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RATE LIMITING PROCESSES IN THE BOHR SHIFT IN HUMAN RED CELLS

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SUMMARY

1. The rates of the Bohr shift of human red cells and some of its constituent reactions have been studied with a modified Hartridge–Roughton rapid reaction apparatus using an oxygen electrode to measure the progress of the reaction.

2. The rate of the Bohr shift was compatible with the hypothesis that the transfer of H^+ across the membrane by means of CO_2 exchange and reaction with buffers is generally the rate-limiting step.

(*a*) When the Bohr off-reaction was produced by a marked increase in P_{CO_2} around the cells, the half-time at 37° C was 0·12 sec. In this case CO_2 was available initially to diffuse into the cells, the process being predominantly limited by the rate of intracellular CO_2 hydration.

(*b*) When the Bohr off-shift was produced by an increase of $[H^+]$ outside the cell, P_{CO_2} being low and equal within and outside the cells, the half time became 0·31 sec. In this case, even at the start, the H_2CO_3 formed by the almost instantaneous neutralization reaction of H^+ and HCO_3^- had to dehydrate to form CO_2 and this in turn had to diffuse into and react within the red cell before the $[HbO_2]$ could change. When a carbonic anhydrase inhibitor was added to slow the CO_2 reaction inside the cell, the half-time rose to 10 sec.

(*c*) The Bohr off-shift in a haemolysed cell suspension produced by an increase in P_{CO_2} appeared to be limited by the rate at which the CO_2 could hydrate to form H^+.

3. The Bohr off-shift has an average Q_{10} of 2·5 between 42·5 and 28° C with an activation energy of 8000 cal.

4. The pronounced importance of the CO_2-bicarbonate system for rapid intracellular pH changes is discussed in connexion with some physiological situations.

INTRODUCTION

The Bohr shift* plays an important role in respiratory gas exchange. Craw, Constantine, Morello & Forster (1963) measured the rate of the Bohr shift of human red cells at 37° C under conditions where the pH was changed by changing the external P_{CO_2}. In the present study we have extended their observations using the rapid-flow technique, with the aim of (1) measuring the rate of the Bohr shift under a variety of conditions which may approach those in the organism, and (2) measuring the rate-limiting effect of parts of the Bohr shift.

To this end we have measured the rate of the Bohr shift at different temperatures and under conditions where the pH has been changed by another acid than CO_2, notably by lactic acid. In this latter case the relative importance of CO_2 and hydrogen ions as transmitters of acidity across the red cell membrane was studied by adding carbonic anhydrase or an inhibitor of this enzyme to the reaction mixture. We have also tried to evaluate the rate-limiting influence of the volume of the intracellular water space by measuring the rate of the Bohr shift in osmotically swollen and shrunken red cells.

METHODS

General. These experiments were carried out with the same modified Hartridge–Roughton rapid reaction apparatus (Hartridge & Roughton, 1923*a*) that was originally used by Craw *et al.* (1963) (Fig. 1). The object of the method is to cause an abrupt change in chemical composition of the extracellular fluid and measure the rate of the resultant appearance or disappearance of dissolved O_2. This is accomplished in the following way. A red cell suspension at a certain known pH, P_{CO_2} and P_{O_2} is mixed with a buffer solution of different pH and possibly P_{CO_2} but of the same P_{O_2}, and travels at a known, constant speed through a uniform bore observation tube to the P_{O_2} electrode. By using observation tubes of different lengths the time interval from mixing (i.e. from changing the pH) can be varied. The time course of the Bohr shift and thereby its rate can then be obtained.

The rate of oxygenation or deoxygenation was studied with the same method, but in these experiments the P_{O_2} of the solutions were different while the pH and P_{CO_2} were the same. The P_{O_2} of the mixture immediately after mixing, which will be the weighted average of the P_{O_2} of the two solutions, will not be in equilibrium with the haemoglobin. As the chemical reaction $Hb + O_2 \rightleftharpoons HbO_2$ proceeds a new P_{O_2} will be established in the mixture at a measurable rate.

* Since Bohr, Hasselbalch & Krogh (1904) described the effect of variations in P_{CO_2} and not just the effect of the molecular species of CO_2 upon the O_2 affinity of blood, it seems reasonable to the authors that the term 'Bohr shift' should include effects on the O_2 affinity of hydrogen ions, regardless of the anion. In this article, therefore, 'Bohr shift' will mean the effect of variations in pH upon the O_2 affinity of haemoglobin. The shift can occur in two directions in that pH can either increase or decrease. Since it is convenient to refer to these cases separately, we have chosen to call the phenomenon connected with acidification a Bohr off-shift, while the opposite is termed a Bohr on-shift, 'off' and 'on' referring to the direction of O_2 movement relative to Hb (or to O_2-affinity). In the lungs therefore, we have a Bohr on-shift while in the tissue a Bohr off-shift.

Red cell suspension. About 50 ml. of venous blood was drawn from one of six healthy young male subjects into a sterile syringe whose dead space contained heparin, and then added to 1·75 l. of Solution no. 1 of composition (mM): NaCl 115; KCl 3·5, KH_2PO_4 0·25, Na_2HPO_4 2·5, $NaHCO_3$ 25·7. The resulting red cell suspension was placed in a 2 l. bottle in a bath thermo-regulated to within 0·5° C. The suspension was kept in equilibrium with a known (Scholander, 1947) gas mixture by bubbling through an aquarium stone. This procedure was shown to give a constant P_{O_2} within ½ hr and to maintain it throughout the experiment. No haemolysis was observed although foaming was profuse.

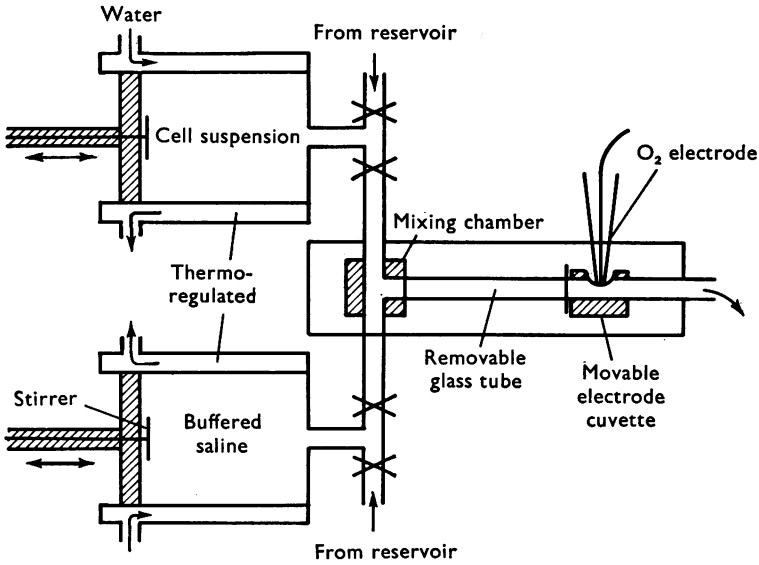

Fig. 1. Schematic drawing of the rapid reaction apparatus used in this investigation. The shading indicates solid material, plastic or stainless-steel.

Buffer solution. A similar volume of Solution no. 2, of composition (mM): NaCl 125·7, KCl 3·5, KH_2PO_4 0·25, Na_2HPO_4 2·5, $NaHCO_3$ 15, without red cells was equilibrated in the same way with a known gas mixture. In some experiments one or both solutions were modified to give the desired pH by addition of different volumes of 0·17 M lactic acid. The osmolarity of all solutions was within 2·5% of average human plasma and the concentration of individual electrolytes were similar to those of human plasma. The concentration of bicarbonate in the buffer solution was reduced below that in the cell suspension (Solution 1) so that the pH of the mixture would be within physiological range. The exact composition of the solutions and of the mixture will depend upon the pH and P_{CO_2}. Sufficient information is presented (Table 1) to calculate the exact composition at any steady state situation.

