Biochem. J. (1976) **155**, 503–510
Printed in Great Britain

The Rate of Reaction of Superoxide Radical Ion with Oxyhaemoglobin and Methaemoglobin

By HARRY C. SUTTON,* PETER B. ROBERTS*
and CHRISTINE C. WINTERBOURN†
**Institute of Nuclear Sciences, D.S.I.R., Lower Hutt, New Zealand,*
and †Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

(*Received* 8 *October* 1975)

Superoxide radical ions ($O_2^{-\cdot}$) produced by the radiolytic reduction of oxygenated formate solutions and by the xanthine oxidase-catalysed oxidation of xanthine were shown to oxidize the haem groups in oxyhaemoglobin and reduce those in methaemoglobin as in reactions (1) and (2):

$$2H^+ + O_2^{-\cdot} + \text{haem–}Fe^{2+}\text{–}O_2 \rightarrow \text{haem–}Fe^{3+} + H_2O_2 + O_2 \quad (1)$$

$$O_2^{-\cdot} + \text{haem–}Fe^{3+} \rightarrow \text{haem–}Fe^{2+}\text{–}O_2 \quad (2)$$

$$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

Reaction (1) is suppressed by reaction (8) when $[O_2^{-\cdot}]$ exceeds 10μM, but consumes all the $O_2^{-\cdot}$ generated in oxyhaemoglobin solutions when [oxyhaemoglobin] $\geq$ 160μM and $[O_2^{-\cdot}] \leq$ 1nM at pH7. The yield of reaction (2) is also maximal in methaemoglobin solutions under similar conditions, but less than one haem group is reduced per $O_2^{-\cdot}$ radical. From studies of (*a*) the yield of reactions (1) and (2) at variable [haemoglobin] and rates of production of $O_2^{-\cdot}$, (*b*) their suppression by superoxide dismutase, and (*c*) equilibria observed with mixtures of oxyhaemoglobin and methaemoglobin, it is shown that $k_1/k_2 = 0.7 \pm 0.2$ and $k_1 = (4 \pm 1) \times 10^3 M^{-1} \cdot s^{-1}$ at pH7, and k_1 and k_2 decrease with increasing pH. Concentrations and rate constants are expressed in terms of haem-group concentrations. Concentrations of superoxide dismutase observed in normal erythrocytes are sufficient to suppress reactions (1) and (2), and hence prevent the formation of excessive methaemoglobin.

The superoxide radical ion ($O_2^{-\cdot}$) is known to be produced in aerobic cells generally (Fridovich, 1972; Bors *et al.*, 1974) and, in particular, by the spontaneous oxidation of oxyHb (oxyhaemoglobin) in the erythrocyte (Misra & Fridovich, 1972; Wever *et al.*, 1973). Carrell *et al.* (1975) have considered its possible implication in haemolytic diseases associated with increased production of metHb (methaemoglobin) and Winterbourn *et al.* (1976) have shown from qualitative studies with enzymic sources of $O_2^{-\cdot}$ that it can both oxidize the haem groups in oxyHb:

$$O_2^{-\cdot} + 2H^+ + \text{haem–}Fe^{2+}\text{–}O_2 \rightarrow \text{haem–}Fe^{3+} + H_2O_2 + O_2 \quad (1)$$

and also reduce those in metHb:

$$O_2^{-\cdot} + \text{haem–}Fe^{3+} \rightarrow \text{haem–}Fe^{2+}\text{–}O_2 \quad (2)$$

The following studies were performed to determine the rate constants of these reactions and to assess their biological significance. For this purpose we used γ- and electron-beam irradiation of oxygenated solutions of sodium formate to produce known yields of $O_2^{-\cdot}$. In such solutions the radicals $e_{aq.}^{-\cdot}$, H· and OH· produced from water are rapidly converted into $O_2^{-\cdot}$ by the reactions (3)–(7) (Behar *et al.*, 1970):

$$e_{aq.}^{-\cdot} + O_2 \rightarrow O_2^{-\cdot} \quad (3)$$

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot} \quad (4)$$

$$HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{-\cdot}, \; pK = 4.9 \quad (5)$$

$$OH^{\cdot} + HCO_2^- \rightarrow H_2O + CO_2^{-\cdot} \quad (6)$$

$$CO_2^{-\cdot} + O_2 \rightarrow CO_2 + O_2^{-\cdot} \quad (7)$$

H_2O_2 is also produced in a yield that is initially 12% of $[O_2^{-\cdot}]$, and this is supplemented by that resulting from the subsequent dismutation reaction (8):

$$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

This reaction actually occurs predominantly between $O_2^{-\cdot}$ and its protonated form $HO_2^{\cdot}$, with which it is in pH-controlled equilibrium ($pK = 4.9$). For simplicity it is written as shown where k_8 is defined by $-d[O_2^{-\cdot} + HO_2^{\cdot}]/dt = 2k_8[O_2^{-\cdot} + HO_2^{\cdot}]$. Behar *et al.* (1970) have measured k_8 as a function of pH.

