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The Kinetics of Deoxygenation of Human Haemoglobin

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Until 1949, kinetic data for the reactions of haemoglobin with oxygen and carbon monoxide, obtained almost entirely by Hartridge & Roughton (1923, 1925), Roughton (1934) and Millikan (1933) in experiments with sheep haemoglobin, were considered to conform to simple first-order and second-order rate equations, and to be consistent, therefore, with the assumption that the four haems in the molecule behave independently. The best equilibrium data, on the other hand, could only be interpreted by the intermediate-compound theory of Adair (1925) with the assumption of haem interaction (Forbes & Roughton, 1931; Courtice & Douglas, 1947). This situation was reviewed by Roughton (1949), who reported that re-inspection of earlier kinetic data, and new experiments on the deoxygenation of sheep haemoglobin in the presence of sodium dithionite (Legge & Roughton, 1950), gave evidence of haem interaction, notably a significant upward trend in the value of the overall first-order velocity coefficient in the early stages of the deoxygenation reaction. This was confirmed with human haemoglobin under various conditions (cf. Dalziel, 1953, 1954), as will be described here. The further evaluation of the inter-

mediate-compound hypothesis has since been pursued with remarkable success by Roughton, Gibson and their collaborators. For sheep haemoglobin the four equilibrium constants for the oxygen reaction (Roughton, Otis & Lyster, 1955) and the four combination velocity constants for the carbon monoxide reaction (Gibson & Roughton, 1957a) have been determined to within narrow limits. Little further work has been reported for the deoxygenation reaction, however.

For the investigation of the kinetic effects of haem interaction, the oxygen dissociation appeared to be the reaction of choice, since previous work had indicated that the reverse reaction could be eliminated by an excess of sodium dithionite, and that the analysis of consecutive reactions would be simpler than for the reverse, combination reaction. This was the main purpose of the work reported here; a secondary objective was to compare the kinetic properties of haemoglobin from several normal individuals and from persons suffering from various types of anaemia. The work, which was carried out during 1948–53, forms part of a Ph.D. thesis approved by the University of London, and brief accounts have also been published in the literature (Dalziel, 1954, 1955, 1958). It was found that secondary reactions between dithionite-

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oxidation products and the pigment interfered with the spectrophotometric analysis (Dalziel & O'Brien, 1951, 1952, 1957; cf. Legge & Roughton, 1950), and so far as haem interaction effects are concerned the full benefits of the precision of the experimental technique devised for the work (Dalziel, 1953, 1954) could not be realized. Nevertheless, reasonably accurate kinetic data were obtained for the deoxygenation of human haemoglobin under a wide range of conditions of concentration, acidity and temperature.

EXPERIMENTAL

Kinetic measurements

The rapid-reaction apparatus consists essentially of a constant-flow apparatus of the gas-pressure type in conjunction with a Beckman DU photoelectric spectrophotometer, and has been described in detail elsewhere (Dalziel, 1953, 1954). The mixing chamber and observation tube, of 2 mm. bore, was fed by two jets of 1 mm. bore. Total volume flow rates of 5–10 ml./sec., or linear flow rates of 150–300 cm./sec., were used, and mixing of the two reactant solutions was 99% complete in 5–8 msec. Temperature was controlled to within 0.1°.

Either oxyhaemoglobin (HbO₂) reactant or deoxygenated haemoglobin (Hb) calibration fluid could be run from one jet, and either dithionite reactant or HbO₂ calibration fluid from the other. The reactants and calibration fluids were kept in the storage vessels for 20 min. to allow temperature and pressure equilibration and destruction of peroxide formed by exposure of the dithionite solution to air (Dalziel & O'Brien, 1957). For measurements at the usual wavelength of 430 mμ ($\lambda_{\max}$ of Hb) the procedure at each point of observation was: (1) a little HbO₂ reactant was run through jet 1 to displace Hb calibration fluid containing dithionite; (2) with jet 1 closed, HbO₂ calibration fluid was run from jet 2, whilst the spectrophotometer was adjusted to read $E = 0$; (3) with jet 2 closed, Hb calibration fluid was run from jet 1, and $\Delta E_{100\%}$, the extinction change corresponding to complete deoxygenation, was recorded; (4) the two reactants were run together, and the extinction, ΔE_t , was recorded; (5) operations (1) and (2) were repeated to check the stability of the spectrophotometer.

The percentage saturation of the pigment with oxygen at time t after mixing, calculated from the distance of the point of observation from the mixing chamber and the flow rate, was calculated as $Y = 100 (1 - \Delta E_t / \Delta E_{100\%})$.