Acetazolamide (Diamox supplied by courtesy of Lederle Labs, American Cyanamid) was used as a carbonic acid anhydrase inhibitor. It was added to the cell suspension to a concentration of approximately 10^{-3} M. Crystalline carbonic anhydrase (Nutritional Biochemicals) was used in a concentration of 20 mg/l. of solution. Hb-solutions were prepared by haemolysing the blood in a certain volume of distilled water and then adding a similar volume of double strength Solution no. 1.

Mixing of solutions. The two solutions were separately drawn from the reservoirs and into two motor driven syringes of 130 ml. capacity. Their contents were stirred and thermo-

regulated by water jackets to within 0·5° C. They could be emptied into the 4-jet-mixing chamber of this apparatus at constant known speeds. It has been shown previously that mixing, with this chamber at the fluid velocities used, is complete in less than 2 msec (Staub, Bishop & Forster, 1961). From the mixing chamber the mixture travelled, still at a constant known speed, through a 1·69 mm bore glass observation tube and then past the O_2 electrode which was mounted in a methacrylate cuvette providing a measure of the P_{O_2} of the fluid at that point. At the syringe speed selected for these experiments the linear velocity of the mixture was 2·78 m/sec and thus each centimetre of observation tube represented 3·59 msec. By inserting observation tubes of different lengths into the apparatus, changes in P_{O_2} with time could be determined. The end-point for change in P_{O_2} was obtained by mixing the two solutions anaerobically in the desired proportions in one of the syringes and then passing the mixture through the measuring cuvette.

P_{O_2} recording. A 0·05 cm diameter platinum electrode covered with a 0·00125 cm Teflon membrane was used in a modification of the Clark cell with an electrolyte of 3 M-KCl and 1 M-$NaHCO_3$. Current through the oxygen cell was amplified using an amplifier designed and built by Mr George Pierce at the University of Pennsylvania and recorded on a direct-writing potentiometer recorder. Duplicate determinations of the O_2 tension of the reacting mixture were carried out at each point, that is with each observation tube. The single runs were carried out as follows:

One of the two syringes was started at a given speed. When the O_2 potential record had reached a steady plateau (about 7 sec) the other syringe was started at half the speed of the first (Fig. 2). The switch that started the second syringe also reduced the speed of the first one to half of its original value. When the O_2 potential record was again steady the first syringe was stopped and the second syringe alone delivered fluid, now at twice its previous speed. By this procedure the linear velocity of the fluid (to which the electrical signal from the O_2 potential electrode is somewhat sensitive) is kept constant. Seventy to a hundred millilitres of fluid from each syringe were required for a single run.

This procedure has a built-in calibration since the O_2 tensions of the two separate solutions are known from analysis of the equilibration gases and the current through the electrode is a linear function of P_{O_2}. The electrode was further calibrated by sending saline, equilibrated for 1 hr with O_2-free N_2 at the same speed past the electrode. It turned out, not unexpectedly, that the deflexion was identical with that obtained when the electrode was exposed to a stream of N_2-gas. This zero point calibration was frequently checked during the course of the runs. It turned out to be crucial to test the electrode against fluids of the same P_{O_2} but varying P_{CO_2} since the electrode was sometimes sensitive to CO_2. This anomaly could usually be eliminated by changing the electrode fluid. Repeated measurements on the same fluid gave O_2 tensions that usually corresponded within 1 mm Hg. The 100 % response time of the electrode and recording system was about 7 sec. The noise level corresponded to less than 0·05 mm Hg oxygen tension. The record chart could be read to within $\pm 0{\cdot}1$ mm Hg.

Total haemoglobin in the red cell suspension was measured spectrometrically as cyanmethaemoglobin with an accuracy of $\pm 0{\cdot}015$ g/l. pH values within 0·02 units were obtained with a glass pH-meter using a closed electrode chamber into which samples were anaerobically transferred. The measured P_{CO_2} was checked against calculated values obtained by combining the Henderson–Hasselbalch equation with data on the ionic composition of the solutions. Usually the two results agreed within 2 mm Hg and when not, the calculated value is quoted. In the Henderson–Hasselbalch equation we used the solubility coefficients (α) given for 0·155 N-NaCl in *Handbook of Respiration* (1958) and pK equal to $6{\cdot}33-0{\cdot}5\,\mu$, where μ is the ionic strength (Hastings & Sendroy, 1925). At 37° C the pK of carbonic acid in 0·155 N-NaCl becomes 6·10, and it is increased by 0·0078 per °C decrease in temperature.

Calculation of results. A typical record is shown in Fig. 2. In this particular case blood with 145 g Hb/l. was diluted 1:50 with Solution no. 1. The resulting suspension was equilibrated at 31° C with gas in which the P_{O_2} was 26 mm Hg and the P_{CO_2} was 10 mm Hg.

ending up with a pH of 7·96. In the rapid reaction apparatus it was diluted 1:1 with buffer no. 2 which had a P_{O_2} of 25 mm Hg, a P_{CO_2} of 132 mm Hg and a pH of 6·85. The recording is obtained 1·730 sec after mixing and shows a P_{O_2} of 37·4 mm Hg. The pH of the mixture at equilibrium was 7·28, the P_{CO_2} 62 mm Hg and the P_{O_2} 38 mm Hg. The change in P_{O_2} from the moment of mixing to the time the measurement was taken was $\{37{\cdot}4-\frac{1}{2}(25{\cdot}0+26{\cdot}0)\} = 11{\cdot}9$. Note that the delivery rates of the two syringes were adjusted to be equal. This change in P_{O_2} was transformed into a change in percent saturation of haemoglobin

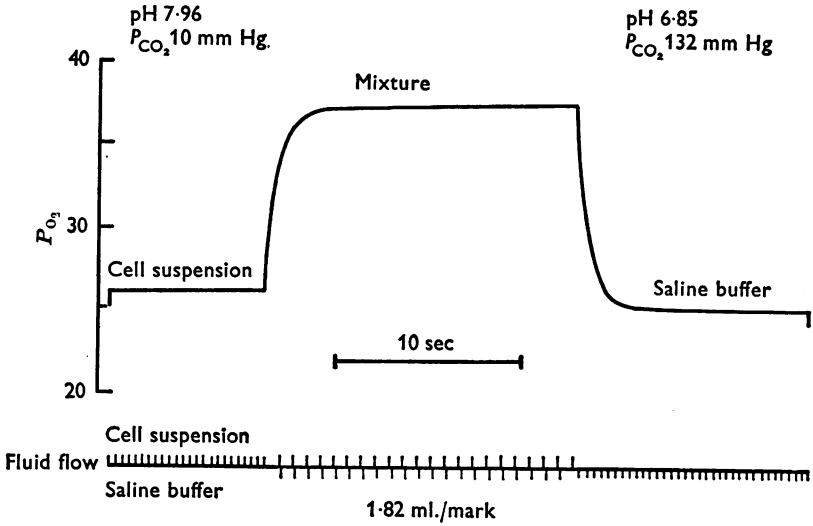

Fig. 2. Example of a typical experimental record (traced). To economize with space, the volume marker has been moved upwards and the zero point for P_{O_2} is therefore not shown. Bohr off-shift; cell suspension at 31° C; elapsed time 1·73 sec.

