# The Oxidation of Myoglobin to Metmyoglobin by Oxygen

## 2. THE RELATION BETWEEN THE FIRST ORDER RATE CONSTANT AND THE PARTIAL PRESSURE OF OXYGEN

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In a previous paper (George & Stratmann, 1952) we showed that the oxidation of myoglobin to metmyoglobin by oxygen at constant oxygen pressure, pH, salt concentration and temperature was first order in unoxidized myoglobin. It thus resembles the autoxidation of haemoglobin investigated by Brooks (1931). In air the oxidation rate is 4·25 times faster than with haemoglobin.

Brooks (1935) showed that the observed first order rate constant for haemoglobin autoxidation was a function of the oxygen pressure, having a well defined maximum value at about 20 mm. In this paper the results of a similar investigation are reported for myoglobin, together with the determination of the equilibrium constant of the myoglobin-oxygen reaction, $K_e$

$$Mb + O_2 \rightleftharpoons MbO_2$$

under the conditions of the experiments. This value of the equilibrium constant is used in an analysis of the results based on a suggestion by George (1949) that the observed first order constant for haemoglobin was a complex function given by

$$k_{obs.} = k \frac{K}{K + [O_2]} \frac{[O_2]}{K + [O_2]}, \qquad (1)$$

where $K$ is the reciprocal of the equilibrium constant for the formation of oxyhaemoglobin. A full comparison is made between the kinetic data for myoglobin and haemoglobin.

## MATERIALS AND APPARATUS

The myoglobin used in these experiments was prepared by the method described by George & Stratmann (1952). The myoglobin solutions were heavily buffered with $K_2HPO_4$ and $KH_2PO_4$, giving a total concentration of phosphate ions of 0·6 M with a pH of 5·69 at 30°.

The flow apparatus was similar to that described by Brooks (1935). The gas mixtures were prepared from cylinders of $O_2$-free $N_2$ and either air or $O_2$ of 'medical' purity. The gases were passed first through cotton wool. The $N_2$ was passed through alkaline pyrogallol solution and the $O_2$ or air through alkaline permanganate solution. In order to prevent deposition of moisture in the capillaries of the flow meters the gases were further washed in half-saturated NaCl. After passing through the flow meters the gases were mixed, passed through a glass coil immersed in the thermostat to preheat it, then passed through a buffer solution like that used for the myoglobin and finally led into the reaction vessel. A similar coil and tube containing buffer solution was used to cool the gas when the flask was immersed in the ice bath employed in the preliminary preparation of the $MbO_2$ described below. The rate of gas flow was 6 l./hr.

### *Kinetic measurements*

The flask used in these experiments was similar to the one used by Brooks (1935) but was boat-shaped like a Meldrum-Roughton boat but without the central partition. A solution of metmyoglobin (MetMb) with a concentration of $2·57 \times 10^{-4}$ M was run into the flask. The optical density of the MetMb having been recorded, a small quantity of sodium dithionite ($Na_2S_2O_4$) was added and the flask gently shaken until the slight excess of $Na_2S_2O_4$ was oxidized and oxymyoglobin formed. The flask was then closed, placed in an ice bath and the gas mixture passed through the flask for 1·5–2 hr. whilst the flask was shaken slowly. A sample of the myoglobin solution was removed, diluted and the optical density measured. The usual sample removed was 1 ml. and diluted with 4 ml. of buffer solution. Details of the spectrophotometric methods of analysis used are given in a previous paper (George & Stratmann, 1952). The flask was then transferred to the thermostat at 30°. At intervals throughout the experiment samples were removed and the optical density measured. During the time taken to measure the optical density of the solutions there was no observable change due to autoxidation. The starting point of the reaction was taken as being the time when the flask was placed in the thermostat. Results were recorded as the percentage of oxymyoglobin present, taking the starting point of the reaction as 100% $MbO_2$. Log percentage $MbO_2$ was plotted against time and the first order rate constant of the reaction ($k$ hr.$^{-1}$) was calculated from the slope of the line.

### *Equilibrium measurements*

The oxygen dissociation curve of myoglobin was determined under the same general conditions as obtained in the kinetic experiments. The reaction flask had a side arm which was of such a size as to fit into the cell carrier of the spectrophotometer. The Pyrex glass used in its construction did not absorb appreciably in the range of wavelengths used, and since the method was a comparative one the small optical density difference between this tube and the reference cell was of no consequence.

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Two slightly different techniques were used. In the first a concentration of $5{\cdot}14 \times 10^{-5}$M-myoglobin was used and the optical densities measured at 540 m$\mu$. In the second, one-tenth of the above concentration was used and the optical densities measured at 420 m$\mu$. In both methods approximately 10 ml. of metmyoglobin were placed in the flask. This was allowed to run into the side arm and the optical density