Precision of the measurements. The precision of the spectrophotometric analysis of the reaction mixture (Dalziel, 1953, 1958) depends upon the constancy of the relative delivery rates, and the molar extinction coefficients of HbO₂ and Hb at the wavelength chosen for measurements. The most suitable wavelength is 430 mμ ($\lambda_{\max}$ of Hb), with 415 mμ ($\lambda_{\max}$ of HbO₂) as second choice. The calculated errors of measurements of the percentage saturation of the pigment are, at 430 mμ, $\pm 0.5\%$ at 95% saturation and $\pm 1.1\%$ at 5% saturation, and, at 415 mμ, $\pm 1.9\%$ at 95% saturation and $\pm 0.9\%$ at 5% saturation. The precision is little affected by variation of $\Delta E_{100\%}$ from 0.2 to 1.2. Nominal band widths of 4–6 mμ were used.

Materials

Oxyhaemoglobin. Haemolysates of washed red cells, diluted with buffer solution, were used without further purification. The HbO₂ solutions contained less than 2% of methaemoglobin, as shown by spectrophotometric analysis for cyanmethaemoglobin. The addition of 3% of methaemoglobin did not affect kinetic data in control experiments.

Clear solutions were obtained by haemolysis with saponin, which had no effect on the kinetics in control experiments. Three experiments with the same sample of washed red cells on three successive days gave identical results, but experiments were usually made not later than the day after the blood was withdrawn. With measurements at 430 mμ, 15 ml. of normal blood was sufficient for a kinetic curve of eighteen points.

Sodium dithionite. Commercial samples (British Drug Houses Ltd.) were used. The solid was stored in a sealed bottle. Dry, freely running samples assayed as 95–98% of Na₂S₂O₄·H₂O (Vogel, 1939).

Buffers. Phosphate and borate buffers were prepared according to Clarke and Lubs (Vogel, 1939).

Preparation of reagents

Oxyhaemoglobin reactant. Red cells were separated by centrifuging from whole blood, to which heparin (0.01 g./100 ml.) had been added, and were washed three times with sodium chloride solution (0.9 g./100 ml.). Saponin solution (3 vol., 0.1 g./100 ml.) was added to 1 vol. of packed red cells, and after 10 min. the haemolysate was centrifuged. The clear supernatant solution was diluted with 1500 ml. of buffer solution. The HbO₂ concentration, expressed as m-equiv./l. (mEq), that is m-moles of haem or combined oxygen/l., was calculated from measurements of $E_{576\text{ m}\mu}$ and a value for ϵ_{mEq} of 15.4 (Dalziel & O'Brien, 1954; Lemberg & Legge, 1949).

Dithionite reactant. This solution was prepared with minimal contact with air, and alkali was added to neutralize the acid formed. For the usual concentration of 0.4 g./100 ml., 5 g. was dissolved in 1225 ml. of buffer plus 25 ml. of 0.2M-NaOH solution in a separating funnel, and the solution was immediately covered with a layer of liquid paraffin. After the Hb calibration fluid had been prepared, the remaining dithionite reactant was run into a storage vessel in the kinetic apparatus and covered with 0.5 cm. of liquid paraffin.

Haemoglobin calibration fluid. To 250 ml. of HbO₂ reactant in a 500 ml. volumetric flask, 250 ml. of dithionite reactant was added from the separating funnel, the mixture was quickly poured into the storage vessel with minimal agitation and at once covered with liquid paraffin.

RESULTS

Variation of the initial oxyhaemoglobin concentration

The time required for the deoxygenation of a given fraction of the oxyhaemoglobin, calculated from spectrophotometric analysis at 430 mμ, is independent of the initial oxyhaemoglobin concentration, and the reaction follows an approximately first-order course (Fig. 1). The first-order plot is strictly linear only beyond 60% saturation, how-

ever; the velocity coefficient, defined by $kY = -dY/dt$, increases significantly during the earlier stages of the reaction (see also Table 2). Values for the first-order velocity coefficient calculated over the last 60% of the reaction, denoted by k_a , are given in Table 1 for a series of experiments at pH 8.4 in which the initial oxyhaemoglobin concentration varies fivefold. Haemoglobin from a single individual was used. When corrected to 18.0° by means of the temperature coefficient given later, the results of these nine experiments show a variation of $\pm 3.5\%$, which confirms the precision of the technique.