The irradiation technique is well established as a source of $O_2^{-\cdot}$ and has been used to measure the rate

constant of the dismutation reaction (8) (Behar *et al.*, 1970) and of its catalysis by the enzyme superoxide dismutase (Rotilio *et al.*, 1972; Klug *et al.*, 1972):

$$2H^+ + 2O_2^{-\cdot} \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2 \qquad (9)$$

This catalysis obeys the kinetics:

$$-d[O_2^{-\cdot}]/dt = k_9[O_2^{-\cdot}][\text{superoxide dismutase}]$$

when $[O_2^{-\cdot}]$ exceeds [superoxide dismutase] and was used in this work to determine the rate of reaction (1) by competition studies.

The generation of $O_2^{-\cdot}$ by the oxidation of xanthine catalysed by xanthine oxidase (McCord & Fridovich, 1969) provided a complementary approach.

Materials and Methods

Oxyhaemoglobin was separated from normal erythrocyte haemolysates by column chromatography on DEAE-Sephadex (Huisman & Dozy, 1965) and was freed from accompanying salts by dialysis before use. Conversion into metHb was effected by the addition of a small excess of potassium ferricyanide, subsequently removed by passage through Sephadex G-25. A trace of the ferrocyanide so produced may accompany the metHb but this reacts very slowly with $O_2^{-\cdot}$ [rate constant $\leqslant k_2$ (Zehavi & Rabani, 1972)], and an equimolar concentration of ferrocyanide did not influence our results. Sodium and potassium phosphate buffers (BDH Chemicals Ltd., Poole, Dorset, U.K.), EDTA (BDH Chemicals Ltd.) and sodium formate (Merck, Darmstadt, Germany) were of A.R. grade and water was triply distilled. Superoxide dimutase from bovine erythrocytes, milk xanthine oxidase and ox liver catalase were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. The absolute activity of a stock superoxide dismutase solution, i.e., the quantity k_9 [superoxide dismutase], was determined at the Institute of Cancer Research, Sutton, Surrey, U.K. by using the pulse-radiolysis method. Concentrations of superoxide dismutase are based on this determination by adopting a value of $k_9 = 2.37 \times 10^9 M^{-1} \cdot s^{-1}$ (Fielden *et al.*, 1974) and a mol.wt. of 32200 (Steinman *et al.*, 1974). Concentrations of oxyHb and metHb were estimated from light-absorption measurements at 576 and 630nm in both cases, and are expressed as concentrations of the corresponding haem groups by using the absorption coefficients of Benesch *et al.* (1973). Changes in concentration observed at the two wavelengths agreed within experimental error and were averaged. Preliminary measurements of the whole visible spectrum confirmed the validity of the method.

Solutions for irradiation with ^{60}Co γ-rays or 1.8MeV electrons contained 10mM-sodium formate buffered at pH7 with a solution of Na_2HPO_4 and KH_2PO_4 of 2mM total concentration unless otherwise stated. Before irradiation these were mixed with oxyHb or metHb to give concentrations normally in the range 10–160μM-haem and equilibrated with O_2 at atmospheric pressure; 0.1mM-EDTA was added in some instances but was without effect. Catalase in concentrations of 4–10μg/ml (approx. 30nM) was also added to the haemoglobin solutions to remove H_2O_2. These solutions retained their catalase activity after irradiation and gave results that were independent of catalase concentrations in the range 0.2–5 times those commonly used. Nevertheless, catalase does not completely suppress a very slow oxidation of oxyHb that was observed in some cases after irradiation, and which was increased by adding H_2O_2, particularly with mixtures of oxyHb and metHb. This may cause our initial yields of reduction of metHb to be underestimated by 5–10% in some instances owing to slow reoxidation of the oxyHb so produced. This slow effect of H_2O_2 became quite significant in prolonged irradiations lasting several days at $0.1 rad \cdot s^{-1}$. Radiation dose rates and hence the rate of $O_2^{-\cdot}$ production were determined by the ferrous sulphate dosimeter (Schuler & Allen, 1956). Yields of $O_2^{-\cdot}$ in the formate system were taken to be 6.0 radicals per 100eV absorbed, as verified previously (Behar *et al.*, 1970; Sutton & Downes, 1972).

Reactions of oxyHb and metHb with $O_2^{-\cdot}$ generated from the xanthine oxidase-catalysed oxidation of xanthine were carried out at 25°C in O_2-saturated 66mM-phosphate buffer at either pH7.0 or 7.9, containing 1.7mM-xanthine, 2mM-EDTA, 35μg of catalase/ml and 0.0125 unit of xanthine oxidase/ml. One unit converts 1μmol of xanthine into uric acid/min at pH7.5 and 25°C.

Results

It should be noted that oxyHb and metHb concentrations and reaction-rate constants are expressed in terms of the concentrations of the corresponding haem groups.