(Δ HbO_2 %) in the following way: The increase in dissolved O_2 represents $(11{\cdot}9/760) \times 0{\cdot}0246 = 0{\cdot}000385$ ml. O_2/ml. solution where 0·0246 is the solubility of O_2 in 0·15 N-NaCl at 1 atm (Handbook of Respiration, 1958) and 11·9 is the change in O_2 tension. In the mixture the Hb concentration was 145/100 = 1·45 g/l. Since the O_2 capacity of haemoglobin is 1·34 ml. O_2/g Hb the O_2 capacity of the mixture is $1{\cdot}45 \times 1{\cdot}34/1000 = 0{\cdot}00177$ ml. O_2/ml. solution. Thus the % fall in HbO_2 is $0{\cdot}000385/0{\cdot}00177 = 21{\cdot}8$ %. Initial values for HbO_2 saturation were obtained from published dissociation curves (*Handbook of Physiology*, 1958) using the P_{O_2} and pH of the cell suspension. Owing to the difference in composition between normal human plasma, to which the published curves apply, and Solution 1, the estimates of HbO_2 are probably not very precise, but in this investigation are relied upon primarily for comparative magnitude.

Experiments

The following groups of experiments were performed:

I. Measurement of the rate of the Bohr off-shift of cells at 42·5, 37, 31 and 23° C mainly by an increase in P_{CO_2}. The cells were suspended in solution with [$NaHCO_3$] equal to approximately 26 mM and mixed with buffer having approximately 16 mM-$NaHCO_3$. These experiments were

performed in two series, one with an initial HbO_2 % of 86–88 %, the other at an initial HbO_2 % of 93–97 %.

II. Measurement of the rate of the Bohr off-shift at 37° C in shrunken and swollen cells. In the first case both solutions were prepared at 2 times their original strength, in the second they were diluted to 2/3 their original ionic strength.

III. Measurement of the rate of the Bohr off-shift at constant P_{CO_2} with external pH changed by addition of lactic acid at 37° C. The cells were suspended in Solution no. 1 and mixed with Solution no. 2, to every litre of which had been added about 150 ml. 0·17 M lactic acid. Such experiments were also performed after the addition of 10^{-3} M acetazolamide.

IV. Measurement of the rate of Bohr off-shift of a solution of human haemoglobin at 37° C. The haemoglobin was dissolved in Solution no. 1, and mixed with buffer no. 2 which had been equilibrated with gas of P_{CO_2} = 150 mm Hg.

V. Measurement of the rate of the Bohr on-shift of cells at 37° C when P_{CO_2} was decreased and external pH was increased. Cells were suspended in buffer no. 1 equilibrated with gas which had a P_{CO_2} of 150 mm Hg. This suspension was then mixed with a solution consisting of buffer no. 2 to every litre of which had been added 10 ml. of 0·17 M-Na_3PO_4 and which had then been equilibrated with a P_{CO_2} of 8 mm Hg. Such experiments were carried out also after addition of 20 mg carbonic anhydrase per litre to both solutions.

VI. Measurements of the rates of deoxygenation and oxygenation of red cell suspensions at 37° C and zero P_{CO_2}. For the first, a cell suspension which had a P_{O_2} of 35 mm Hg was mixed with a buffer with zero P_{O_2}. For the second, a red cell suspension with zero P_{O_2} was mixed with a buffer which had a P_{O_2} of 38 mm Hg. To each litre about 100 ml. lactic acid, 0·17 M, was added to reduce $[HCO_3^-]$ to near zero.

RESULTS

The compositions of the reacting solutions and the mixtures at complete equilibrium and the conditions of the six groups of experiments are given in Table 1. The composition of the suspending fluid in the reacting mixture immediately after mixing but before any reaction involving CO_2 or the movement of chemicals across the red cell membrane has taken place is shown in Table 2. Of particular interest is the 'equilibrated P_{CO_2}' which is the P_{CO_2} that theoretically would be in equilibrium with the $[H_2CO_3]$. It is not immediately obvious from a knowledge of P_{CO_2}, $[H^+]$ and $[HCO_3^-]$ in a given mixture whether they are in equilibrium or not, and if not, whether P_{CO_2} will spontaneously increase or decrease. For this reason it

TABLE 1. Composition of reacting solutions

Type of experiment	Temp. (°C)	No. expts.	Buffer solution (No. 2) P_{CO_2} (mm Hg)	pH	$[HCO_3^-]$ (mM)	P_{O_2} (mm Hg)	Cell suspensions* (No. 1) P_{CO_2} (mm Hg)	pH	$[HCO_3^-]$ (mM)	P_{O_2} (mm Hg)	[Hb] (g/l.)	HbO_2 (%)	Mixture at equilibrium P_{CO_2} (mm Hg)	pH	$[HCO_3^-]$ (mM)	P_{O_2} (mm Hg)	ΔP_{O_2} (mm Hg)	ΔHbO_2 (%)
I. Bohr off-shift: cells ↑ext. P_{CO_2}; ↓ext. pH	42·5	2	158	6·90	16·4	43	9	8·15	25·7	43	3·6	94	62	7·37	21·1	62	−19	−22·0
	42·5	2	165	6·80	16·6	36	9	7·99	25·8	36	3·6	86·5	70	7·22	21·4	57·5	−21	−24·5
	37	1	130	6·70	16·7	32	8	8·05	25·8	39	3·0	97	46	7·23	21·4	57	−18	−24·5
	37	1	150	6·60	16·8	33	8	7·95	25·8	33	4·2	89	64	7·16	21·4	47	−13	−14·8
	31	2	130	6·65	16·8	33	8	7·85	25·9	33	3·3	92·5	58	7·08	21·6	44	−11	−16·0
	31	2	135	6·85	16·5	25	8	7·96	25·8	25	3·2	88	65	7·26	21·3	38	−13	−19·7
	23	2	132	6·67	16·7	36	8	7·84	25·9	35	3·3	98	65	7·05	21·6	43	−7·5	−11·0
	23	2	132	6·60	16·8	18	8	7·84	25·9	18	3·4	87	61	7·10	21·5	29	−11	−17·0
II. Shrunken cells	37	2	160	6·90	34·1	37	10	8·30	51·4	36	4·0	93	70	7·40	42·1	50	−13	−14·0
Swollen cells	37	1	140	6·59	11·2	33	8	7·88	17·3	33	4·3	89	69	7·09	14·4	45	−14	−15·2
III. Constant ext. P_{CO_2}; ↓ext. pH; added lactic acid	37	3	8	2·8	0	37	8	8·12	25·7	37	4·0	94	69	6·80	11·1	62	−25	−30
Added lactic acid + acetazolamide	37	3	8	2·8	0	37	8	8·12	25·7	37	4·0	94	69	6·80	11·1	62	−25	−30
IV. Bohr off-shift: solution ↑ P_{CO_2}; ↓pH	37	1	150	6·80	27·3	36	8	8·10	25·8	37	1·5	96	62	7·22	26·2	44	−6	−20·7
V. Bohr on-shift: cells; ↓ext. P_{CO_2};		1	8	8·10	25·7	35	150	6·93	31·0	35	4·2	39	54	7·33	29·2	25	+10	+10·6
↑ext. pH	37	1	8	8·10	25·7	35	150	6·87	27·5	35	4·2	39	52	7·33	27·7	25	+10	+10·9
+external carbonic anhydrase	37																	
VI. Deoxygenation: cells	37	1	0	8·08	0	0	0	8·08	~0	35	4·1	92	0	8·00	~0	25·8	−9·2	−9·7
VII. Oxygenation: cells	37	1	0	8·08	0	38	0	8·08	~0	0	4·2	0	0	8·08	~0	5·0	+14	+15·9

* In the case of the Bohr off-experiments in solution (IV) this applies to the haemolysate.