Fig. 1. First-order plots for the rate of deoxygenation of oxyhaemoglobin at two concentrations. Concn. of $\text{Na}_2\text{S}_2\text{O}_4$, 0.2 g./100 ml.; pH 8.4. Y, Percentage saturation of the pigment with oxygen. O, Expt. no. 11, 17.7°, 0.017 m-equiv. of $\text{HbO}_2/\text{l.}$; Δ , Expt. no. 14, 17.8°, 0.067 m-equiv. of $\text{HbO}_2/\text{l.}$

Comparison of spectrokinetic measurements at several wavelengths

As a test of the validity of the spectrophotometric analysis, kinetic curves were obtained by measurements at two or more wavelengths with the same blood sample. In the most detailed experiments, with two samples of blood drawn for transfusion and containing citrate and glucose, the five wavelengths of the absorption maxima of oxyhaemoglobin and haemoglobin were used (Fig. 2). Over the whole course of the reaction at pH 8.4 the values of the percentage saturation calculated from measurements at 415 $\text{m}\mu$ were significantly

Fig. 2. Kinetic curves recorded at three wavelengths in experiments with the same sample of oxyhaemoglobin: pH 8.4; concn. of $\text{Na}_2\text{S}_2\text{O}_4$, 0.2 g./100 ml.; temp. 18.2°. O, Expt. 44, 430 $\text{m}\mu$, 0.037 m-equiv. of $\text{HbO}_2/\text{l.}$; $\bullet$, Expt. 45, 415 $\text{m}\mu$, 0.068 m-equiv. of $\text{HbO}_2/\text{l.}$; Δ , Expt. 46, 578 $\text{m}\mu$, 0.33 m-equiv. of $\text{HbO}_2/\text{l.}$

Table 1. Data of nine experiments with oxyhaemoglobin of a normal individual

Conditions were: pH 8.4; concn. of $\text{Na}_2\text{S}_2\text{O}_4$, 0.2 g./100 ml.; λ 430 $\text{m}\mu$. [HbO_2], concn. of oxyhaemoglobin.

Expt. no.	Flow rate (ml./sec.)	[HbO_2] (m-equiv./l.)	Temp.	k_a (sec. ⁻¹)	k_a at 18° (sec. ⁻¹)
9	5.1	0.0325	17.6°	10.2	10.7
10	5.1	0.0380	17.6	10.2	10.7
11	5.1	0.0170	17.7	10.8	11.2
12	5.1	0.079	17.9	11.3	11.4
13	5.1	0.0284	17.7	10.7	11.1
14	5.1	0.067	17.8	10.9	11.2
16	5.1	0.0268	18.4	11.6	11.0
17	9.8	0.0327	18.5	11.8	11.0
19	8.1	0.028	18.5	11.7	10.9
Mean					11.1 $\pm$ 0.4

smaller than those obtained at 430 m μ . The values obtained at 578, 560 and 540 m μ agreed with one another, but were greater over the last half of the

Fig. 3. Time course of deoxygenation at pH 8.4 recorded at 430 m μ (○) and 415 m μ (●). Expt. no. 69, 21.1°, 0.039 m-equiv. of HbO₂/l., concn. of Na₂S₂O₄ 0.2 g./100 ml. The lines are calculated first-order curves fitted to the data in the range Y 60–20%.

Fig. 4. Time course of deoxygenation at pH 6.3 recorded at 430 m μ (○) and 415 m μ (●). Expt. no. 65, 13.8°, 0.038 m-equiv. of HbO₂, concn. of Na₂S₂O₄ 0.2 g./100 ml. The lines are calculated first-order curves fitted to the data in the range Y 60–20%.

reaction than those obtained at 430 m μ . In each case the first-order plot shows a significant increase of slope over the early part of the deoxygenation, but is linear below 60% saturation. Values for k_a calculated from the slopes are 11.8 sec.⁻¹ at 415 m μ , 10.8 sec.⁻¹ at 430 m μ and 8.9 sec.⁻¹ at 578 m μ .

In all subsequent experiments with fresh blood, under various conditions of concentration, temperature and pH, measurements were made at both 415 and 430 m μ . The results obtained at pH 8.4 (Fig. 3), and at pH 9.5, confirmed those just described. Under all conditions the apparent time course of the reaction was similar at the two wavelengths. The differences between the calculated percentage saturation decreased with decrease of pH and at pH 6.3 identical values were obtained at the two wavelengths (Fig. 4).

These experiments show that the assumption that the reaction mixture contains only two absorbing species, characterized by the absorption spectra of the initial reactant and final product, is not strictly valid at neutral and alkaline pH.

