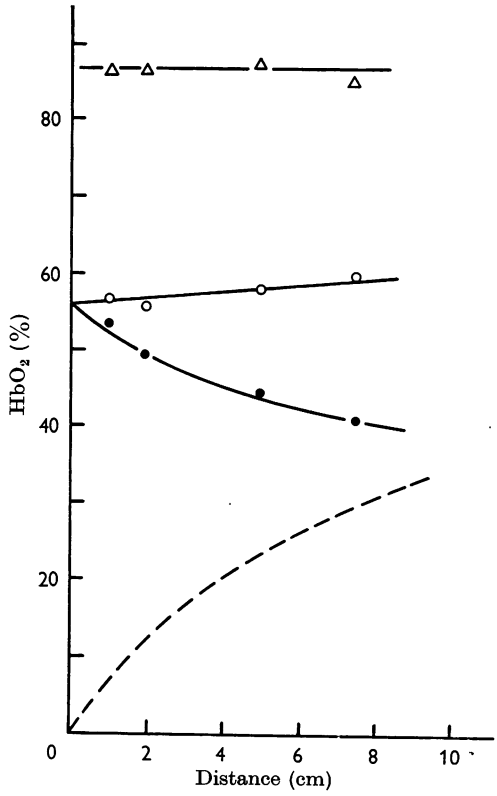

Fig. 3. Complete curves obtained at various points along the observation tube with the constant-flow technique and blood cells in all the solutions, as stated in the text. △ *x*% HbO_2; ○ uptake of O_2; ● egress of O_2 after mixing with $Na_2S_2O_4$. Light filters 560 and 542 mμ, flow rate 198 cm sec^{-1}, temperature 23° C, pH 7·4. From the initial slope r = 14·2 sec^{-1}. The interrupted line corresponds to the formation of Hb, after mixing with $Na_2S_2O_4$, on a 100 % scale.

In principle the difficulty of measuring the % HbO_2 in the cells, using reference suspensions of different optical homogeneity, is avoided with the stopped-flow procedure. The complete reaction is observed in this case on the same fraction of cells and in a uniform suspending medium. The major problem with this type of experiment, however, was how to obtain a comparative calibration curve of % HbO_2 against deflexion. Once $Na_2S_2O_4$ is added to the solution, as in the stopped-flow reaction, no means exists of obtaining various constant % HbO_2 conditions for equivalent

calibration. To assess this factor the concentration of cells in the suspension was varied and measurements made with different combinations of light filters. Normally, when equal quantities of cell suspension and reacting solution are mixed together, in the absence of $Na_2S_2O_4$, agreement can be obtained at initial blood dilutions below 1:200 when light filter combinations of 432 and 480 mμ are compared with 560 and 542 mμ. In the presence of dithionite this did not occur and even at an initial dilution of