TABLE 2. Solution composition immediately after mixing, but before any CO_2 or O_2 has reacted

Type of experiment	Temp. (°C)	No.	Outside cells: P_{CO_2} (mm Hg)	Outside cells: Equil. P_{CO_2} (mm Hg)	Outside cells: pH	Inside cells: $[HCO_3^-]$ (mM)	Inside cells: P_{O_2} (mm Hg)	Inside cells: Equil. P_{CO_2} (mm Hg)
I. Bohr off-shift: cells ↑ ext. P_{CO_2}; ↓ ext. pH	42·5	2	84	35	7·40	21·08	43	9
	42·5	2	87	42	7·33	21·2	36	9
	37	1	69	48	7·27	22·8	36	8
	37	1	79	51	7·22	21·4	33	8
	31	2	69	48	7·22	21·4	33	8
	31	2	67	39	7·32	21·2	25	8
	23	2	70	46	7·22	21·4	36	8
	23	2	70	46	7·22	21·4	18	8
II. Shrunken cells	37	2	85	62	7·43	42·7	36	10
Swollen cells	37	1	74	36	7·20	14·3	33	8
III. Constant ext. P_{CO_2}; ↓ ext. pH; added lactic acid	37	3	8	5000	5·2	12·1	37	8
Added lactic acid + acetazoleamide	37	3	8	5000	5·2	12·1	37	8
IV. Bohr off-shift: solution ↑ ext. P_{CO_2}; ↓ ext. pH	37	1	79	42	7·43	25·7	36	8
V. Bohr on-shift: cells ↓ ext. P_{CO_2}; ↑ ext. pH	37	1	79	49	7·35	28·3	35	150
+ ext. carbonic anhydrase	37	1	79	52	7·30	26·6	35	150
VI. Deoxygenation: cells	37	1	0	0	8·08	0	17·5	0
VII. Oxygenation: cells	37	1	0	0	8·08	0	17·5	0

The chemical composition of the cell interior can be estimated but only the equilibrated P_{CO_2} is accurately known, so it alone has been included. Equilibrated P_{CO_2} is that which would be in equilibrium with the existing $[H_2CO_3]$.

is useful to calculate the P_{CO_2} that would be in equilibrium with the existing $[H^+]$ and $[HCO_3^-]$ as follows:

$$P_{CO_2} = \frac{[H^+]\,[HCO_3^-]}{\alpha K'},$$

where α is the solubility of CO_2 at 37° C, $3{\cdot}2 \times 10^{-5}$ M/mm Hg; and K' is the combined equilibrium constant $7{\cdot}9 \times 10^{-7}$ M^{-1} at 37° C in 0·145 M saline (Hastings & Sendroy, 1925). By comparison of equilibrated P_{CO_2} with P_{CO_2} one can tell the state of the equilibrium; if they are equal, equilibrium exists; if equilibrated P_{CO_2} is greater than P_{CO_2}, H_2CO_3 will be dehydrated, if the converse, CO_2 will be hydrated. Graphs of the change in HbO_2 with time during the reactions are given in Figs. 3–8.

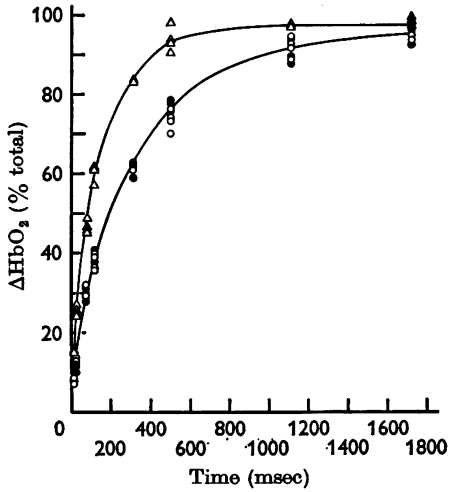

Fig. 3. Time course of the Bohr off-shift with the initial percentage of HbO_2 between 86·5 and 89 at three temperatures. External P_{CO_2} ↑ and pH ↓ (Exp. type I) (Δ) 42·5° C; (●) 31° C; (○) 23° C.

Figures 3 and 4 are plots of the change in $[HbO_2]$ with time following an increase of P_{CO_2} and external $[H^+]$ at 42·5° C, 31° C and 23° C when the initial $[HbO_2]$ percentage was 86–89 and 93–94 respectively (Experiments type I). All of the data points from all experiments are plotted in the figures. The ordinate is normalized to represent the fraction of the total change in $[HbO_2]$ in order that the speed of the various experiments may be compared more conveniently, since the total change in $[HbO_2]$ was not the same in different experiments. The data at 37° C were not plotted because the graphs are already crowded and because the results were similar to those of Craw *et al.* (1963). Figure 5 gives the time course of the Bohr off-shift at 37° C only, when osmotically shrunken and

swollen cells were exposed to a sudden increase in P_{CO_2} and $[H^+]$. Figure 6 presents analogous results to those of Fig. 4 but the cells were exposed to an increase in external $[H^+]$ only, the P_{CO_2} being maintained constant. In addition in one group of experiments acetazolamide was added. In Fig. 7 are presented graphs of increase in $[HbO_2]$, the Bohr on-shift, when

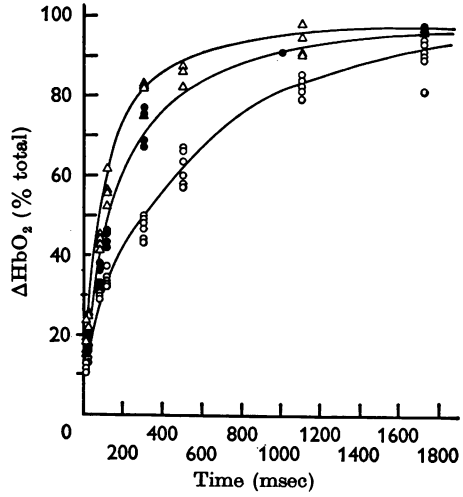

Fig. 4. Time course of the Bohr off-shift with the initial percentage of HbO_2 between 92·5 and 98 at three temperatures. External P_{CO_2} ↑ and pH ↓ (Exp. type I) (Δ) 42·5° C; (●) 31° C; (○) 23° C.

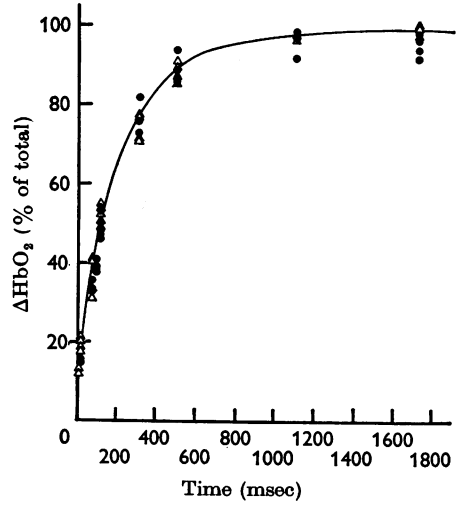

Fig. 5. Time course of Bohr off-shift at 37° C of osmotically swollen and shrunken red cells. External P_{CO_2} ↑ and pH ↓ (Exp. type II) (●) shrunken cells; (Δ) swollen cells.

external P_{CO_2} and external [H^+] were decreased. In some of these experiments carbonic anhydrase was added to increase the rate of CO_2 dehydration. Finally, Fig. 8 presents the time course of deoxygenation and

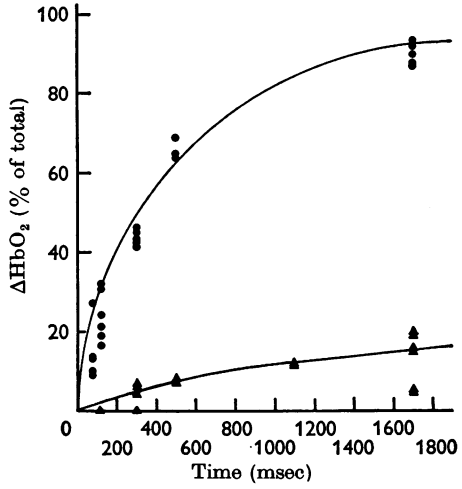

Fig. 6. Time course of Bohr off-shift at 37° C created by adding lactic acid to the suspending fluid while keeping P_{CO_2} constant, with (▲) and without (●) 10^{-3} M acetazoleamide.