Variation of dithionite concentration. The results in Fig. 5 show that deoxygenation is preceded by an induction period which decreases from about 30 msec. with a dithionite concentration of 0.05 g./100 ml. to less than 5 msec. with dithionite concentrations greater than 0.2 g./100 ml. These experiments were made at pH 8.4 and 18°, with measurements at 430 m μ . Similar results were

Fig. 5. Kinetic curves at 430 m μ with various dithionite concentrations: ▲, 0.05; ●, 0.1; ○, 0.2; △, 0.4; ■, 0.8 g./100 ml. Expt. nos. 20–24, pH 8.4, temp. 18°, 0.031–0.036 m-equiv. of HbO₂/l.

obtained with measurements at 415 m μ , and also at pH 7.0, where the induction period was shorter than at pH 8.4. The value of k_a increases slightly with dithionite concentration over the range tried, but the upward trend of k during the first 40 % of the reaction persists. This is shown in Table 2 by values of k calculated from pairs of successive measurements in the experiments with dithionite concentrations of 0.05 and 0.8 g./100 ml. A 16-fold increase of dithionite concentration, which practically eliminates the induction period, causes only a small increase in the rate of haemoglobin formation, and does not accelerate the early stages of the reaction, from Y 90 to 60 %, to a greater extent

Table 2. Variation of velocity coefficient during the deoxygenation of oxyhaemoglobin in the presence of dithionite at pH 8.4

Velocity coefficient, $k = 2.3 \Delta \log Y / \Delta t$.			
Expt. no. 20 0.05 g. of Na ₂ S ₂ O ₄ /100 ml.		Expt. no. 23 0.8 g. of Na ₂ S ₂ O ₄ /100 ml.	
Mean value of Y (%)	k (sec. ⁻¹)	Mean value of Y (%)	k (sec. ⁻¹)
92	6.2	90	7.4
83	7.9	83	10.0
78	6.9	76	9.8
70	8.7	68	11.6
57	9.9	60	12.2
45	10.5	54	12.6
39	9.9	48	12.1
34	10.6	40	12.0
30	10.1	31	12.8
		23	13.2
		18.5	12.8
		16	12.0
		13	12.2

Fig. 6. Variation of the first-order velocity coefficient with pH at 18° for the deoxygenation of oxyhaemoglobin samples from K.D. (●), and three other individuals (○, □, △). The curve was calculated from $pK = 7.0$, k_a for acidic form = 55 sec.⁻¹ and k_a for basic form = 10 sec.⁻¹. 0.03–0.04 m-equiv. of HbO₂/l.; concn. of Na₂S₂O₄, 0.2 g./100 ml.

than the later stages, and the initial upward trend in k remains. Similar results were obtained with haemoglobin from another individual, and also with a second sample of sodium dithionite.

In all the following experiments, the dithionite concentration was 0.2 g./100 ml.

Variation of pH. The reaction was studied at several pH values from 5.9 to 9.4, at temperatures from 18 to 21°, and with spectrophotometric measurements at both 415 and 430 m μ . The buffer solutions were Clarke and Lubs borate (pH 8.0–9.4) and phosphate (pH 5.9–7.65), and the ionic strengths of the reaction mixtures varied from 0.1 to 0.17. Experiments could not be made in more acid solutions because of turbidity; even at pH 5.9, the light absorption of the product changed slowly on standing, but at pH 6.3 the product was stable.

Plots of $\log Y$ against time were linear from 60 % down to 20 % saturation at every pH value. At pH > 7.0, linearity extended to less than 5 % saturation, but in slightly acid solutions there was evidence of slight deviations towards slower rates below 20 % saturation (cf. Fig. 4). The gradual increase in the first-order velocity coefficient in the early stages of the reaction was evident over the whole pH range, but became progressively less marked with decrease of pH: at pH 6.3, the maximum value k_a was reached at 70 % saturation.

From studies of the effect of temperature on the reaction rate, described below, values for k_a at 18° were calculated, and are plotted against pH in Fig. 6.

Variation of temperature. Values of $\log k_a$ obtained from measurements at 430 m μ in experiments with oxyhaemoglobin of one individual, at various pH values and temperatures from 15 to 30°, are plotted against the reciprocal of the absolute temperature in Fig. 7. The activation energy calculated from the slopes increases slightly with increasing acidity, and the difference between the values of 22 000 cal./mole at pH 6.3 and 26 000 cal./mole at pH 8.4 is outside the random error of the measurements.

To test whether the rate during the first 40 % of deoxygenation, during which k increases, varies with temperature to the same extent as the later stages, $\log Y$ was plotted against $t.k_a/2.3$ for experiments at pH 8.4 in the temperature range 15–30°, over which k_a increases ninefold. The slopes of these plots (Fig. 8) are equal to k/k_a , and their constancy shows that this ratio is independent of temperature. The average value of k/k_a is about 0.7 in the range Y 80–95 %. The curves for the several experiments are not exactly superimposed because differences of flow rate affect the rate of mixing, and temperature differences alter the duration of the induction period.