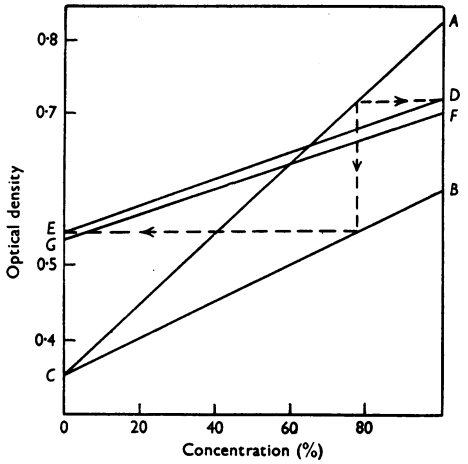


Fig. 1. Diagram used to facilitate calculation in the determination of the equilibrium constant of the reaction: $Mb + O_2 \rightleftharpoons MbO_2$ at 30° in 0·6M-phosphate buffer of pH 5·69. The points *A*, *B* and *C* represent the optical densities (at a wavelength of 540 m$\mu$.) of solutions containing 100% $MbO_2$, 100% Mb and 100% MetMb respectively. The line *AC* relates optical density to the percentage oxidation. The line *BC* relates optical density to the proportion of Mb and MetMb present in the solution if no $MbO_2$ is present. The point *D* gives the amount of MetMb formation which has occurred by autoxidation in the time taken for equilibration of the solution with the gas mixture. This point enables the line *DE*, which relates optical density to the proportion of Mb and $MbO_2$ present, after oxidation has been taken into account, to be drawn. The dotted line shows how the points *D* and *E* are derived. The measured optical density of the solution, equilibrated with the gas mixture, is used to determine the percentage of $MbO_2$ in the mixture by simply reading off the corresponding value on the line *DE*. The line *FG* represents a second equilibration experiment, using the same solution, when further oxidation has taken place.

was measured. The flask was removed from the spectrophotometer, placed in an ice bath and the solution run back into the body of the flask. A small quantity of $Na_2S_2O_4$ was added and the flask gently shaken until reduced myoglobin was formed. The optical density was again measured. The flask was again returned to the ice bath and shaken until all excess $Na_2S_2O_4$ was oxidized and oxymyoglobin formed. The optical density of this solution was measured. In order to facilitate calculation a diagram shown in Fig. 1 was constructed which related optical density to the percentages of $MbO_2$, Mb and MetMb present in the solution. *A* represents the optical density of a solution containing 100% $MbO_2$, while *B* and *C* represent the optical densities of solutions containing 100% Mb and MetMb respectively. Lines were drawn connecting *A* to *C* and *B* to *C*. The line *AC* relates the optical density of the solution with the proportion of $MbO_2$ and MetMb present in it or, in other words, the percentage oxidation. The line *BC* relates the optical density of the solution to the proportion of Mb and MetMb present in the mixture if no $MbO_2$ is present. A gas mixture of the desired partial pressure of $O_2$ was then passed through the flask which was shaken slowly in an ice bath for a period of 1 hr. The flask was then placed in the thermostat at 30° and shaken for 10–15 min. The flask was then quickly removed from the thermostat and the optical density of the solution measured. It was immediately replaced in the ice bath, opened to the air, the solution equilibrated with air and the optical density again measured. After equilibration with air the measured optical density (the point *D* on the diagram) gives the amount of oxidation of the mixture during the time taken for equilibration for the particular partial pressure of $O_2$, and enables the line *DE* to be drawn, which relates the optical density of the solution with the proportion of Mb and $MbO_2$ present, after oxidation has been taken into consideration. The dotted line on the diagram shows how this point *E* is derived. The measured optical density of the solution equilibrated with the gas mixture can now be used to determine the percentage $MbO_2$ in the solution by simply reading off the value which corresponds to the measured optical density on the line *DE*. The whole cycle was repeated once or twice. The line *FG* represents a second equilibration experiment, using the same solution, when further oxidation had occurred.

In the second method the technique was essentially the same. A wavelength of 420 m$\mu$. was chosen at which MetMb and Mb are isobestic and the diagram is in consequence simplified. As the solutions absorbed to a much greater extent at this wavelength it was necessary to use a solution approximately ten times more dilute than that used in the experiments described above.

### *Denaturation check*

The azide-metmyoglobin check for denaturation was carried out at 2 mm. $O_2$ pressure, it having been found previously that at 152 mm. $O_2$ pressure no detectable denaturation occurred (George & Stratmann, 1952).

## RESULTS

### *Denaturation check*

In 3 hr. and at a partial pressure of 2 mm. of oxygen 77·5% of metmyoglobin was formed from oxymyoglobin by autoxidation. Fig. 2 shows that some traces of denaturation of the protein are evident. This amounts, however, to no more than 1·5–1·7% of the myoglobin originally present, and it was not considered necessary to take this into consideration in the calculation of the rate constant.

### *Oxidation kinetics*

The metmyoglobin formation was measured at 10 and 20 min. intervals and at many partial pressures of oxygen ranging from 0·3 to 760 mm. Fig. 3 shows four typical first order plots at the following oxygen pressures: 760, 1·0, 0·6 and 0·3 mm.

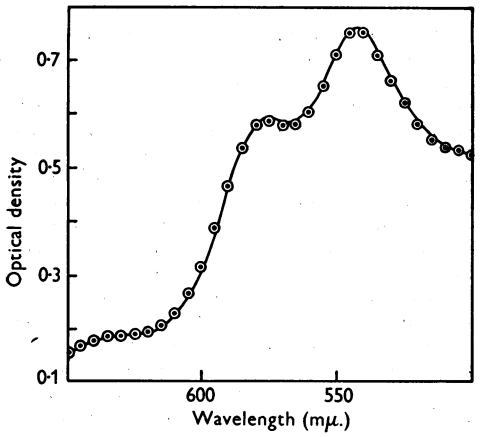


Fig. 2. Spectra of the azide-metmyoglobin complex formed before and after autoxidation of myglobin at 30° in 0·6 M-phosphate buffer of pH 5·69 at a partial pressure of oxygen of 2 mm. ——, from a solution of metmyoglobin before autoxidation; ⊙, from a solution of metmyoglobin of the same concentration after autoxidation for 3 hr.