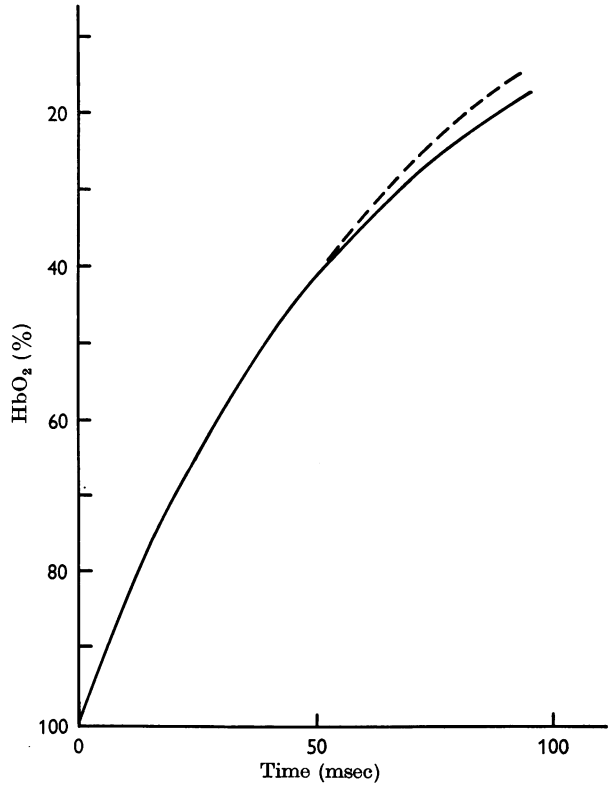

Fig. 4. A comparison of the rate of egress after mixing and 1:20 dilution in Ringer–Locke equilibrated with 100 cm P_{CO} (interrupted curve) and after dilution in an isotonic solution of $Na_2S_2O_4$ (4 g/l.) (continuous curve). Stopped-flow, pH 7·4, 25° C. Each curve is the mean of six determinations at two different combinations of light filters, which were made concurrently with the same HbO_2 erythrocyte suspension.

whole blood of 1:250 the 432 and 480 mμ filter combination gave a value of '*r*' 20 % higher than with the 560 and 542 mμ filters. A similar problem was encountered previously with sheep's blood (Sirs, 1966*d*). To minimize this effect the present results have been obtained by combining the $Na_2S_2O_4$ action with the dilution technique. Under these circumstances,

immediately after rapid mixing, the $Na_2S_2O_4$ concentration is only reduced from 0·4 to 0·38 %, but the external oxygen concentration is physically diluted 1:20. This means that any initial phase lag of $Na_2S_2O_4$ removing the oxygen is avoided and the concentration of O_2 to be removed, and in turn the acidic by-products formed, are minimal. A greater measure of agreement was then obtained with the above light filter combinations. A series of comparisons were made with six of the normal individuals mentioned previously of the rate of displacement of HbO_2 to HbCO after mixing with an isotonic solution equilibrated with 110 cm P_{CO}, followed by mixing and dilution with the solution of $Na_2S_2O_4$ (4 g/l.). A typical

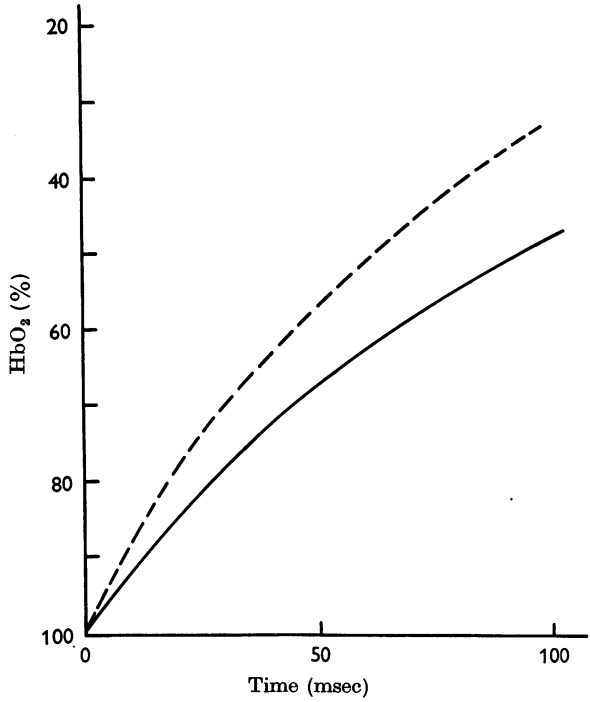

Fig. 5. A comparison of the rate of egress after mixing and diluting with P_{CO} (interrupted curve) and $Na_2S_2O_4$ (continuous curve) at pH 8·2. Temperature 23·9° C. The rate constant 'r' for the P_{CO} curve, calculated from the half-time, is 11·1 sec^{-1}.