Yields of reactions (1) *and* (2)

In the first experiments, superoxide radicals in 15μM concentration were produced by electron-beam irradiation of aerated sodium formate solutions and mixed 5ms after irradiation with 40μM solutions of oxyHb or metHb at pH7, by using techniques described elsewhere (Sutton & Downes, 1972). Less than 1% oxidation or reduction was observed. We conclude that reactions (1) and (2) are completely suppressed at these high $O_2^{-\cdot}$ concentrations by the dismutation reaction (8), which is second order in $[O_2^{-\cdot}]$ with $k = 0.8 \times 10^6 M^{-1} \cdot s^{-1}$ at pH7 (Behar *et al.*, 1970). Hence k_1 and k_2 must be less than $1 \times 10^4 M^{-1} \cdot s^{-1}$.

We therefore turned to the more complex method described in the experimental procedure in which

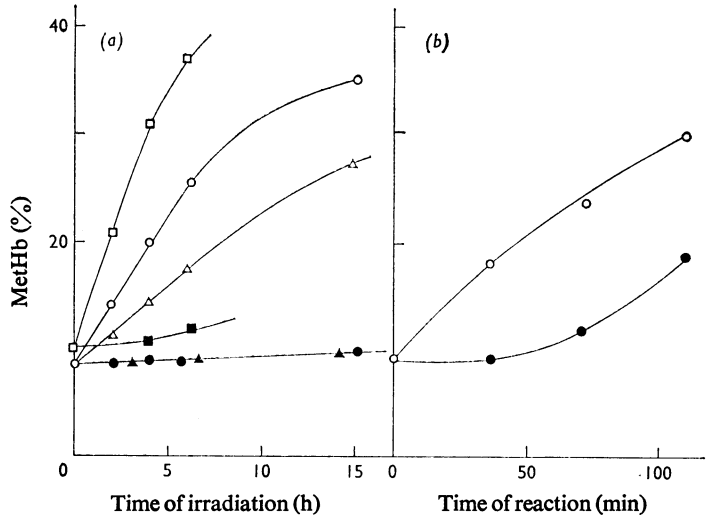

Fig. 1. *Oxidation of oxyHb by $O_2^{-\cdot}$ at pH7*

Conversion of oxyHb into metHb was calculated from the averaged changes in absorbance at 576 and 630nm. Solutions of oxyHb contained 8–10% metHb initially. $O_2^{-\cdot}$ was generated in Fig. 1(*a*) by the radiolytic procedure and in Fig. 1(*b*) by the enzyme-catalysed oxidation of xanthine as outlined in the text in the presence of: ■, □, 10μM-haem groups; ●, ○, 40μM-haem groups; ▲, △, 160μM-haem groups. Closed symbols refer to $O_2^{-\cdot}$ generation in the presence of 0.32μg of superoxide dismutase/ml (10nM). Radiolytic generation of $O_2^{-\cdot}$ was at a dose rate of 0.12rad·s^{-1}, equivalent to an $O_2^{-\cdot}$ production rate of 7.6×10^{-10}M·s^{-1}.

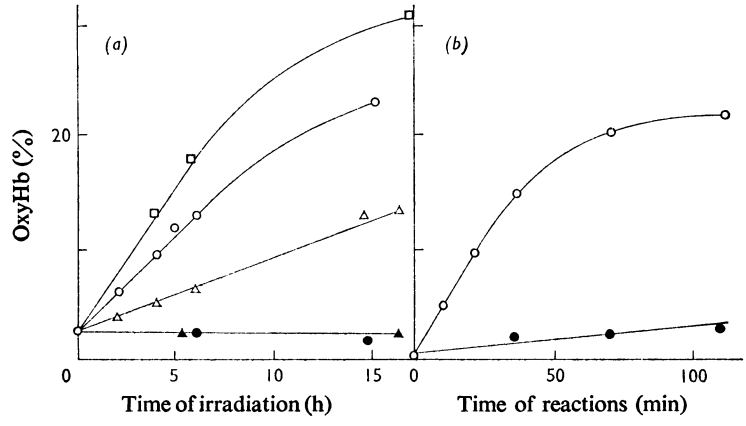

Fig. 2. *Reduction of metHb by $O_2^{-\cdot}$ at pH7*

The data for the conversion of metHb into oxyHb was obtained under conditions identical with those of Figs. 1(*a*) and 1(*b*). The symbols used identify similar concentrations and conditions.

pre-mixed solutions of protein and formate were irradiated to obtain low steady-state concentrations of $O_2^{-\cdot}$ determined by the dose rate. Such concentrations correspond more closely to those expected *in vivo*. Figs. 1(*a*) and 2(*a*) illustrate the result obtained at the unusually low dose rate of 0.12rad·s^{-1} which produces $O_2^{-\cdot}$ in a yield equivalent to 7.6×10^{-10}M·s^{-1}. The possibility that these effects are caused by reaction with haemoglobin of the radical precursors of $O_2^{-\cdot}$, namely $e^-_{aq.}$, H· and OH·,