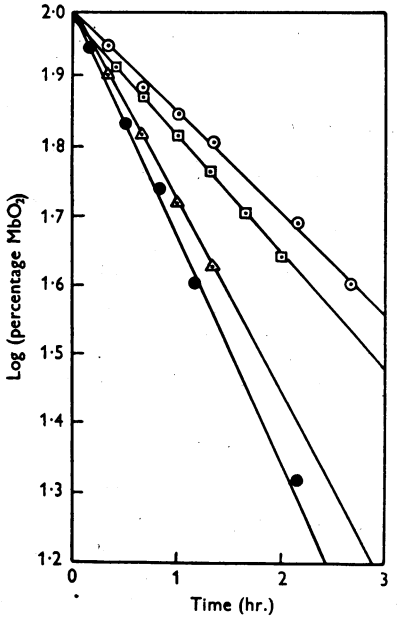


Fig. 3. Typical first order plots for the autoxidation of myoglobin to metmyoglobin in 0·6 M-phosphate buffer of pH 5·69 at 30° at four different partial pressures of oxygen. ⊙, partial pressure of oxygen of 760 mm.; ●, partial pressure of oxygen of 1·0 mm.; △, partial pressure of oxygen of 0·6 mm.; ⊡, partial pressure of oxygen of 0·3 mm.

The first order rate constants at these oxygen pressures were: 0·33, 0·75, 0·65 and 0·39 hr.$^{-1}$ respectively. Fig. 4 shows the relationship between the partial pressure of oxygen and the first order rate constant over the entire range of oxygen pressures studied. A clearly defined maximum was observed at a partial pressure of about 1 mm. oxygen. Increase of the oxygen pressure above approximately 30 mm. was found to have very little effect on the rate constant, its value at these pressures being around 0·30 hr.$^{-1}$. All these results were obtained with the same sample of myoglobin.

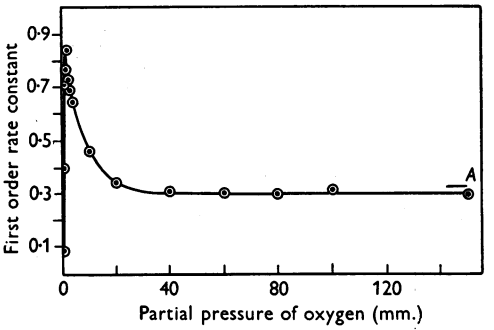


Fig. 4. The relationship between the partial pressure of oxygen and the first order rate constant for the autoxidation of myoglobin to metmyoglobin at 30° in 0·6 M-phosphate buffer, over the entire range of oxygen pressures studied. $A$ = the first order rate constant at 760 mm. $p_{O_2}$.

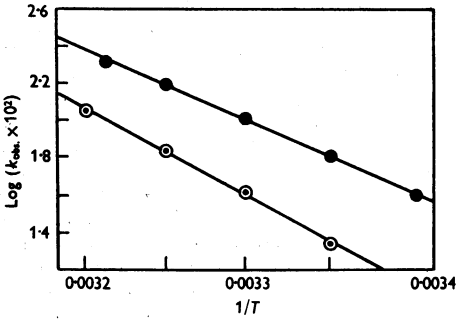


Fig. 5. Results of experiments to determine the activation energy of the autoxidation of myoglobin to metmyoglobin in 0·6 M-phosphate buffer of pH 5·69 at 4 and 760 mm. partial pressures of oxygen. ●, partial pressure of oxygen = 4 mm.; ⊙, partial pressure of oxygen = 760 mm.

*The activation energy of the autoxidation*

The activation energy of the reaction was determined at two partial pressures of oxygen, namely 4 and 760 mm. A range of temperatures from 22 to 38° was studied and the rate constant was determined at 4° intervals over this range. Fig. 5 shows the results of these experiments. Logarithm of the rate constant is plotted against $1/T$. Calculations from this data gives the activation energy, $E$, for the reaction at 760 mm. oxygen pressure as 25·0 ± 1 kg. cal., and at 4 mm. as 19 ± 1 kg.cal. The temperature

coefficient of the buffer solution used in these experiments is −0·03 pH units for an increase of 10° (Cohn, 1927). The rate constant would be changed by this decrease of pH over 16° by about 10%, by analogy with Brooks's (1931) results with haemoglobin. If this is the case then the true values of the activation energies are within 1 kg.cal. of the above values.

It was found to be impossible to obtain a satisfactory value for the rate constant in an experiment carried out at 4 mm. oxygen pressure and at 38° owing to rapid denaturation of the protein at this temperature and oxygen pressure. It would appear that reduced myoglobin is more easily denatured by heat than is oxymyoglobin, since the experiment at 38° and 760 mm. oxygen pressure was reproducible and there were no detectable traces of denaturation.