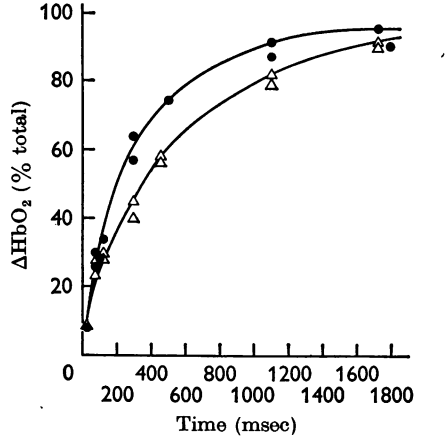

Fig. 7. Time course of Bohr on-shift at 37° C with (●) and without (Δ) extracellular carbonic anhydrase. (Exp. type V.)

oxygenation of a cell suspension at 37° C produced by lowering and raising, respectively, P_{O_2} in the medium. No CO_2 was present. Also included in Fig. 8 is a graph of the Bohr off-shift in a solution of haemolysed cells.

Although the displacement of O_2 from HbO_2 by H^+ involves a sequence

of processes that cannot in theory or practice be described by a simple exponential curve, it is useful to describe the over-all speed of the phenomenon by the half-time, and to calculate an exponential constant from it. This is the same as considering the Bohr shift as described by the equation

$$\frac{[HbO_2]_t - [HbO_2]_\infty}{[HbO_2]_0 - [HbO_2]_\infty} = e^{-kt}, \quad (1)$$

where k is the velocity constant, $[HbO_2]_0$ is the concentration of oxyhaemoglobin at the start of the reaction, $[HbO_2]_t$ is that at any time thereafter and $[HbO_2]_\infty$ is that at time infinity, that is when chemical

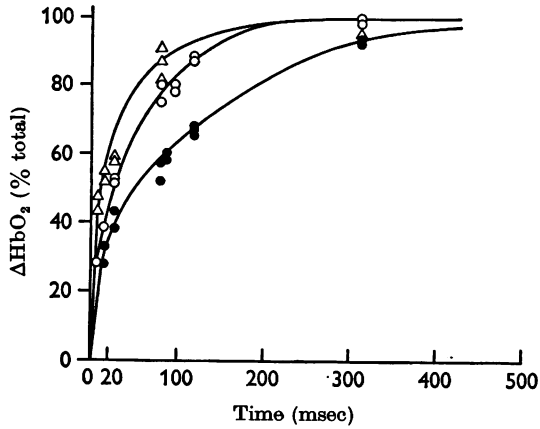

Fig. 8. Time course of deoxygenation (Δ) and oxygenation (○) of intact cells and of Bohr off-shift (●) in solution of haemolysed blood at 37° C. (Exp. type IV, VI and VII.)

equilibrium has been achieved. In their paper on the rate of the Bohr off-shift Craw *et al.* (1963) treated this process in the same manner. However, when this equation is applied to our data k turns out to be greater when calculated from the early part of the process than when calculated from the later part. This indicates that the Bohr shift is not a simple exponential process in general. Half-time ($t_{\frac{1}{2}}$) was obtained from the graphs and k in turn calculated from it. The results for the combined data are given in Table 3. One possible reason for the shape of the reaction curves is heterogeneity of the red cell population.

DISCUSSION

The accuracy of the basic measurements has been discussed earlier but there are particular errors of the rapid reaction apparatus that should be considered. The P_{O_2} electrode was consistently linear, was maintained

at a constant temperature and measurements were always made with the fluid flowing past it at a constant linear velocity. We believe the relative O_2 tensions are reliable to within less than $\pm 0{\cdot}5$ mm Hg. The calculated elapsed time for the fluid seen by the electrode at any instant is not as precise. The four jet mixing chamber mixes the two fluids

TABLE 3. Summary of half-times and apparent velocity constants

Type of experiment	Temp. (°C)	Initial $[HbO_2]$ (%)	ΔP_{O_2} (mm Hg)	$\Delta[HbO_2]$ (%)	$t_{\frac{1}{2}}$ (sec)	k (sec^{-1})
I. Bohr off-shift: cells ↑ext. P_{CO_2}; ↓ext. pH	42·5	94	−19	−22·0	0·090	7·7
	42·5	86·5	−21	−24·5	0·090	7·7
	37	97	−18	−24·5	0·120	5·8
	37	89	−13	−14·8	0·120	5·8
	31	92·5	−11	−16·0	0·140	5·0
	31	88	−13	−19·7	0·190	4·3
	23	98	−7·5	−11·0	0·310	2·2
	23	87	−11	−17·0	0·190	4·3
II. Shrunken cells	37	93	−13	−14·0	0·120	5·8
Swollen cells	37	89	−14	−15·2	0·120	5·8
III. Constant ext. P_{CO_2}; ↓ext. pH; added lactic acid	37	94	−25	−30·0	0·310	2·2
Added lactic acid + acetazoleamide	37	94	−25	−30·0	10·0	0·069
IV. Bohr off-shift: solution ↑ext. P_{CO_2}; ↓ext. pH	37	96	−6	−20·7	0·00	13·8
V. Bohr on-shift; cells ↓ext. P_{CO_2}; ↑ext. pH	37	39	+10	+10·6	0·350	2·0
+ carbonic anhydrase	37	39	+10	+10·9	0·200	3·5
VI. Deoxygenation: cells	37	92	−9·2	−9·7	0·012	58
VII. Oxygenation: cells	37	0	+14	+15·9	0·023	30

completely in less than 0·002 sec as can be shown by photometric measurements of the reaction of H^+ and phenolphthalein (Staub *et al.* 1961). However, theoretically the layer of fluid nearest the electrode membrane moves more slowly than the average velocity of the mixture which we used to calculate the elapsed time. Thus the electrode might be expected to 'see' fluid in which the chemical reaction was further advanced than in the main stream. Further experience with several electrodes in flowing reacting mixtures, O_2, CO_2 and pH (H. H. Rotman, R. A. Klocke and R. E. Forster, unpublished observation) suggests that there is an apparent delay in the fluid corresponding to about 0·005 sec in the present apparatus. This must be taken into account in the more rapid reactions.

For the reacting mixture to travel in a relatively square front so as to permit adequate time resolution, the Reynolds number in the flowing stream must exceed 2000. However the longest observation tube that would fit our apparatus was 25 cm, corresponding to 0·119 sec. In order

to study slower reactions, we resorted to observation tubes constructed of much larger bore but spiral in shape (made for us by Mr James Graham) which extended the elapsed time out to 1·7 sec. However, flow was certainly not turbulent through them; marked blurring of a square front could be seen with the naked eye using coloured fluid, and the estimate of elapsed time was imprecise. These spirals were only used in the later parts of the reactions, where the rate of change of P_{O_2} was much less, and to determine the final end-point of the reaction, for which latter purpose they were entirely adequate.

The Bohr shift is a decrease in O_2 affinity, by definition at equilibrium, produced by a reaction of increased $[H^+]$ with haemoglobin which results in an increased dissociation velocity constant (Hartridge & Roughton, 1923*b*). CO_2 can react with the NH_2 groups of the haemoglobin leading to the formation of carbamate (Roughton, 1964), which in turn produces H^+ by ionization, providing a type of shunt around the hydration mechanism. Although there may be an effect of P_{CO_2} independent of $[H^+]$ on the HbO_2 dissociation curve, an increase in P_{CO_2} without a change in intracellular $[H^+]$ produces a negligible dissociation of HbO_2 (Craw *et al.* 1963), in agreement with the concept that $[H^+]$ is the important factor.