curve is shown in Fig. 4. It will be noted that the simple egress and change of HbO_2 to Hb follows the chemical rate of dissociation, determined by k_4, over at least 50 % of the reaction. In some cases the two curves are almost identical over the whole range of reaction but this is difficult to establish decisively as it is very dependent on the exact line taken as the completely reacted (100 % Hb) condition. At pH 8·2, obtained by making up the solution without equilibrating with carbon dioxide, the chemical

reaction rate ($k_4/4$), as determined by mixing with CO, is slightly lowered, consistent with the small reduction found in solution (Gibson *et al.* 1955). The rate of change of HbO_2 to Hb after mixing with $Na_2S_2O_4$ is, however, slower still, as is indicated in Fig. 5. This is believed to be due to a slight decrease of flexibility of the erythrocyte in alkaline pH.

With normal cells at pH 7·4, no variation of the rate of egress occurred upon mixing with $Na_2S_2O_4$ when the cells were equilibrated with 4 cm P_{O_2} or 12 cm P_{O_2} at 20–22° C. Provided the whole blood is quickly cooled and stored at 4° C, the rate of egress after mixing with $Na_2S_2O_4$ is normally constant over a period of 4 hr following the start of the experiment and equivalent to the dissociation rate of HbO_2 to HbCO determined on the same suspension in parallel experiments.

DISCUSSION

An analysis of the process determining the rate of egress of HbO_2, when the oxygenated cells are mixed with an isotonic solution equilibrated with a high P_{CO} has been given by Sirs (1966*b*). This suggested that the rate is determined simply by equation (1) given previously. Physically this can be explained on the basis that as oxygen is released from HbO_2 its place is almost always taken by carbon monoxide, providing the relative concentration of O_2 to CO is small. This is normally the case, as very rapid equilibration of CO occurs when the cell initially contains 100 % HbO_2. Thus O_2 diffuses out of the cell with negligible chance of reacting again, and in this sense is akin to an inert gas such as N_2. The diffusion time in these circumstances, even allowing for the most adverse experimental data reported for the diffusion coefficient in haemoglobin solutions and membrane permeability is less than 2 msec. If, however, diffusion through the internal haemoglobin and membrane are involved, the concentration of oxygen in the cell interior will be correspondingly raised and, if significant, will reduce 'r' by increasing the ratio $(O_2)/(CO)$. The magnitude of the oxygen concentration within the cell is dependent on the haemoglobin concentration, y_0, the cell thickness, $2b_1$, and the diffusion constant of oxygen through haemoglobin, D_1, and can be calculated (Sirs, 1966*b*) from

$$C = \frac{ry_0}{2D_1}[b_1^2(1+2/\lambda)-x^2], \tag{2}$$

where $$\lambda = \frac{\text{permeability of membrane}}{\text{permeability of interior Hb}} = \frac{D_2/b_2}{D_1/b_1}.$$

The initial rate constants 'r' obtained with red blood cell suspensions are comparable with those obtained with solutions of haemoglobin and suggest

that they are a measure of $k_4/4$ within the cell. This is further supported by calculation of the oxygen pressure that is possible within the cell on the basis of equation (2). If the values of

$$D_1 = 4{\cdot}76 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1},\ y_0 = 20 \text{ mM},\ b_1 = 9{\cdot}85 \times 10^{-5} \text{ cm}$$