*The oxygen dissociation curve of myoglobin*

The fraction of myoglobin in the form of oxymyoglobin at a given oxygen pressure was obtained by the method described above. Spectrophotometric analysis at 420 mμ. was used in addition to the measurements at 540 mμ. in the hope that more consistent results would be obtained. In Table 1,

Table 1. *Measurement of equilibrium constant for* $Mb + O_2$ *at* 30° *in* 0·6 M-*phosphate buffer*, pH 5·69 *by spectrophotometric analysis at* 540 *and* 420 mμ.

| Wavelength (mμ.) | $p_{O_2}$ (mm.) | Fraction $MbO_2$ present $(1-\alpha)$ | Mean $1-\alpha$ | $K_e=\frac{(1-\alpha)}{\alpha p_{O_2}}$ |
|---|---|---|---|---|
| 540 | 0·6 | 0·37 | 0·38 | 1·02 |
| | | 0·38 | | |
| 540 | 1·0 | 0·55 | 0·55 | 1·08 |
| | | 0·53 | | |
| | | 0·48 | | |
| 540 | 2·0 | 0·60 | 0·61 | 0·78 |
| | | 0·62 | | |
| 420 | 3·0 | 0·65 | 0·66 | 0·65 |
| | | 0·67 | | |
| 420 | 5·0 | 0·84 | 0·81 | 0·85 |
| | | 0·81 | | |
| | | 0·78 | | |
| 420 | 10·0 | 0·95 | 0·90 | 0·90 |
| | | 0·90 | | |
| | | 0·85 | | |

Mean $K_e = 0{\cdot}88 \pm 0{\cdot}12$ mm.$^{-1}$

Therefore partial pressure of oxygen for half saturation = 1·00–1·32 mm., mean **1·16**.

which records the results at a number of different oxygen pressures, it is seen that there is little to choose between the two methods. The value of the equilibrium constant $K_e$ under the conditions of the experiments, at 30°, is $0{\cdot}88 \pm 0{\cdot}12$ from which it follows that the oxygen pressure for half saturation of the myoglobin is 1·00–1·32 mm.

## ANALYSIS OF THE RESULTS

The most striking feature of the results is that myoglobin, like haemoglobin, shows a maximum oxidation rate at low oxygen pressures. In the case of haemoglobin the oxygen pressure for the maximum rate corresponds closely to that required to half saturate the haemoglobin. This also holds for myoglobin as illustrated in Fig. 6.

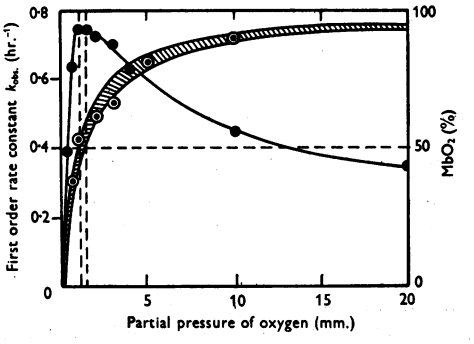


Fig. 6. The relationship between the oxygen pressure variation of the first order rate constant for the autoxidation of myglobin to metmyoglobin and the oxygen dissociation curve of myoglobin at 30° in 0·6 M-phosphate buffer of pH 5·69. ●, experimental values of the first order rate constant at partial pressures of oxygen over the range 0–20 mm. ⊙, experimental points in the determination of the oxygen dissociation curve of myglobin. The true dissociation curve lies within the shaded area, the two lines being the limits of experimental error. ----, the partial pressure of oxygen required for half saturation of the myoglobin.

George (1949) suggested that the oxygen pressure function responsible for this behaviour in the case of haemoglobin was that given in eqn. 1.

$$k_{obs.} = k \frac{K}{K+[O_2]} \frac{[O_2]}{K+[O_2]}. \qquad (1)$$

Inspection of Fig. 4 shows that this equation alone cannot adequately account for the myoglobin data. At oxygen pressures above 30 mm., $k_{obs.}$ reaches a constant value and does not continuously diminish as $p_{O_2}$ is increased in accord with this equation. It does, however, account for the maximum value of $k_{obs.}$ at the oxygen pressure required for half saturation of the myoglobin, which suggests that the correct function for $k_{obs.}$ may be of the form:

$$k_{obs.} = k_1 \frac{K}{K+[O_2]} \frac{[O_2]}{K+[O_2]} + Y. \qquad (2)$$

The additional oxygen pressure function $Y$ can be numerically small at low oxygen pressures and in consequence $k_{obs.}$ can be given, in effect, by Eqn. 1 and, in addition, at high oxygen pressures $Y$ can be such that a constant value for $k_{obs.}$ is obtained. The

actual form of $Y$ in the present data can be found in the following way. $\frac{K}{K+[O_2]}$ and $\frac{[O_2]}{K+[O_2]}$ are the values of $\alpha$ and $(1-\alpha)$, the fractions of uncombined myoglobin and oxymyoglobin respectively, so that eqn. 2 amounts to

$$k_{obs.} = k_1\alpha(1-\alpha) + Y. \quad (3)$$

If $k_1$ can be evaluated then the variation of $Y$ with oxygen pressure is given by $k_{obs.} - k_1\alpha(1-\alpha)$. eqn. 3 may be rewritten

$$\frac{k_{obs.}}{\alpha(1-\alpha)} = k_1 + \frac{Y}{\alpha(1-\alpha)}.$$

The problem is thus one of chosing a suitable oxygen pressure function to plot $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against so that $k_1$ may be obtained by extrapolation. Plotting against $p_{O_2}$ itself is not satisfactory since a definite curve is obtained. The functions $(1-\alpha)/\alpha$ and $1/\alpha$ both give plots which are reasonably linear as shown in Fig.7, the first point lying off the line in each case. Values of $k_1$ obtained from the intercept are 2·62 and 2·30 hr.$^{-1}$ respectively. Table 2 gives the calculation of $Y$ based on these two values using eqn. 3. It can be seen that the variation of $Y$ with oxygen pressure is of the type anticipated above. The linearity shown in Fig. 7 when $\frac{k_{obs.}}{\alpha(1-\alpha)}$ is plotted against $(1-\alpha)/\alpha$ implies that $Y$ is of the form $k_2(1-\alpha)^2$, and for the plot against $1/\alpha$ that $Y$ is of the form $k_2(1-\alpha)$. It seems that the experimental data are not precise enough to decide between these two possibilities. This is understandable for the uncertainty in the velocity constant is about $\pm$ 0·02 hr.$^{-1}$ in each case. The slopes of the two lines are identical for their numerical value should be equal to $k_{obs.}$ at high oxygen pressures, hence $k_2 = 0{\cdot}30$ hr.$^{-1}$. For myoglobin autoxidation we may thus summarize the data in the two possible expressions:

$$k_{obs.} = 2{\cdot}62\alpha(1-\alpha) + 0{\cdot}30(1-\alpha)^2, \quad (4a)$$

$$k_{obs.} = 2{\cdot}30\alpha(1-\alpha) + 0{\cdot}30(1-\alpha). \quad (4b)$$

It is of interest now to examine Brooks's (1935) data on haemoglobin autoxidation to see whether there is any evidence of similar behaviour. The analysis previously referred to is given in Table 3 (George, 1949). The ratio $\frac{k_{obs.}}{\alpha(1-\alpha)}$ is not in fact constant but shows a small steady increase as $p_{O_2}$ increases. The overall variation of this ratio, however, is far less than that obtained using Legge's

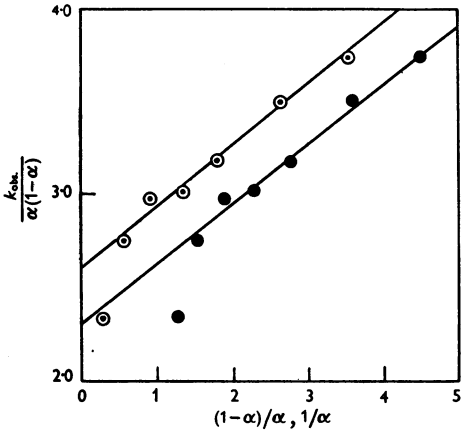


Fig. 7. Plot of $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against $(1-\alpha)/\alpha$ (⊙); and $1/\alpha$ (●) in the case of myglobin.

mechanism (Legge, 1942), assuming methaemoglobin formation by the unique decomposition of $Hb_4(O_2)_2$, and for this reason analysis of the results in terms of eqn. 1 was suggested. If now $\frac{k_{obs.}}{\alpha(1-\alpha)}$ is plotted against $(1-\alpha)/\alpha$ and $1/\alpha$ as has been done in Fig. 7 for myoglobin, the two lines in Fig. 8 are obtained, thus showing that haemoglobin autoxidation follows the same pattern. In this case the two possible equations are

$$k_{obs.} = 0{\cdot}68\alpha(1-\alpha) + 4\times10^{-2}(1-\alpha)^2, \quad (5a)$$

$$k_{obs.} = 0{\cdot}64\alpha(1-\alpha) + 4\times10^{-2}(1-\alpha). \quad (5b)$$

Table 2. *Calculations of Y, the high pressure component of the overall first order constant for myoglobin autoxidation*

($Y = k_{obs.} - k_1\alpha(1-\alpha)$, where $k_1 = 2{\cdot}62$ or $2{\cdot}30$ hr.$^{-1}$
$\alpha = K/K+O_2$, $1-\alpha = O_2/(K+O_2)$, where $K = 1{\cdot}16$ mm.)

| $p_{O_2}$ (mm.) | $1-\alpha$ | $\alpha$ | $\alpha(1-\alpha)$ | $k_{obs.}$ | $k_{obs.} - 2{\cdot}62\,\alpha(1-\alpha)$ | $k_{obs.} - 2{\cdot}30\times\alpha(1-\alpha)$ |
|---|---|---|---|---|---|---|
| 0·3 | 0·21 | 0·79 | 0·166 | 0·39 | (−·07) | (−0·01) |
| 0·6 | 0·35 | 0·65 | 0·228 | 0·635 | 0·035 | 0·105 |
| 1·0 | 0·47 | 0·53 | 0·249 | 0·745 | 0·085 | 0·165 |
| 1·5 | 0·57 | 0·43 | 0·245 | 0·74 | 0·100 | 0·180 |
| 2·0 | 0·64 | 0·36 | 0·230 | 0·74 | 0·130 | 0·200 |
| 3·0 | 0·725 | 0·275 | 0·199 | 0·70 | 0·180 | 0·240 |
| 4·0 | 0·78 | 0·22 | 0·172 | 0·635 | 0·205 | 0·245 |
| 100 | 0·99 | 0·011 | 0·011 | 0·32 | 0·29 | 0·29 |
| 160 | 0·99 | 0·007 | 0·007 | 0·30 | 0·28 | 0·28 |
| 760 | 1·00 | 0·0015 | 0·0015 | 0·33 | 0·33 | 0·33 |

The points corresponding to the two lowest oxygen pressures do not lie on the lines, which may be attributed to uncertainty in the precise value for $p_{O_2}$; a small uncertainty in this region leads to large discrepancies in the functions plotted. It again appears that the experimental data as a whole do not permit a choice between the two possible equations for $k_{obs.}$.