Variation of ionic strength. The effect of ionic strength on k_a was studied over a range of pH by varying the buffer concentration and by the addition of sodium chloride. At pH 8.5 and 9.5, the buffer was borate-potassium chloride-sodium hydroxide and, at pH 7.0 and 6.3, sodium dihydrogen phosphate-sodium hydroxide. At pH 8.0, both borate and phosphate buffers were used in two concentrations, 0.05 and 0.2M. There was no

evidence of specific ion effects. With increase of ionic strength, k_a increased at pH 8.0-9.5, decreased at pH 6.3, and varied only slightly at pH 7.0. The results shown in Fig. 9 were obtained from measurements at 430 m μ . The salt effects at pH 6.3 and 9.5 were confirmed in measurements at 415 m μ .

Comparative data for oxyhaemoglobin of several individuals. Values for k_a at 18.0° and pH 8.4 for haemoglobin samples from five normal individuals, calculated from measurements at 430 m μ , were 11.1, 11.2, 11.5, 11.8 and 11.8 sec.⁻¹. The first of these is the mean of nine experiments (cf. Table 1), and the difference of 6% between this and the last two values is significant. Some of these samples were also studied at lower pH values (Fig. 6), but for these the differences were within the limits of experimental error. Samples from patients suffering from polycythaemia, untreated pernicious anaemia and acute haemolytic anaemia (acholuric jaundice) gave values of 11.3, 11.4 and 11.3 sec.⁻¹ for k_a at pH 8.4 and 18.0°.

Kinetic studies over the pH range 6.5-8.4 were also made with haemoglobin samples from a patient suffering from sickle-cell anaemia and an individual showing sickle-cell trait. These experiments were made at an early stage of the research, and are less reliable than the later work, but the results did not differ significantly from those of control experiments with normal haemoglobin, and showed the same pH effect on the rate and the usual upward trend in k . The values for k_a were smaller, by about 15%, than those obtained in the later work. This was probably due to the use of a poor sample of dithionite and to neglect of sources of systematic error unsuspected at that time.

Effects of salts related to dithionite. Possible effects of oxidation products and impurities in the dithionite were sought in experiments at pH 8.5. Sodium sulphate, metabisulphite, thiosulphate and

Fig. 7. Arrhenius plots for the deoxygenation of oxyhaemoglobin (K.D.) at several pH values: Δ , pH 6.3; $\square$, pH 7.35; $\circ$, pH 8.4; $\bullet$, pH 9.4. 0.03-0.04 m-equiv. of $\text{HbO}_2/\text{l.}$; concn. of $\text{Na}_2\text{S}_2\text{O}_4$, 0.2 g./100 ml.

Fig. 8. Plots of $\log Y$ against $t.k_a/2.3$ for five experiments at temperatures from 15 to 30°, pH 8.4, 0.03-0.04 m-equiv. of $\text{HbO}_2/\text{l.}$, concn. of $\text{Na}_2\text{S}_2\text{O}_4$, 0.2 g./100 ml. k_a is the first-order velocity coefficient calculated from measurements in the range Y 60-20%, and t is time in sec.

Fig. 9. Variation of the first-order velocity coefficient with ionic strength: $\blacksquare$, pH 9.5; $\circ$, pH 8.5; $\bullet$, pH 8.0; $\blacktriangle$, pH 7.0; $\triangle$, pH 6.3. 0.03-0.04 m-equiv. of $\text{HbO}_2/\text{l.}$, concn. of $\text{Na}_2\text{S}_2\text{O}_4$, 0.2 g./100 ml.

sulphide added to the dithionite reactant (0.4 g./100 ml.) in concentrations of from 0.08 to 0.2 g./100 ml. had no effect on kinetic measurements at 415 and 430 m μ .

DISCUSSION

The validity of the optical methods which have been used to measure the percentage saturation of haemoglobin with various gases in kinetic studies depends upon the assumption that the reacting mixture contains only two absorbing species, characterized by the absorption curves of the unreacted and fully reacted mixture. This assumption has been tested by the comparison of measurements at several wavelengths, and the results show that it is not strictly true for the deoxygenation reaction in the presence of sodium dithionite. The apparent percentage saturation, after a given time, varied with the wavelength over a range of about 8% in slightly alkaline solutions. Therefore, one or more additional absorbing species must be formed, either as intermediates in the reaction or in secondary reactions. There will in fact be five molecular species in a partially saturated mixture, according to the intermediate-compound theory, but if each of the four haems in the haemoglobin molecule contributes equally and independently to the light absorption, regardless of the presence of a ligand on the iron atoms of the other three, there will be effectively only two absorbing species, oxygenated and unoxxygenated haems, and the light absorption should be linearly related to the percentage saturation of the pigment. Hartridge & Roughton (1925) found this to be true within the error of their spectroscopic method of analysis, and it has been stated that tests with more sensitive spectrophotometric methods also support this assumption (Roughton, 1955; Gibson & Roughton, 1957*a*).