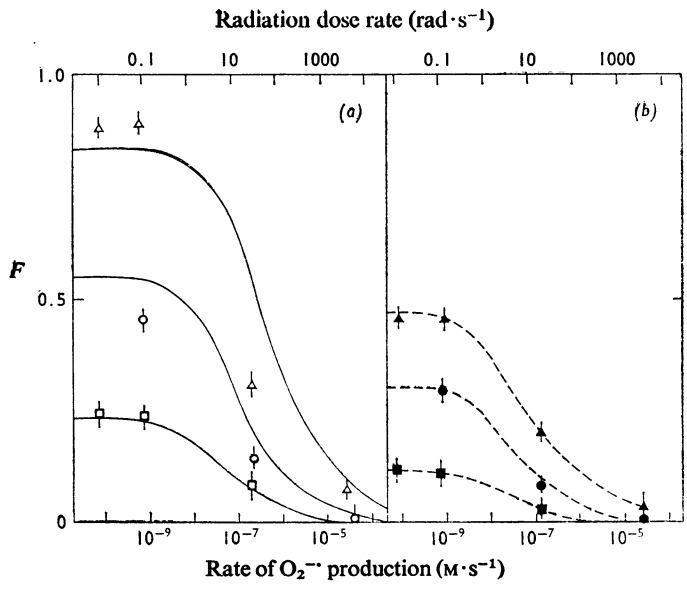

Fig. 3. *Effect of haem concentration and rate of generation of $O_2^{-\cdot}$ on haem oxidation or reduction yields at pH7*

The fractional yield (*F*) denotes the yield of haem groups oxidized or reduced by $O_2^{-\cdot}$ expressed as a fraction of the corresponding yield of $O_2^{-\cdot}$ radical ions produced by irradiation with 1.8 MeV electrons at 5000 rad·s^{-1}, or by ^{60}Co γ-rays at all other dose rates. Irradiated solutions contained O_2-saturated 10 mM-sodium formate to generate $O_2^{-\cdot}$ radiolytically in a yield of 6.2 μM/krad (equivalent to 6.0 radicals per 100 eV) and also oxyHb in (*a*) at concentrations of: □, 10 μM; ○, 40 μM; △, 160 μM; (*b*) gives corresponding data for metHb with closed symbols. Solid lines in (*a*) are calculated from eqn. (12) in the text; interrupted lines in (*b*) express the experimental data only.

rather than by $O_2^{-\cdot}$ produced in reactions (3)–(7), is discounted by two considerations. First, with each experiment a control mixture was irradiated containing 10 nM-superoxide dismutase to catalyse the removal of $O_2^{-\cdot}$ in reaction (9) and suppress the reaction with haemoglobin. More than 98% of the observed effect was suppressed in this way, as shown by Figs. 1(*a*) and 2(*a*), and the remaining small percentage was subtracted in estimating effects owing to $O_2^{-\cdot}$. Corresponding data for the enzymic source of $O_2^{-\cdot}$ are shown in Figs. 1(*b*) and 2(*b*) in which suppression by superoxide dismutase is nearly complete for metHb and for small conversions of oxyHb. Secondly, from the relative rates of reactions of the radical precursors with O_2 (1.3 mM) and formate (10 mM) on the one hand, and protein on the other, it is estimated from rate constants available in the literature (Anbar & Neta, 1967) that less than 4% of $e^-_{aq.}$, 0.2% of H· and 5% of OH· react directly with 160 μM-oxyHb or -metHb, which is the highest haem concentration normally used, and correspondingly less at lower concentrations.

The initial slopes of the curves in Figs. 1(*a*) and 2(*a*) measure the yields of reactions (1) and (2) and are conveniently expressed as a fraction (*F*) of the known yield of $O_2^{-\cdot}$ (i.e. *F* is the number of haem groups oxidized or reduced/number of $O_2^{-\cdot}$ radicals produced).

F values for the oxidation of oxyHb varied with dose rate and [oxyHb], as shown by Fig. 3(*a*) and tend to approach unity at high [oxyHb] and low dose rate, implying that reaction (1) becomes quantitative for each haem group in oxyHb under these conditions. With increasing dose rate, and hence, $O_2^{-\cdot}$ concentration, reaction (8) consumes more $O_2^{-\cdot}$ and *F* decreases to practically zero at 5000 rad·s^{-1}. This dose rate was obtained by continuous irradiation with 1.8 MeV electrons and produced steady-state concentrations of $O_2^{-\cdot}$ which are estimated to be 4.2 μM. Measurements of small *F* values at dose rates exceeding 10 rad·s^{-1} are subject to considerable error because of the relatively greater influence of radical precursors of $O_2^{-\cdot}$, i.e. of effects that are not suppressed by superoxide dismutase. These latter effects are proportional to dose, but independent of dose rate; they caused nearly all the effects observed with necessarily large doses at 5000 rad·s^{-1}.