Table 3. *Analysis of Brooks's (1935) results for haemoglobin autoxidation in 0·6 M-phosphate buffer at 30°*

(The observed first order constants are divided by the oxygen pressure function $\alpha(1-\alpha)$ based on equilibrium measurements on the haemoglobin-oxygen reaction under the same experimental conditions.)

| $p_{O_2}$ | $k_{obs.}$ (hr.$^{-1}$ × 10²) | $\alpha$ | $1-\alpha$ | $k_{obs.}/\alpha(1-\alpha)$ |
|---|---|---|---|---|
| 4·5 | 9·9 | 0·89 | 0·11 | 1·01 |
| 6·0 | 12·6 | 0·85 | 0·15 | 0·98 |
| 13·7 | 15·9 | 0·84 | 0·36 | 0·69 |
| 16·0 | 17·6 | 0·57 | 0·43 | 0·72 |
| 25·8 | 17·7 | 0·39 | 0·61 | 0·74 |
| 33·3 | 14·5 | 0·29 | 0·71 | 0·71 |
| 63·1 | 10·3 | 0·12 | 0·88 | 0·97 |
| 92·9 | 8·45 | 0·08 | 0·92 | 1·16 |
| 122 | 7·40 | 0·06 | 0·94 | 1·30 |
| 152 | 6·36 | 0·048 | 0·95 | 1·39 |
| 435 | 5·17 | 0·035 | 0·965 | 1·52 |
| 723 | 4·61 | 0·030 | 0·97 | 1·59 |

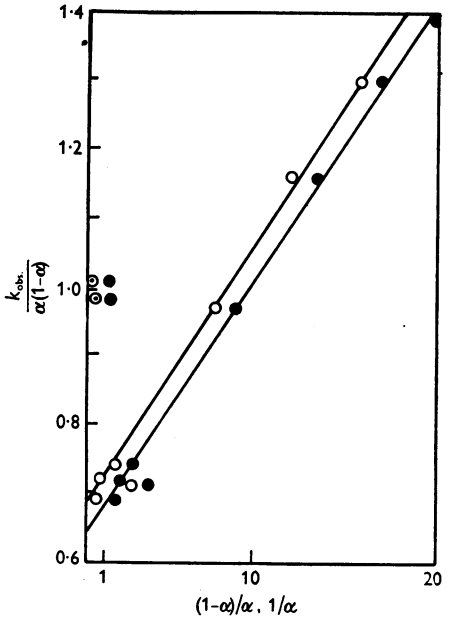


Fig. 8. Plot of $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against $(1-\alpha)/\alpha$ (⊙) and $1/\alpha$ (●) in the case of haemoglobin. (From Brooks's (1935) data.)

The empirical equation suggested by Brooks (1931)

$$k_{obs.} = k'\alpha \frac{bp}{1+bp}$$

proved very successful in accounting for the haemoglobin data, but it does not fit the myoglobin data so well as eqns. 4*a* and 4*b* at high oxygen pressures, for Brooks's equation like the single term $k_1\alpha(1-\alpha)$ in eqn. 1 diminishes continuously as $p_{O_2}$ increases. The chief distinction between the haemoglobin and myoglobin systems is that the same range of oxygen pressures, 0·3–760 mm. covers a far wider range in the fraction of unoxygenated haemoprotein in the case of myoglobin than in the case of haemoglobin. This is well brought out by comparison of Tables 2 and 3.

It is probable that other oxygen-pressure functions could be found to account equally well for $k_{obs.}$, particularly if more than one single function is used, as in eqn. 3 above. The merit of the present analysis is that it gives a close fit with the data using only oxygen-pressure functions directly connected with known possible reactants, i.e. $\alpha$ and $(1-\alpha)$ represent the fractions of unoxidized haemoprotein present in the reduced state and in combination with oxygen respectively.

## DISCUSSION

The kinetic data on myoglobin and haemoglobin autoxidation reveal the same anomaly at low oxygen pressures. Eqns. 4*a* and 4*b*, 5*a* and 5*b* show that the observed rate constant shows a complex second order variation with oxygen pressure according to the product of the concentrations of Mb and $MbO_2$, Hb and $HbO_2$, whilst it is obtained from a first order reaction; for if $Fe_p^{2+}$ represents the total ferrous protoporphyrin concentration and $\alpha$ and $(1-\alpha)$ the fractions of it free and combined with oxygen respectively, then the observed oxygen pressure relationship would be expected to derive from the kinetic equation

$$-\frac{dFe_p^{2+}}{dt} = k(1-\alpha)\ Fe_p^{2+}\ \alpha Fe_p^{2+},$$

i.e. second order in $Fe_p^{2+}$.

A possible explanation for this anomaly is that the autoxidation is a complicated sequence of reactions, such that the total concentration of unoxidized haemoprotein appears in the denominator of a rate equation of the form

$$-\frac{dFe_p^{2+}}{dt} = \frac{k_a(1-\alpha)\ Fe_p^{2+}\ k_b\alpha Fe_p^{2+}}{k_c Fe_p^{2+}} = \frac{k_a k_b}{k_c}(1-\alpha)\ (\alpha)\ Fe_p^{2+}, \qquad (6)$$

where $k_a$, $k_b$ and $k_c$ are the rate constants for certain steps in the mechanism. The numerator of the equation suggests that in step $a$ $MbO_2$ or $HbO_2$ is involved, giving the term $k_a(1-\alpha)\,Fe_p^{2+}$ and in step $b$ the free Mb or Hb, giving the term $k_b\alpha Fe_p^{2+}$.