The discrepancies between measurements at different wavelengths can be explained by secondary reactions between haemoglobin and hydrogen peroxide formed in the auto-oxidation of dithionite, in which a small proportion of the haemoglobin is irreversibly degraded (Dalziel & O'Brien, 1957). The discrepancies are not large. At every wavelength used, the measurements indicated a similar time course for the reaction, and below about 60% saturation they conformed to the

requirements of a simple first-order dissociation. The velocity coefficient, k_a , varied by $\pm 15\%$ about the value obtained at 430 m μ . It is unlikely that the results obtained for the rate of reaction and its variation with temperature, pH and ionic strength are seriously in error.

A reproducibility of $\pm 3.5\%$ in k_a was realized consistently in experiments with single blood samples and with samples withdrawn from the same individual at intervals over a period of many months. There were no greater variations between the rates of deoxygenation, at several pH values, of haemoglobin from seven individuals, including three suffering from blood disorders, except at pH 8.4, where the highest and lowest values of k_a , 11.8 and 11.1 sec.⁻¹, differed significantly. It is of special interest that no significant differences were detected in the kinetic behaviour, at several pH values, of haemoglobins from a normal individual, one with sickle-cell trait, and a patient suffering from sickle-cell anaemia. Evidently the differences between the globins (Itano & Pauling, 1949) do not affect the haem-linked acidic groups.

In contrast, Gibson, Kreuzer, Meda & Roughton (1955) reported considerable differences between the rates of deoxygenation of three samples of human haemoglobin; for example, at pH 7.1 rate constants of 21, 26 and 36 sec.⁻¹ were obtained. Unfortunately, no evidence of the reproducibility of measurements with a single sample of blood was given. Even greater individual variations have, however, been reported for the rates of combination of human and sheep haemoglobin with oxygen and carbon monoxide (Hartridge & Roughton, 1925; Gibson *et al.* 1955; Gibson & Roughton, 1957*a*).

In Table 3, values for k_a at 18° obtained in the present work from measurements at 430 m μ are compared with specific reaction rates for human haemoglobin reported by other workers, corrected where necessary to 18° with the temperature coefficient reported here. The consistency of the data is remarkably good in view of the differences in technique. Millikan (1933) used a constant-flow method with colorimetric analysis, and his results were consistent with a simple first-order course throughout. Dubois (1941) used the deoxygenation of a mixed blood sample as a test of his constant-flow apparatus. Gibson *et al.* (1955) made measurements over the first third of the reaction only, with

Table 3. *Specific rates of deoxygenation of human haemoglobin at 18°*

Source	Specific reaction rate (sec. ⁻¹)			
	pH 6.0	pH 7.1	pH 8.5	pH 9.1-9.8
Present work, k_a	51	29	11	11
Millikan (1933)	44	24	10	10
Dubois (1941)	—	—	11	—
Gibson, Kreuzer, Meda & Roughton (1955)	—	28	—	11

a stopped-flow method, and the values given in the Table are averages of those reported for three blood samples.

Hartridge & Roughton (1923) found the rate of deoxygenation of sheep haemoglobin to be independent of the dithionite concentration above 0.05 g./100 ml., and concluded that the role of the dithionite is that of a passive oxygen absorbent, which, after an induction period during which the oxygen concentration is reduced sufficiently, permits the dissociation of oxyhaemoglobin and eliminates the reverse reaction. These findings have been generally confirmed here, although the greater precision of the method has revealed an increase of about 10% in the rate of deoxygenation, at pH 8.4, for an increase of dithionite concentration from 0.05 to 0.8 g./100 ml. This small increase can be accounted for as a neutral-salt effect, however. The results establish that deoxygenation by second-order reaction of oxyhaemoglobin with dithionite is very slow, if it occurs at all, but do not of course exclude the possibility that dithionite may have some influence on the rate of dissociation, for example by reaction with the globin.

The variation of the rate of deoxygenation of human haemoglobin with pH is very similar to that of sheep haemoglobin, and can be explained by the assumption (Henderson, 1920) that oxyhaemoglobin contains an oxy-labile acidic group with pK 7.0 and that the deoxygenation of the unionized form is five times as fast as that of the basic form. The corresponding data of Hartridge & Roughton (1923) for sheep haemoglobin were pK 6.7, and a ratio of 7 between the rates of deoxygenation of acidic and basic forms; the differences are within the experimental error. The pH effects on the rates of dissociation of the first carbon monoxide and nitric oxide molecule respectively from the carbon monoxide and nitric oxide compounds of sheep haemoglobin are also accounted for by the assumption of a haem-linked group with pK 6.7 (Gibson & Roughton, 1957*b*, *c*). These kinetic effects may all be attributed to the weaker of the two haem-linked groups revealed by pH effects on the dissociation curve (Ferry & Green, 1929) and differential titration of oxyhaemoglobin and haemoglobin (German & Wyman, 1937), for which pK values of 6.7 and 5.8 have been estimated for horse haemoglobin (Wyman & Ingalls, 1941; Wyman, 1948).