The Bohr shift in red cells is a sequence of chemical reaction and diffusion steps presumably as follows:

Outside the cell

1. $H^+ + HCO_3^- \rightleftharpoons H_2CO_3$. The neutralization reaction is several orders of magnitude faster than the Bohr shift and can be considered instantaneous in comparison.
2. $H_2CO_3 \underset{k_u}{\overset{k_v}{\rightleftharpoons}} CO_2$. Normally there is no carbonic anhydrase outside the cell, so the dissociation constant, k_v, is 89 sec^{-1} at 37° C, and the association constant, k_u, is 0·12 sec^{-1} (Roughton, 1964).

Across the wall

3. Diffusion of CO_2. Un-ionized acids such as H_2CO_3 and lactic acid do not appear to cross the membrane with significant rates in terms of present studies (Booth, 1938; Giebel & Passow, 1960; Green, 1949). HCO_3^- can exchange for Cl^-; this, however, has a minimal effect on pH because of the relatively large $[HCO_3^-]$ present.

Inside the cell

4. $CO_2 \underset{k_v}{\overset{k_u}{\rightleftharpoons}} H_2CO_3$. There is a large amount of carbonic anhydrase inside the cell and so this reaction velocity constant would be of the order of 13,000 times faster than the uncatalysed values (Roughton, 1964).

Owing to the finite thickness of the cell CO_2 has to move throughout it by diffusion, so that this process takes place simultaneously with the chemical reaction.

5. $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$. The neutralization reaction is practically instantaneous as remarked above.
6. Increased $[H^+]$ acts on the haemoglobin to increase the reaction velocity constant for the dissociation of O_2 from HbO_2. This is probably of the same relative velocity as neutralization.
7. $HbO_2 \rightleftharpoons Hb + O_2$ and the gas diffuses out of the cell. Owing to the fact that chemical reaction and diffusion occur simultaneously throughout the cell, the two processes cannot be separated (Forster, 1964), but are about 10 times faster than the Bohr shift.

Although for purposes of discussion these steps have been listed separately this by no means implies that one step must be completed before the next takes place; to the contrary they all undoubtedly take place to some extent at the same time (see Table 2).

Bohr off-shift in solution

In this experiment a solution of lysed cells at a P_{CO_2} of 8 mm Hg was mixed with solution with greater P_{CO_2} and $[H^+]$. This means the HbO_2 environment suddenly became more acid (from pH 8·1 to 7·43). In view of the findings of H. H. Rotman, R. A. Klocke and R. E. Forster (unpublished observations) the reaction of H^+ with the haemoglobin will be extremely fast and the dissociation constant of HbO_2 should have changed in less than several milliseconds. Therefore the velocity of the first part of the observed reaction should be limited only by the rate of dissociation of O_2 from HbO_2. This reaction has a rate constant of 200 sec^{-1} at 37° C corresponding to a half time of 0·0035 sec (Lawson, Holland & Forster, 1965). Thus there should be an initial rapid decrease in $[HbO_2]$ owing to the change in $[H^+]$ which is complete in less than 0·10 sec. This appears to be shown in Fig. 8, where the initial rate of fall in $[HbO_2]$ is more rapid than that from 20 % to about 0 %.

Although the deoxygenated HbO_2 will bind an increased amount of H^+, the haemoglobin concentration is so small that the phosphate buffers will predominate and pH will remain essentially at 7·43, if no other reactions take place. This means that at the conclusion of the initial rapid phase of the Bohr shift resulting from the effect of the change in $[H^+]$ itself, the pH will still be approximately 7·43. Since there has as yet been no marked hydration of the dissolved CO_2, P_{CO_2} will approximate 79 mm Hg. Similarly $[HCO_3^-]$ will not have increased yet and equilibrated P_{CO_2} will be unchanged, namely 42 mm Hg (Table 2). From this time on, the rate of dissociation of HbO_2 should be limited by the rate of increase

of $[H^+]$ which will be proportional to the rate of hydration of CO_2. This reaction is described by the equation

$$\frac{d[CO_2]}{dt} = -k_u[CO_2] + \frac{k_v[H^+]\,[HCO_3^-]}{K}. \quad (2)$$

$[CO_2]$ is the concentration of dissolved CO_2 in moles/l. t is time in seconds, k_u is the velocity constant for the hydration of CO_2 and k_v is that for dehydration, both in sec^{-1}. K is the equilibrium constant for the ionization of H_2CO_3 and equals $3{\cdot}5 \times 10^{-4}$ M at 36° C (Roughton, 1964). $[HCO_3^-]$ is the concentration of carbonate ion, which is 25·7 mM initially and rises only to 26·2 mM at chemical equilibrium and so may be considered constant as an approximation. $[H^+]$, however, increases from 4×10^{-8} M to 6×10^{-8}M, corresponding to a pH change from 7·4 to 7·22. The CO_2 hydrated liberates an equal amount of H^+ because the H_2CO_3 is almost entirely ionized. Therefore we can state

$$[H^+] \approx [H^+]_0 + \Delta[CO_2]/12 \times 10^3, \quad (3)$$

where $[H^+]_0$ is the original $[H^+]$ in the mixture, $\Delta[CO_2]$ is the amount of CO_2 that has disappeared and 12×10^3 is the buffering capacity of the mixture in increment of $[H^+]$ bound/increment in $[H^+]$. This value was calculated from composite titration curves of the buffer pairs contained in the mixture. The total change in $[H^+]$ bound/total change in $[H^+]$ happens also to be the average value of d$[H^+]$ bound/d$[H^+]$ over the range. This expression (3) for $[H^+]$ can be substituted in equation (2), which then can be solved in terms of $[CO_2]$, giving a simple exponential relationship with an exponential constant equal to

$$k_u + k_v[HCO_3^-]/(K \times 12{,}000). \quad (4)$$

Substituting the numerical values of 0·12 sec^{-1} for k_u and 89 sec^{-1} for k_v (Roughton, 1964), we obtain a value of 0·67 sec^{-1}. If we assume from visual approximation of Fig. 8 that the rapid phase is over when 20 % of the total fall in $[HbO_2]$ has taken place, we obtain an excellent linear semi-log plot for the remainder of the process, giving an exponent of 7·7 sec^{-1}. There is of course carbonic anhydrase present along with haemoglobin in the haemolysate, sufficient in the cell to accelerate the hydration reactions 13,000-fold (Kernohan, Forrest & Roughton, 1963). The cell contents have been diluted from an original intracellular haemoglobin concentration of 300 g/l. to 0·75 g/l. in the mixture, so that the reaction rate should have only been accelerated $0{\cdot}75/300 \times 13{,}000$ or 32·5-fold. The experimental reaction rate compared to that expected if there were no enzyme present was increased 7·7/0·67 or eleven-fold, an acceptable agreement.