This gives an indication as to what may be the operative rate equation at high oxygen pressures, whether it corresponds to 4*a* and 5*a* or 4*b* and 5*b*. As the oxygen pressure is increased Mb and Hb will decrease in concentration and $MbO_2$ and $HbO_2$ increase. Thus if both the free and oxygenated forms can undergo the same chemical reaction signified in the rate constant $k_b$, then the high oxygen pressure relationship would be given by

$$-\frac{dFe_p^{2+}}{dt}=\frac{k_a(1-\alpha)\,Fe_p^{2+}\,k_b'(1-\alpha)\,Fe_p^{2+}}{k_cFe_p^{2+}}, \qquad (7)$$

and the ratio of $k_b$ to $k_b'$ would be given by 2·62/0·30 for myoglobin in eqn. 4*a* and by 0·68/0·04 in the case of haemoglobin in eqn. 5*a*. The fact that, with myoglobin, the activation energy at 760 mm. $p_{O_2}$ is greater than that at 4 mm., as shown in Fig. 5, supports this interpretation, for when $MbO_2$ reacts additional energy required to break the iron-oxygen bond would be expected to appear as an increase in the activation energy. This is one simple way of accounting for the transition between the kinetics at low and high oxygen pressures. Attempts to account for the order of the reaction and the transition of the kinetics in terms of an equation of the form 4*b* and 5*b* have so far failed, and for this reason interpretation of the kinetics in terms of the rate eqns. 6 and 7 can profitably be explored.

Equations of this type are reminiscent of those derived from free radical mechanisms in which two valency states of a metal ion compete for the same free radical. The mechanism suggested by Weiss (1935) for the autoxidation of ferrous ion, in which the radicals $O_2^-$, $HO_2^{\cdot}$, $H_2O_2$ and the OH radical are intermediates, is:

$$Fe^{2+}+O_2 \xrightarrow{1} Fe^{3+}+O_2^-$$
$$O_2^-+H^+ \rightleftharpoons HO_2^{\cdot}$$
$$Fe^{2+}+HO_2 \xrightarrow{2} Fe^{3+}+HO_2^-$$
$$Fe^{3+}+O_2^- \xrightarrow{3} Fe^{2+}+O_2$$
$$HO_2^-+H^+ \rightleftharpoons H_2O_2$$
$$2Fe^{2+}+H_2O_2 \longrightarrow 2Fe^{3+}+2OH^-.$$

From the appropriate stationary state equations it can be shown that the oxidation rate should be

$$-\frac{dFe^{2+}}{dt}=4k_1Fe^{2+}\,O_2\,\frac{k_2Fe^{2+}}{k_2Fe^{2+}+k_3Fe^{3+}}.$$

The numerator in this equation is similar to those in eqns. 6 and 7, but the denominator does not correspond, in fact here the predicted rate is not first order with respect to the ferrous ion concentration. However, a free radical mechanism which can account for the myoglobin and haemoglobin kinetics can be derived in the following way.

Barb, Baxendale, George & Hargrave (1949, 1951), in an extensive study of the ionic iron-hydrogen peroxide system, showed that cupric ions are able to catalyse the reaction of $Fe^{3+}$ with $O_2^-$ through the two steps

$$Cu^{2+}+O_2^- \xrightarrow[\text{determining}]{\text{rate-}} Cu^+ + O_2,$$
$$Cu^+ + Fe^{3+} \xrightarrow[\text{rapid}]{\text{very}} Cu^{2+}+Fe^{2+}.$$

Now if it is assumed that there is an auxiliary electron-accepting group in the unoxidized forms of myoglobin and haemoglobin that can act as the cupric ion does, this enables the following reaction scheme to be formulated:

$$0\text{-}Fe_p^{2+}\,O_2 \underset{3}{\overset{1}{\rightleftharpoons}} 0\text{-}Fe_p^{3+}+O_2^-$$
$$0\text{-}Fe_p^{2+}+HO_2 \xrightarrow{2} 0\text{-}Fe_p^{3+}+O_2H^-$$
$$\begin{Bmatrix}0\text{-}Fe_p^{2+}\\0\text{-}Fe_p^{2+}\,O_2\end{Bmatrix}+O_2^- \xrightarrow{4} \begin{Bmatrix}ø\text{-}Fe_p^{2+}\\ø\text{-}Fe_p^{2+}\,O_2\end{Bmatrix}+O_2$$
$$\begin{Bmatrix}ø\text{-}Fe_p^{2+}\\ø\text{-}Fe_p^{2+}\,O_2\end{Bmatrix}+0\text{-}Fe_p^{3+} \xrightarrow[\text{rapid}]{\text{very}} \begin{Bmatrix}0\text{-}Fe_p^{2+}\\0\text{-}Fe_p^{2+}\,O_2\end{Bmatrix}+0\text{-}Fe_p^{2+}$$

followed by the reaction of the $H_2O_2$ with two more myoglobin or haemoglobin molecules. In this scheme $0\text{-}Fe_p^{2+}$, $0\text{-}Fe_p^{2+}\,O_2$ and $0\text{-}Fe_p^{3+}$ represent, for instance, Mb, $MbO_2$ and MetMb with the electron-accepting group in its oxidized state, $ø\text{-}Fe_p^{2+}$ and $ø\text{-}Fe_p^{2+}\,O_2$, when the group in Mb and $MbO_2$ has accepted an electron. The solution of the stationary state equations gives

$$-\frac{dFe^{2+}}{dt}=\frac{4k_1(1-\alpha)\,Fe_p^{2+}k_2\alpha Fe_p^{2+}}{k_4Fe_p^{2+}+k_2\alpha Fe_p^{2+}+k_3Fe_p^{3+}}.$$

If step 4, which is the back reaction regenerating Mb, predominates, then this equation reduces to

$$-\frac{dFe_p^{2+}}{dt}=\frac{4k_1k_2}{k_4}(1-\alpha)\,(\alpha)\,Fe_p^{2+} \qquad (8)$$

which is identical in form with eqn. 6.