Salt effects on the rate of deoxygenation have not been recorded hitherto. The increase of rate with ionic strength at pH 8.4 and 9.4, and the reverse effect at pH 6.3, cannot be explained by a secondary salt effect on the dissociation of a haem-linked acidic group. A difference of total charge between the oxyhaemoglobin molecule and

the activated complex is not consistent with the fact, referred to below, that the entropy of activation appears to be greater in alkaline solution than in acid solution, and the absence of any evidence that the isoelectric points of oxyhaemoglobin and haemoglobin differ significantly. A more likely explanation of the salt effects, perhaps, is that disorganization of the protein structure accompanies the formation of the activated complex (cf. Eley, 1943) and that concomitant redistribution of charge and fission of hydrogen bonds is influenced by the relatively high salt concentrations used here. The situation is complicated by the fact that the processes involved in haem interaction may also be influenced by salts. It would be of interest to investigate salt effects on the dissociation of the first of the four oxygen molecules alone, the rate of which can be determined in the absence of dithionite (Gibson & Roughton, 1955), and also to make similar studies of the deoxygenation of myoglobin, in which haem interaction cannot occur.

The activation energies for the overall deoxygenation, 22 000 cal. at pH 6.3 and 26 000 cal. at pH 8.4, agree very well with the value of 22 000 cal. obtained by Hartridge & Roughton (1923) for sheep haemoglobin at pH 9.0. The latter workers also recorded some measurements at pH 5.6 which indicated a slightly greater activation energy of 25 000 cal., but the difference, which is evidently within the experimental error, was not commented upon. Nevertheless, Eley (1943) regarded the difference as significant, and, on the basis of the transition-state theory, calculated entropies of activation of 36 and 24 cal./degree/mole at pH 5.6 and pH 9.1 respectively. The pH effect on the activation energy indicated by the present experiments, which give entropies of activation of 25 cal./degree/mole at pH 6.3 and 36 cal./degree/mole at pH 8.4, is in the opposite direction, and suggests that the higher rate of deoxygenation in acid solution is due to a lower activation energy and not to a larger positive entropy of activation, as Eley concluded for sheep haemoglobin.

Evidence that the deoxygenation of sheep oxyhaemoglobin does not follow a simple first-order course throughout, as was concluded by Millikan (1933), was first obtained by Legge & Roughton (1950). Their conclusion that an initial upward trend in the velocity coefficient is a genuine feature of the kinetics of the overall reaction is strongly supported by the present experiments, in which the trend is far outside the experimental error and has been demonstrated under various conditions. Experiments in which the dithionite concentration was varied 16-fold, the initial oxyhaemoglobin concentration sixfold, and the flow rate twofold, together with experimental controls of the tech-

nique itself which included kinetic studies of the dehydration of carbonic acid (Dalziel, 1953), established that the effect is not due to insufficiently rapid removal of dissolved oxygen by the dithionite, incomplete mixing, flow anomalies or other technical errors. It is especially important, in view of the occurrence of secondary reactions which interfered with the colorimetric analysis by Legge & Roughton (1950), that the deviations from a simple first-order course have been recorded by measurements with monochromatic light of five wavelengths, comprising the absorption maxima of reactant and product. On the intermediate-compound theory, this feature may be attributed to the fact that k_4 , the velocity constant for the dissociation of the first of the four oxygen molecules from oxyhaemoglobin, is smaller, relative to the rate constants for the three succeeding dissociations, than would be expected on a statistical basis. This interpretation is consistent with the equilibrium data (Roughton, 1949), and convincing kinetic evidence in its support was obtained by Gibson & Roughton (1955).

Direct measurements of the individual constant k_4 , by a method which does not involve the use of dithionite, have now been reported for both human and sheep haemoglobin, and preliminary comparisons of these data with the overall kinetics in the presence of dithionite have been made (Gibson & Roughton, 1955; Gibson *et al.* 1955). The present findings will be briefly considered in the light of these discussions. First, the overall velocity coefficient k_a is greater at pH 7.1, by a factor of 2.5, than at pH 9.1. Over this pH range, the overall velocity coefficient in the very earliest stages of the reaction is estimated, by extrapolation to zero time, as $< 0.6 k_a$, and should be equal to $0.25 k_4$. On the other hand, direct measurements showed that k_4 is independent of pH over this range. Although this is not inconsistent with the marked pH effect on k_a , which could be put down to an effect on one or more of the other three individual rate constants, the initial upward trend of k should in this case be considerably more marked at pH 7.1 than at pH 9.1. There was no evidence of this in the present experiments; indeed there were some indications of the reverse effect.