The corresponding yields for metHb reduction are shown in Fig. 3(*b*) and display the same variation with dose rate and haem concentration but with smaller *F* values, which tend to an upper limit of approx. 0.5 at 160 μM-metHb. Less than 5% increase

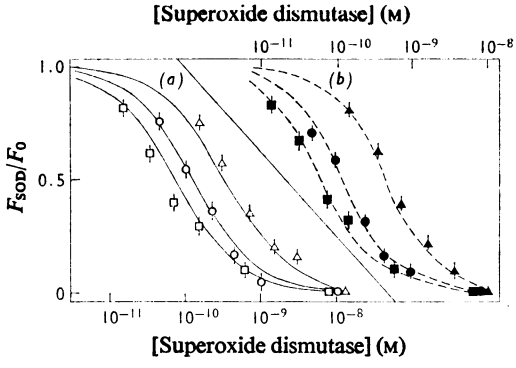

Fig. 4. *Inhibition by superoxide dismutase of $O_2^{-\cdot}$-induced oxidation or reduction of haem groups at pH7*

The effect of increasing superoxide dismutase on haem conversion yields is expressed as F_{SOD}/F_0, where % inhibition $= 100 \times (1-F_{SOD}/F_0)$ and F_{SOD} and F_0 are fractional yields in the presence and absence of superoxide dismutase as defined in the text. $O_2^{-\cdot}$ was generated by radiolysis as in Fig. 3 at $0.12 rad \cdot s^{-1}$. (*a*) Oxidation of oxyHb at haem concentrations of: □, 10μM; ○, 40μM; △, 160μM, and the full curves have been calculated from eqn. (13) in the text. (*b*) Data for corresponding concentrations of metHb with closed symbols and with the interrupted lines expressing only the experimental data. The bottom axis refers to (*a*) and the top axis to (*b*).

of F resulted from doubling this concentration. F values for metHb reduction were not as reproducible from one sample to another as those for oxidation of oxyHb and those shown in Fig. 3(*b*) relate to one preparation. Nevertheless, with due allowance for the 5–10% errors owing to H_2O_2 mentioned above, and for the variation of F with [metHb]/[$O_2^{-\cdot}$] we are confident that the maximum value of F does not exceed 0.6 under optimum conditions, which is significantly less than for oxidation of oxyHb.

It is not possible to obtain F values for enzymic sources of $O_2^{-\cdot}$ because the yields of $O_2^{-\cdot}$ obtained by this method cannot be determined reliably. An attempt was made by substituting the more efficient $O_2^{-\cdot}$ scavenger cytochrome *c* for haemoglobin and observing the rate of its reduction in the standard xanthine–xanthine oxidase system. On this basis, we obtained F values that were approximately one-tenth of those obtained by the radiolytic method at comparable rates of formation of $O_2^{-\cdot}$ ($1 \times 10^{-7} M \cdot s^{-1}$). The yield of $O_2^{-\cdot}$ produced radiolytically in the formate system has been well established (Behar *et al.*, 1970, and references quoted therein; Sutton & Downes, 1972) and is confirmed in these studies by the observation that F approaches unity under appropriate conditions for oxyHb solutions. Thus it appears that the yield of $O_2^{-\cdot}$ that is available in the enzymic system varies with the substrate and is higher for cytochrome *c* than for oxyHb or metHb. This might occur because the smaller cytochrome *c* molecule has readier access to the site on the enzyme at which $O_2^{-\cdot}$ is produced and might therefore react with $O_2^{-\cdot}$ which would otherwise have reacted to form H_2O_2 before escaping.

Kinetic studies

The inhibition by superoxide dismutase of the oxidation of oxyHb by $O_2^{-\cdot}$ was studied quantitatively at $0.12 rad \cdot s^{-1}$, to compare the rates of reactions (1) and (9). Fig. 4 shows the effect of increasing [superoxide dismutase] on F values at three haem concentrations. The results are expressed as relative yields, F_{SOD}/F_0, where % inhibition $= 100 \times (1-F_{SOD}/F_0)$. Corresponding data for the reduction of metHb are also shown.