If $MbO_2$ can also react like Mb in reaction 2 but less rapidly, i.e.

$$0\text{-}Fe_p^{2+}\,O_2+HO_2^{\cdot} \xrightarrow{2'} 0\text{-}Fe_p^{2+}+O_2+O_2H^-,$$

and this reaction replaces reaction 2 at high oxygen pressures, then eqn. 8 becomes

$$-\frac{dFe_p^{2+}}{dt}=\frac{4k_1k_2'}{k_4}(1-\alpha)\,(1-\alpha)\,Fe_p^{2+}, \qquad (9)$$

which is identical with Eqn. 7.

Although this mechanism can account entirely for the kinetic behaviour obtained from spectrophotometric measurements of the metmyoglobin formation, it leaves unexplained the abnormal consumption of oxygen during the reaction. In a previous paper (George & Stratmann, 1952) it was shown that $2{\cdot}5 \pm 0{\cdot}3$ moles of oxygen are used for each mole of metmyoglobin formed. The stoicheiometry in the above mechanism requires the ratio to be 0·25 or 0·5 if the peroxide is utilized in a secondary oxidation. Without detailed knowledge of which groups on the protein molecule are involved no further progress can be made in establishing the precise mechanism. Even though the simple reaction scheme leading to eqns. 8 and 9 is thus inadequate in detail there is good reason to believe it to be correct in principle in so far as the kinetics derive from competition reactions, for such reactions account readily for the form of the rate equation.

## SUMMARY

1. The oxidation of myoglobin to metmyoglobin by molecular oxygen at 30° in 0·6M-phosphate buffer, pH 5·69, is shown to be first order in unoxidized myoglobin over a range of oxygen pressures from 0·3 to 760 mm.

2. The observed first order rate constant at first increases with increasing oxygen pressures, shows a well defined maximum value at 1–1·4 mm. partial pressure of oxygen and then decreases to a constant value above 30 mm.

3. The determination of the equilibrium constant for the myoglobin-oxygen reaction under the conditions of the oxidation experiments at 30° gave $K_e = 0{\cdot}88 \pm 0{\cdot}12$ mm.$^{-1}$. Hence the partial pressure for half saturation is 1–1·31 mm. and thus $k_{obs.}$ has its maximum value at half saturation.

4. Using this value of $K_e$ the variation of $k_{obs.}$ with oxygen pressure is shown to be of the form

$$k_{obs.} = 2{\cdot}62\alpha(1-\alpha) + 0{\cdot}30(1-\alpha)^2, \quad (a)$$

or

$$k_{obs.} = 2{\cdot}30\alpha(1-\alpha) + 0{\cdot}30(1-\alpha), \quad (b)$$

where $\alpha$ and $(1-\alpha)$ are the fractions of Mb and $MbO_2$ respectively.

5. The autoxidation of haemoglobin shows the same kinetic characteristics and Brooks's (1935) data can be represented by the equations:

$$k_{obs.} = 0{\cdot}68\alpha(1-\alpha) + 0{\cdot}04(1-\alpha)^2 \quad (a)$$

or

$$k_{obs.} = 0{\cdot}64\alpha(1-\alpha) + 0{\cdot}04(1-\alpha). \quad (b)$$

6. The activation energy at low oxygen pressures (4 mm.) is $19 \pm 1$ kg.cal. and at high oxygen pressures (760 mm.) is $25 \pm 1$ kg.cal. At these extremes the operative form of the equation for $k_{obs.}$ involves only the first and only the last term respectively.

7. Neither the present data for myoglobin nor Brooks's data for haemoglobin permit a choice between equations *a* and *b* but a consideration of the chemical mechanism including the different activation energies at the extremes of oxygen pressure favour equation *a*.

8. A free radical mechanism for the autoxidation is discussed which involves the participation of an auxiliary electron-accepting group on the protein molecule acting as a catalyst in a reaction regenerating the unoxidized haemoprotein and thus serving to 'protect' the haem from oxidation. However, this mechanism is not complete for it does not account for the additional consumption of oxygen above that required for oxidizing the haem group (George & Stratmann, 1952).

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## REFERENCES

Barb, W. G., Baxendale, J. H., George, P. & Hargrave, K. R. (1949). *Nature, Lond.*, **163**, 692.

Barb, W. G., Baxendale, J. H., George P. & Hargrave, K. R. (1951). *Trans. Faraday Soc.* **47**, 462, 591.

Brooks, J. (1931). *Proc. roy. Soc.* B, **109**, 35.

Brooks, J. (1935). *Proc. roy. Soc.* B, **118**, 560.

Cohn, E. J. (1927). *J. Amer. chem. Soc.* **49**, 173.

George, P. (1949). *1st Int. Congr. Biochem.* (Abstr.), p. 365.

George, P. & Stratmann, C. J. (1952). *Biochem. J.* **51**, 203.

Legge, J. W. (1942). *Proc. roy. Soc. N.S. Wales*, **76**, 47.

Weiss, J. (1935). *Naturwissenschaften*, **23**, 64.