(Roughton, 1959) and the experimental value of $r = 10{\cdot}4 \text{ sec}^{-1}$ at 20° C are used, the mean partial pressure throughout the cell interior can be calculated as approximately 6·5 cm Hg. Inserting this value into equation (1), with P_{CO} of 95 cm Hg after mixing, allowing for the difference of solubility and taking the ratio k_4/l_4 as three, the value of 'r' would be 21·5 % lower than $k_4/4$. If this difference was present, however, lowering the CO concentration by half would increase this to 35·5 %. As no significant difference, within the experimental error of ± 5 %, was observed on lowering the P_{CO} from 110 to 50 cm Hg, 'r' must be a valid measure of $k_4/4$. A difference of 5 % between the 110 and 50 cm P_{CO} rates would correspond to a mean partial pressure of only 1·2 cm Hg within the cell. More extensive measurements with sheep blood cells indicated that an even lower limit of not greater than 0·4 cm P_{O_2} was involved (Sirs, 1966*b*). In order to account for this in terms of equation (2), D_1 must be increased by an order of magnitude and λ be infinite. The rate of egress obtained after mixing the human cells with dithionite supports this conclusion. Though the oxygen pressure external to the cells must be close to zero in these circumstances, if the diffusion of oxygen through the internal haemoglobin and cell membrane were significant factors, the oxygen concentration within the cell would be finite. The rate of egress would then be much slower than given by $k_4/4$, and the back reaction of oxygen with reduced haemoglobin within the cell would also become a limiting factor, as has been shown by Nicolson & Roughton (1951). The rates of egress after mixing with CO and $Na_2S_2O_4$ are closely in agreement, which must imply that neither diffusion of oxygen through the membrane or internal haemoglobin significantly limits the rate of exchange. It may be noted, however, that the constant-flow experiment does not follow the chemical reaction rate for more than 25 % of the dissociation. This is believed to be due to the inhibitory effect of turbulence (Sirs, 1966*d*). That the contents of the erythrocyte are effectively mixed by oscillatory movement of water, as the cells are flexed and distorted, has previously been suggested by Sirs (1964). Prothero & Burton (1961) considered that stirring of the internal contents was likely, as the cell shape altered, but their interpretation did not take into account that at the high concentration of Hb within the cell the molecules must be touching (Perutz, 1948) and therefore be immobile relative to each other. The effect of alkaline pH is to slightly reduce the flexibility of the cell (Teitel & Nicolau, 1964) and

correspondingly lowers the rate of egress after mixing with $Na_2S_2O_4$, as indicated in Fig. 5. The rate constant 'r' obtained at pH 8·2 is consistent with the effect of alkalinity on $k_4/4$ observed with sheep and human haemoglobin in solution (Gibson & Roughton, 1955; Gibson *et al.* 1955). A reduction of the rate of egress of O_2 by human erythrocytes occurs after decreasing the cell flexibility by adding formaldehyde or heating (Sirs, 1966*a*). The lowered rates obtained, after mixing the erythrocytes with CO, reported for the twelfth individual, are also consistent with the erythrocytes being inflexible in this case. A number of factors are now thought to alter the red cell flexibility *in vivo* but the origin of the effect in this particular individual is not known.

When the HbO_2 cells are mixed and diluted in an isotonic solution containing a high concentration of CO, a plot of log HbO_2 with time is linear (Fig. 1), as would be expected with a process determined by a first-order dissociation. From the slope of this line 'r' can be calculated and hence, by multiplying by four, k_4. The activation energy of this constant can be obtained from the slope of a plot of log k_4 against the reciprocal of the absolute temperature, using the values given in Fig. 2. For haemoglobin in solution this was 19·3 kcal $\pm$ 5 %, and in the red blood cell 18 kcal $\pm$ 7 %. The value in solution is in agreement with that previously obtained by Gibson *et al.* (1955). A decrease of the energy of activation within the red blood cell was also found with sheep cells (Sirs, 1966*c*). The effect of haemolysis and dilution is thus not particularly detrimental, as was in fact suggested by the earlier O_2 equilibrium data, but calculation of the entropy of activation suggests that haem–haem interaction is less within the cell (Sirs, 1966*c*).

A general conclusion can therefore be drawn that the data and implications obtained with sheep's blood are supported by the present experiments on human erythrocytes. In normal circumstances, the cell membrane offers little or no resistance to the passage of oxygen and the cell contents are effectively mixed. The rate of egress is then determined by the chemical reaction of dissociation of HbO_2 within the cell, the velocity constant of which is similar to that obtained with haemoglobin in solution.

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