Prolonged treatment of either oxyHb or metHb by $O_2^{-\cdot}$ should lead to an equilibrium in which [metHb]/[oxyHb] $= k_1/k_2$. Such studies are impractical with both radiolytic and enzymic sources of $O_2^{-\cdot}$ owing to interfering processes, so an alternative approach was adopted in which brief treatments, capable of complete suppression by superoxide dismutase, were given to mixtures of oxyHb and metHb of different initial composition. The results shown in Fig. 5 indicate that these brief treatments are without observable effect at pH7 on mixtures containing 60±5% metHb for radiolytically produced $O_2^{-\cdot}$, and 42±5% for enzymically produced $O_2^{-\cdot}$. However, the irradiated solutions contained 10mM-sodium formate, which complexes with the haem groups in metHb, as shown by a small effect on the absorption peaks at 576 and 630nm, but does not complex with oxyHb. The maximum effect observed in more concentrated formate solutions corresponded to an 18% increased absorption at 576nm with a slight shift of the absorption peak to lower wavelengths, and to a 24% increased absorption at 630nm with a shift in the absorption peak to 621nm. In 10mM-formate solutions, the effects observed corresponded to approx. 20% of these maxima. The formate–metHb complex evidently reacts less rapidly with $O_2^{-\cdot}$ than does free metHb because the addition of 10mM-formate to the xanthine oxidase system shifted the equilibrium composition in oxyHb–metHb mixtures to 54±5% metHb, which agrees with the result with radiolytically produced $O_2^{-\cdot}$ under the same conditions. Formate addition also slightly decreased the rate of conversion of metHb observed as in Fig. 2(*b*), but was without effect on oxyHb conversion (Fig. 1*b*). We conclude that $k_1/k_2 = 0.7 \pm 0.2$ at pH7, whereas the corresponding ratio for metHb in the presence of 10mM-formate is about 1.5.

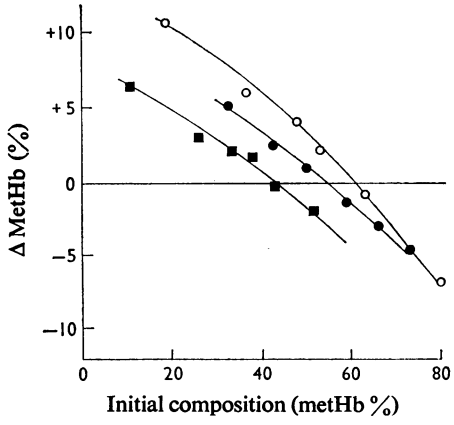

Fig. 5. *Change in percentage of metHb in mixtures of oxyHb and metHb exposed to $O_2^{-\cdot}$ at pH7*

The ordinate represents the change in composition of a mixture of oxyHb and metHb after exposure to $O_2^{-\cdot}$, expressed in terms of percentage of metHb. Initial and final [metHb] were obtained by using the absorbance data of Benesch *et al.* (1973) at 576 and 630nm and averaging the results at the two wavelengths. The total concentration of haem groups was 40μM. Generation of $O_2^{-\cdot}$ was either by radiolysis of oxygenated 10mM-sodium formate (○), or enzymically in the absence (■) and presence (●) of 10mM-sodium formate. Radiolytic generation was at a dose rate of 0.12rad·s^{-1} for 6h and exposure to enzymically produced $O_2^{-\cdot}$ was for 1h. Control mixtures demonstrated the stability of the mixtures and the complete suppressability of the changes by superoxide dismutase.

Effect of pH

Yields of oxidation of oxyHb and reduction of metHb by radiolytically produced $O_2^{-\cdot}$ were independent of pH in the range 6.3–7, but declined rapidly above pH7 to undetectable values at pH9, despite the constancy of the radiolytic yield of $O_2^{-\cdot}$ in the formate system throughout this pH range. Similar trends were observed with enzymically produced $O_2^{-\cdot}$ but to differing relative extents. These decreasing yields cannot be related to the known deceleration of reaction (8) with increasing pH (Behar *et al.*, 1970) as this would cause a reverse effect; they indicate that k_1 and k_2 have maximum values at pH7 and decrease with increasing pH presumably owing to changes in the conformation of the protein or the ligand co-ordination of the haem groups, thus allowing other reactions to consume $O_2^{-\cdot}$.

Equilibrium compositions obtained as in Fig. 5 correspond to 60 and 55% metHb for radiolytically produced $O_2^{-\cdot}$ at pH7 and 7.9 respectively, as compared with 42% at pH7 and 25% at pH7.9 for enzymically produced $O_2^{-\cdot}$; the enzymic source of $O_2^{-\cdot}$ in the presence of formate-complexed metHb gave 41% at pH7.9. Measurements near pH8 are difficult because of the small extent of the superoxide dismutase-suppressible reaction for brief treatment times.

Discussion

The major features of our observations on oxyHb solutions may be expressed in terms of the oxidizing reaction (1), the spontaneous dismutation of $O_2^{-\cdot}$ in reaction (8), its catalysis in reaction (9) and the additional reaction:

$$O_2^{-\cdot} \rightarrow \text{first-order decay} \qquad (10)$$

Reaction (10) is included to express the spontaneous first-order decay of $O_2^{-\cdot}$ that occurs in competition with reaction (8) at micromolar concentrations of $O_2^{-\cdot}$ in formate solutions at pH7 (Behar *et al.*, 1970). This reaction would be expected to predominate when $[O_2^{-\cdot}] \leqslant 1$nM, a concentration that corresponds with dose rates of 0.1rad·s^{-1} in our case, and it accounts for the decrease in F with decreasing [oxyHb] which is observed in the region where F is independent of dose rate (see Fig. 3*a*).