Secondly, the finding that the initial trend in the overall velocity coefficient is not significantly altered by temperature change, from 15° to 30°, indicates that the activation energy for k_4 is not greatly different from that calculated for k_a . In this case, the overall data are consistent with direct determinations of k_4 : for human haemoglobin at pH 7.1, Gibson *et al.* (1955) obtained an activation energy of 19 600 cal. for k_4 , compared with the value of 23 000 cal. for k_a found here. The difference is not great enough to produce a significant tem-

perature effect on the time course of overall deoxygenation.

It is evident that further direct comparisons of the overall deoxygenation kinetics and direct measurements of k_4 on the same blood sample are needed. The value of k_4 appears to vary considerably from sample to sample (Gibson *et al.* 1955), but the reproducibility of the data for a single sample has not been demonstrated. Secondary reactions accompanying the overall deoxygenation in the presence of dithionite have so far discouraged attempts to evaluate all four individual dissociation velocity constants, as has been done for the carbon monoxide reaction (Gibson & Roughton, 1957*a*). These secondary reactions may be minimized by using high concentrations of pigment and dithionite (Dalziel & O'Brien, 1957), and controlled by making measurements at two or more wavelengths corresponding to the absorption maxima of haemoglobin and oxyhaemoglobin. It may also be possible to obtain evidence of the nature and magnitude of the errors from this source by spectrokinetic studies of the reduction of methaemoglobin by dithionite in the presence and absence of dissolved oxygen, since the absorption spectrum of methaemoglobin in the Soret region is similar to that of oxyhaemoglobin.

It is unlikely, however, that the marked differences between the pH effects on k_a and k_4 can be due to secondary reactions of the type demonstrated so far. Dithionite may affect the rate of deoxygenation by reaction with the globin (cf. Gibson & Roughton, 1955). It is known that dithionite can react with amino groups, and Roughton (1944) has suggested that the weaker of the two oxy-labile groups may be an amino group which can bind carbon dioxide. In this connexion studies of the effect of carbon dioxide on the kinetics of deoxygenation would be of interest.

SUMMARY

1. Spectrokinetic studies of the deoxygenation of human haemoglobin in the presence of sodium dithionite have been made under various conditions with a constant-flow rapid-reaction apparatus and a photoelectric spectrophotometer. A reproducibility of $\pm 3.5\%$ in estimates of the first-order velocity coefficient has been demonstrated.

2. No significant differences were detected between the kinetic behaviour at several pH values of oxyhaemoglobin samples from normal individuals and from persons suffering from several types of anaemia, including sickle-cell anaemia. The greatest difference observed between two haemoglobin samples was 6% in the specific reaction rate.

3. Significant differences between the results of analyses of the reaction mixture, by measurements

of light absorption at several wavelengths, show that the deoxygenation is accompanied by secondary reactions involving the pigment. The errors thereby introduced are not large, but realization of the full benefits of the precision of the technique in the interpretation of the detailed time course of the reaction is restricted.

4. The time course of the overall reaction cannot be accurately described by the simple first-order relation, $d[\text{HbO}_2]/dt = k[\text{HbO}_2]$. The calculated value of k increases from an initial value of $< 0.6k_a$ to a constant value of k_a when reaction is 40 % complete. This initial trend in k is not affected by wide variations of the initial oxyhaemoglobin and dithionite concentrations, and is evident in measurements at five wavelengths at pH 8.4 and two wavelengths at pH 6.3–9.5, and is therefore considered to be a genuine kinetic feature of the deoxygenation. This confirms the conclusion of Legge & Roughton (1950) for the deoxygenation of sheep haemoglobin, and is in agreement with other recent work on the kinetics and equilibria of sheep haemoglobin and with the intermediate-compound theory.

5. Variations of the rate of deoxygenation with pH are consistent with the presence of an oxy-labile group with pK 7.0. The specific reaction rate is five times as great at pH 6.0 as at pH 8.5–9.5. There was no evidence of a pH effect on the initial upward trend in the specific reaction rate.

6. Activation energies of 22 000 cal. at pH 6.3 and 26 000 cal. at pH 8.4 have been calculated from the variation of the rate of deoxygenation with temperature. There was no evidence that the early and late stages of the overall deoxygenation were differently affected by temperature change.

7. With increase of ionic strength, the specific reaction rate decreases at pH values below 7.0 and increases at pH values above 7.0.

8. The results are considered in relation to other recent work, and certain anomalies are discussed.

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