Bohr off-reaction in cell suspensions

The Bohr off-reaction in a cell suspension is more complicated than that in a haemolysate because any changes in the chemical environment of the cell must be transferred across the cell membrane to affect the haemoglobin. The major mechanism of transport into the cell is by means of diffusion of CO_2 across the membrane with dehydration and hydration occurring simultaneously outside and inside the cell respectively, the 'catalysed diffusion' mechanism (Jacobs & Stewart, 1942). In the experiments where P_{CO_2} and $[H^+]$ were both increased in the suspending fluid (Table 1 and 2, expts. I) the P_{CO_2} is greater than the equilibrated P_{CO_2} inside and therefore CO_2 will diffuse into the cell and hydrate, producing H^+. HCO_3^- will diffuse out of the cell in exchange for Cl^- but this process has *per se* a minimal effect on intracellular $[H^+]$. Removal of HCO_3^- from the bicarbonate buffer system inside the cells, at constant P_{CO_2}, will produce a percentage increase in $[H^+]$ less than proportional to the percentage decrease in $[HCO_3^-]$ because of the strong buffer capacity of the haemoglobin. The total change in intracellular $[HCO_3^-]$ was of the order of -16% (the extracellular $[HCO_3^-]$ decreased from 25·8 to 22·0 mM), while the increase in intracellular $[H^+]$ was 600 %, so that the importance of HCO_3^- movement on the Bohr shift is negligible. H^+ itself presumably cannot exchange across the cell membrane over the time period of these experiments, at least because there are no mobile cations to exchange for it. At the start of the process the dissolved P_{CO_2} is greater than the equilibrated P_{CO_2} inside the cell so that hydration of CO_2 will immediately proceed more rapidly where there is carbonic anhydrase. The half-times of the Bohr shift ranged from 0·09 to 0·3 sec, decreasing with increased temperature. The value at 37° C was 0·12 sec, the same as found by Craw *et al.* (1963).

The Bohr off-reactions in cell suspensions at constant P_{CO_2} (Type III) are similar in that CO_2 must move across the cell wall to affect the internal pH, but at the start of the reaction there is no P_{CO_2} gradient. Since the external equilibrated P_{CO_2} is greater than the P_{CO_2}, CO_2 will be formed from H_2CO_3 and bicarbonate outside the cells, but this will take time, being uncatalysed, making the process somewhat slower than in the previous experiments (Type I). The rate constant for the conversion of HCO_3 to CO_2 can be calculated using equation (4). $[HCO_3^-]$ may be assumed constant; actually it changes from 12·1 to 11·1 mM. As an approximation the buffering capacity of the mixture may also be assumed constant at 300 moles H^+ bound/mole change in $[H^+]$. This buffering capacity was obtained by the same kind of calculation as for the value in equation 4. It is worth pointing out that this value for buffering capacity

increases markedly with increasing pH, which increases the half-time of the reaction *pari passu*. The exponential constant is thus equal to

$$0{\cdot}12+\frac{89\times 12\times 10^{-3}}{3{\cdot}5\times 10^{-4}\times 300}=10$$

and gives a half-time of 0·69/10 = 0·069 sec. The half-time of the total process is 0·310 sec and includes conversion of H_2CO_3 to CO_2, with its 0·069 sec half-time, plus the diffusion and reaction of the gas to produce the Bohr shift with a half-time of 0·12 sec. However the half-time of the total process is not a simple function of the half-times of the two steps.

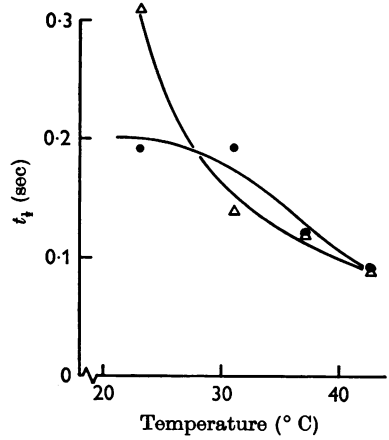

Fig. 9. The $t_{\frac{1}{2}}$ for Bohr off-shifts versus temperature for two levels of initial HbO_2 saturation in (Δ) 93–98 % [HbO_2]; (●) 86–88 % [HbO_2].

Reducing the temperature from 42·5 to 23° C decreased the apparent exponential rate constant of the Bohr off-shift (Series 1, Table 3) from 7·7 to 2·2 sec^{-1} (Fig. 9). The $\log_{10}k$ is plotted against log $1/T$ (absolute temperature) in Fig. 10 and corresponds to an average activation energy of about 8000 cal. No significant effect of change in initial oxyhaemoglobin saturation was seen. Admittedly k is not a true reaction velocity constant and different values are obtained depending on the exact method of its calculation, for example, from the initial slope or from the $t_{\frac{1}{2}}$. However, the values of the activation energy obtained are a reasonable estimate.

It is of interest to compare the activation energy of the Bohr shift with that of several of the individual processes producing the Bohr shift. The apparent activation energy of the dissociation of O_2 from human erythrocytes over the temperature range of 7–42° C was 16,473 cal (Lawson *et al.* 1965). The hydration and dehydration of CO_2 in connexion with the Bohr shift occur predominantly within the red cell and are

catalysed by carbonic anhydrase. The average Q_{10} of this enzyme catalysed reaction in the range from 0 to 35° C is 1·4 (Roughton & Clark, 1951), corresponding to an activation energy of 5970 cal. Thus the experimentally determined activation energy of the Bohr shift corresponds most closely to that of the enzyme catalysed reactions of CO_2 and water; that of the

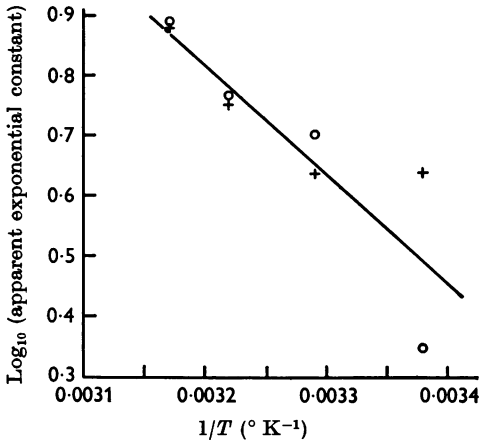

Fig. 10. $\text{Log}_{10}k$ plotted against 1/absolute temperature. (+) [HbO_2] about 86 % initially: (O) [HbO_2] about 94 % initially.

O_2 dissociation from HbO_2 is too great. This agrees with the conclusion that the rate of the Bohr shift is normally limited by the catalysed hydration of CO_2.

Effect of change in cell volume

The velocity constant, k, for the Bohr off-shift at 37° C is the same, namely 5·8 sec^{-1}, for normal volume cells, cells with a 40 % increase in volume and cells with a 50 % decrease in volume (series II). This suggests that diffusion through the cell interior is not an important rate-limiting process, since it should change under these conditions, but that the results are again compatible with the assumption that the rate-limiting process is primarily the reaction of CO_2 with the buffer systems.

However, the rate of diffusion + reaction in the red cell interior is theoretically approximately proportional to 1/[radius (concentration of reactant)$^{\frac{1}{3}}$] (Forster, 1964), considering the cell as a sphere. The concentration of cell contents will vary reciprocally with changes in cell volume. The radius will vary directly as (volume)$^{\frac{1}{3}}$. For small changes in cell volume these effects may cancel out, and no detectable change in the rate of gas exchange of the cells would be expected. These experiments do show that the change in cell volume resulting from water movements, such as occur in the capillary beds, do not change the rate of the Bohr shift.

Bohr on-shift in cells

Measurements of the Bohr on-shift (Series V, Tables 1 and Fig. 7) produced by a decrease in P_{CO_2} provided a half-time of 0·350 sec, but the time course was not precisely exponential and it is difficult to interpret this figure in relation to the processes known to be proceeding. The red cells start the reaction with their contents in chemical equilibrium with a P_{CO_2} of 150 mm Hg, while the external fluid has a P_{CO_2} of 79 mm Hg and a buffer system which is in chemical equilibrium with a P_{CO_2} of 49 mm Hg. At the start of the reaction, inside the cell H_2CO_3 will dehydrate to form CO_2 which will diffuse out as long as the external P_{CO_2} is less than the internal *equilibrated* P_{CO_2}. The external P_{CO_2} will be little affected by the amounts of CO_2 moving out of the cells because of the small cell volume/total fluid volume. Therefore as the internal equilibrated P_{CO_2} drops from 150 to about 79 mm Hg, the process will be rapid because the rate is limited only by the dehydration of H_2CO_3 inside the cell and this is catalysed by carbonic anhydrase. At the same time CO_2 is being hydrated in the suspending fluid to form H_2CO_3 because the P_{CO_2} is more than the equilibrated P_{CO_2} of the extracellular buffers, but this process is much slower because there is no carbonic anhydrase present. Once the intracellular equilibrated P_{CO_2} has fallen to about 79 mm Hg, this extracellular hydration of CO_2 becomes the rate limiting reaction and the whole process slows down as the intracellular P_{CO_2} drops from approximately 79 mm Hg to the final equilibrium value of 52–54 mm Hg.