We may then write for the stationary state where $O_2^{-\cdot}$ decays as fast as it is produced:

$$2k_8[O_2^{-\cdot}]^2 + k_1[O_2^{-\cdot}][\text{oxyHb}] + k_9[O_2^{-\cdot}][\text{superoxide dismutase}] + k_{10}[O_2^{-\cdot}] = GR \qquad (11)$$

where G is the yield of $O_2^{-\cdot}$ produced radiolytically (6 radicals per 100eV) and R is the dose rate in appropriate units. Hence, for experiments in the absence of superoxide dismutase:

$$F_{\text{calc.}} = k_1[\text{oxyHb}] / (2k_8[O_2^{-\cdot}] + k_1[\text{oxyHb}] + k_{10}) \qquad (12)$$

and for the superoxide dismutase (SOD) competition experiments at 0.12rad·s^{-1} where $2k_8[O_2^{-\cdot}] < k_{10}$:

$$\left(\frac{F_{\text{SOD}}}{F_0}\right)_{\text{calc.}} = \frac{1 + (k_{10}/k_1[\text{oxyHb}])}{1 + (k_{10}/k_1[\text{oxyHb}]) + (k_9[\text{superoxide dismutase}]/k_1\text{oxyHb}])} \qquad (13)$$

Taking the known values of k_8 and k_9 as quoted above, the two adjustable parameters k_1 and k_{10} can be calculated and have been assigned the values $k_1 = 4\times10^3$M^{-1}·s^{-1} and $k_{10} = 0.13$s^{-1}. k_1 is largely determined by the superoxide dismutase competition data at 160μM-oxyHb where $k_{10} < k_1[\text{oxyHb}]$. Calculated values of F and F_{SOD}/F_0 obtained in this way are shown as the full curves in Figs. 3(*a*) and 4(*a*).

Agreement with the experimental points is satisfactory in Fig. 4(*a*) with regard to both the shapes of the competition curves and their displacement with [oxyHb]. The general trend of the data in Fig. 3(*a*) also agrees with that calculated, though there is some discrepancy in the intermediate dose-rate region at high [oxyHb] which is only partially accounted for by the uncertainties in measuring F in this region. It follows from eqn. (12) that $F_{calc.}$ tends to unity at sufficiently high [oxyHb] and low [$O_2^{-\cdot}$], but this region is not accessible to experimental study.

It is difficult to understand the nearly twofold effect on k_2 of 10mM-formate which the equilibria data imply, since only 20% of the metHb appears to be complexed at this concentration. Even if the formate complex is totally unreactive to $O_2^{-\cdot}$ one would expect only a 20% decrease in the overall rate observed for the equilibrium mixture of free and complexed metHb. Therefore we do not attempt a detailed interpretation of the metHb–formate system but it should be noted that the equilibria data in Fig. 5 are consistent with the superoxide dismutase suppression data in Fig. 4(*b*) with regard to the magnitude of k_1/k_2 for formate-containing metHb solution and that the interpretation of Fig. 3(*b*) is clearly analogous with that of Fig. 3(*a*) for oxyHb.

Although the rate of reaction of $O_2^{-\cdot}$ with metHb is clearly slower in the presence of formate, the eventual yield of reduction should not be altered. $O_2^{-\cdot}$ does not react with formate; no evidence was found for oxidation of thiol groups in haemoglobin (Winterbourn *et al.*, 1976) and peptide 'mapping' and specific staining failed to detect any changes caused by $O_2^{-\cdot}$. Further, one would expect that any effect in the globin moiety which could operate for metHb could also occur with oxyHb. Thus there is no evidence for $O_2^{-\cdot}$ reacting with metHb in formate-containing solutions to give products other than oxyHb. The small limiting value of F at pH7 for metHb, which implies that approximately 2 $O_2^{-\cdot}$ radicals are consumed during the reduction of each haem group, is therefore unexpected, and may warrant further study.