The importance of the rate of hydration of CO_2 is demonstrated by the increased rate of the over-all process produced by the simple addition of carbonic anhydrase to the external fluid, the half-time reducing to 0·2 sec. Presumably in the absence of this enzyme externally, CO_2 is produced inside the cells more rapidly than it can be hydrated by the buffer system externally, and the external and internal P_{CO_2} become nearly equal, only falling slowly as the uncatalysed reaction proceeds externally.

Nakamura & Staub (1964) also determined the rate of the Bohr on-shift without external carbonic anhydrase and obtained a value of 0·2 sec, considerably less than our finding. However, the pertinent conditions may not be comparable; the initial $[HbO_2]$ in our work was much higher. They also studied the effect of producing a decrease in P_{O_2} in a suspension of human red cells simultaneously with an increase in P_{CO_2}. They claimed a synergistic action on the rate of the combined processes, because they stated that the over-all process was faster than either deoxygenation brought about by reducing P_{O_2} alone or by increasing P_{CO_2} alone. However, we disagree that the combined reaction was faster. Their method of

calculating the pseudo rate-constant for the deoxygenation, k_c in sec^{-1}, was to use the equation

$$\frac{d[HbO_2]}{dt} = -k_c[HbO_2]_0, \qquad t = 0, \tag{5}$$

where $[HbO_2]$ is the concentration of oxygenated haemoglobin and t is time in seconds, the subscript $_0$ indicating the start of the reaction. We believe it would have been more correct to use the relation

$$\frac{d[HbO_2]}{dt} = k_c([HbO_2]_0 - [HbO_2]_e), \qquad t = 0, \tag{6}$$

where $[HbO_2]_e$ is the concentration of HbO_2 that would exist at equilibration with the actual P_{O_2} in the external solution. It is necessary to take some account of the existence of a finite P_{O_2} outside the red cells, because it is the P_{O_2} gradient between the interior and exterior of the cells that actually causes the gas to move. If this method of calculating the exponential rate constant for the over-all process is used, the value of k_c is at least as great, if not greater, than the value for the combined reaction. This is also brought out by a comparison of the half-times: those for the deoxygenation reaction in general (Table 1, Nakamura & Staub, 1964) appear faster than the combined reaction process. We do not believe that they were justified in comparing the rates of the combined reaction with those of the Bohr shift alone, because the latter were Bohr on-reactions while the deoxygenation and combined reactions were Bohr off-reactions. Comparison would only be possible if the rate limiting steps were considered and the pertinent values of the reactants calculated. This epitomizes the difficulty of considering the Bohr shift as a single process, instead of a series of processes.

Deoxygenation and oxygenation reactions

The rates of simple deoxygenation and oxygenation of red cell suspensions were measured for comparison with the data on the Bohr shift. These experiments consisted of suddenly decreasing the P_{O_2} around highly oxygenated cells or increasing the P_{O_2} around deoxygenated cells. Temperature was 37 °C and P_{CO_2} was zero. The half-time of these processes was one fifth to one-half that for the Bohr shift at the same temperature indicating that the rate of O_2 exchange by the cells was presumably not the rate-limiting process in the Bohr shift. Comparisons are difficult though, because of the complication of diffusion + chemical reaction in the cell interior and because the O_2 and haemoglobin reactions are going on simultaneously with a

series of other reactions in the Bohr shift. The deoxygenation appears a little faster than oxygenation, as might be expected since the chemical kinetics are generally faster when the HbO_2 saturation is higher (Roughton, 1964). The curves in Fig. 8 are not completely satisfactory for calculation of the reaction velocity constant of association, k'_c, and dissociation, k_c, but the values obtained from the two sets of data are similar to the more reliable results of Staub *et al.* (1961) and of Lawson *et al.* (1965).

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REFERENCES

BOHR, C., HASSELBALCH, K. & KROGH, A. (1904). Ueber einen in biologisches Beziehung wichtigen Einfluss, den die Kohlensäurespannung des Blutes auf dessen Sauerstoffbindung übt. *Skand. Arch. Physiol.* **16**, 402–412.

BOOTH, V. H. (1938). Carbonic anhydrase activity inside corpuscles. Enzyme-substrate accessibility factors. *J. Physiol.* **93**, 117–128.

CRAW, MARGOT R., CONSTANTINE, H. P., MORELLO, J. A. & FORSTER, R. E. (1963). Rate of the Bohr shift in human red cell suspension. *J. appl. Physiol.* **18**, 317–324.

FORSTER, R. E. (1964). Rate of gas uptake by red cells. In *Handbook of Physiology, Section* 3, *Respiration*, vol. 1, ch. 32, ed. FENN, W. O. & RAHN, H. Washington: American Physiological Society.

GIEBEL, O. & PASSOW, H. (1960). Die Permeabilität der Erthyrocytenmembran für organische Anionen. *Pflügers Arch. ges. Physiol.* **271**, 378–388.

GREEN, J. W. (1949). The relative rate of penetration of the lower saturated monocarboxylic acids into mammalian erythrocytes. *J. cell. comp. Physiol.* **33**, 247–265.

Handbook of Respiration (1958). Ed. DITTMER, D. S. & GREBE, R. M., pp. 6–10, Washington: National Academy of Sciences.

HARTRIDGE, H. & ROUGHTON, F. J. W. (1923*a*). A method of measuring the velocity of very rapid chemical reactions. *Proc. R. Soc.* A **104**, 376–394.

HARTRIDGE, H. & ROUGHTON, F. J. W. (1923*b*). The kinetics of hemoglobin. II. The velocity with which oxygen dissociates from its combination with hemoglobin. *Proc. R. Soc.* A **104**, 395–412.

HASTINGS, A. B. & SENDROY, J. JR. (1925). Effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid. *J. biol. Chem.* **65**, 445–455.

JACOBS, M. H. & STEWART, DOROTHY R. (1942). The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. *J. gen. Physiol.* **25**, 539–552.

KERNOHAN, J. C., FORREST, W. W. & ROUGHTON, F. J. W. (1963). The activity of concentrated solutions of carbonic anhydrase. *Biochim. biophys. Acta* **67**, 31–41.

LAWSON, W. H., JR., HOLLAND, R. A. B. & FORSTER, R. E. (1965). Effect of temperature on deoxygenation rate of human red cells. *J. appl. Physiol.* **20**, 912–918.

NAKAMURA, T. & STAUB, N. C. (1964). Synergism in the kinetic reactions of O_2 and CO_2 with human red blood cells. *J. Physiol.* **173**, 161–177.

ROUGHTON, F. J. W. (1964). Transport of oxygen and carbon dioxide. In *Handbook of Physiology, Section* 3, *Respiration*, vol. 1, ch. 31, ed. FENN, W. O. & RAHN, H., pp. 767–825. Washington: American Physiological Society.

ROUGHTON, F. J. W. & CLARK, A. M. (1951). Carbonic anhydrase. In *The Enzymes*, vol. I, part 2, ch. 43, pp. 1250–1265. New York: Academic Press.

SCHOLANDER, P. F. (1947). Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. biol. Chem.* **167**, 235–259.

STAUB, N. C., BISHOP, J. M. & FORSTER, R. E. (1961). Velocity of O_2 uptake by human red blood cells. *J. appl. Physiol.* **16**, 511–516.