The superoxide radical and its protonated form, $HO_2^{\cdot}$, commonly exhibit both oxidizing and reducing properties analogous to those occurring in reactions (1) and (2). Chemical (Rao & Hayon, 1975; Wood, 1974) and electrochemical (P. L. Airey & H. C. Sutton, unpublished work) studies show that these tend to occur with equal rate at pH7 in systems with reduction potentials in the range of +0.1 to 0.3V. The oxyHb–metHb reduction potential, E, has not been measured, but may be estimated from the relation $E = E_m + (0.059/n)\log([\text{oxyHb}]/[\text{deoxyHb}])$, where E_m is the reduction potential observed for the deoxyHb–metHb system, namely, +0.15V at pH7 (Antonini *et al.*, 1964), [oxyHb]/[deoxyHb] is the ratio of the concentrations of oxyHb and deoxyHb that co-exist at equilibrium in 1mM-oxygen at pH7 (approx. 1×10^3), and n is an experimentally observed parameter that has the value 1.6 for the deoxyHb–metHb system at pH7 (Antonini *et al.*, 1964). On this basis, E is approx. +0.27 at pH7, which lies within the range quoted above. The oxidation–reduction equilibria that we observe ($k_1/k_2 = 0.7$ at pH7) are therefore consistent with the known chemistry of $O_2^{-\cdot}$. Although our value of k_2, obtained from measurements of k_1/k_2 and of k_1, is only $6\times10^3\,M^{-1}\cdot s^{-1}$, which is far below diffusion-controlled rate constants of typically $1\times10^{10}\,M^{-1}\cdot s^{-1}$, and less than that for $O_2^{-\cdot}$ reduction of cytochrome *c* [$1.1\times10^5\,M^{-1}\cdot s^{-1}$ at pH8.5 (Land & Swallow, 1971)], it is nevertheless consistent with rate constants observed for reactions of $O_2^{-\cdot}$ with other iron salts. For example, the reduction of ferricyanide by $O_2^{-\cdot}$ occurs with $k = 270\,M^{-1}\cdot s^{-1}$ (Zehavi & Rabani, 1972), and the rate with positively charged iron in metHb would be expected to exceed this.

Carrell *et al.* (1975) have pointed out the stress that is imposed on the erythrocyte by spontaneous formation of methaemoglobin and the need for its subsequent reduction to oxyhaemoglobin. It follows from these studies that this stress would be augmented in the absence of superoxide dismutase by concomitant formation of $O_2^{-\cdot}$, because its predominant reaction in the erythrocyte would cause further oxidation of oxyhaemoglobin rather than reduction of relatively small concentrations of methaemoglobin. However, $O_2^{-\cdot}$ is decomposed by superoxide dismutase at the enzyme concentrations commonly found in erythrocytes of about 0.5mg/g of haemoglobin or approx. 5μM (Stansell & Deutsch, 1966; Winterbourn *et al.*, 1975) some 100 times faster than it reacts with oxyhaemoglobin. This conclusion follows from comparison of the product of rate constant and concentration of reactant (kC) for reaction of $O_2^{-\cdot}$ with superoxide dismutase ($kC = 2.3\times10^9\times5\times10^{-6} = 1.2\times10^{-4}\,s^{-1}$) with the corresponding quantity for the 30% concentration of oxyhaemoglobin (i.e., about 18mM-haem groups) occurring in erythrocytes, for which $kC = 4\times10^3\times1.8\times10^{-2} = 72\,s^{-1}$. Insofar as homogeneous kinetics can be applied to the contents of the erythrocyte, the controlling or suppressing role of superoxide dismutase is thus demonstrated, but the ubiquitous presence of high concentrations of this enzyme in practically all aerobic cells (McCord *et al.*, 1971) implies that $O_2^{-\cdot}$ has other damaging biochemical properties which require suppression. If these also occur in the erythrocyte then they must either have kC products of the order $1\times10^2\,s^{-1}$ in order to compete with superoxide dismutase and haemoglobin for $O_2^{-\cdot}$, which is unlikely in view of the limited bond-breaking ability of $O_2^{-\cdot}$, or else occur at selective sites that are peculiarly accessible to $O_2^{-\cdot}$ at the point of its generation.

We are indebted to Dr. E. M. Fielden and Dr. M. McGrath (Division of Physics, Institute of Cancer Re-

search, Sutton, U.K.) for their determination by pulse radiolysis of the active [superoxide dismutase] in our sample. Valuable discussions were held with Dr. R. W. Carrell. Miss L. Windsor and Mrs. M. Brian provided excellent technical assistance. This work was supported in part by a grant from the Medical Research Council of New Zealand.

References

Anbar, M. & Neta, P. (1967) *J. Appl. Radiat. Isotopes* **18**, 493–523

Antonini, E., Wyman, J., Brunori, M., Taylor, J. F., Rossi-Fanelli, A. & Caputo, A. (1964) *J. Biol. Chem.* **239**, 907–912

Behar, D., Czapski, G., Rabani, J., Dorfman, L. M. & Schwarz, H. A. (1970) *J. Phys. Chem.* **74**, 3209–3213

Benesch, R. E., Benesch, R. & Yung, S. (1973) *Anal. Biochem.* **55**, 245–248

Bors, W., Saran, M., Lengfelder, E., Spottl, R. & Michel, C. (1974) *Curr. Top. Radiat. Res. Q.* **9**, 247–309

Carrell, R. W., Winterbourn, C. C. & Rachmilewitz, E. A. (1975) *Br. J. Haematol.* **30**, 259–264

Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G. & Calabrese, L. (1974) *Biochem. J.* **139**, 49–60

Fridovich, I. (1972) *Acc. Chem. Res.* **6**, 321–326

Huisman, T. H. J. & Dozy, A. M. (1965) *J. Chromatogr.* **19**, 160–169

Klug, D., Rabani, J. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 4839–4842

Land, E. J. & Swallow, A. J. (1971) *Arch. Biochem. Biophys.* **145**, 365–375

McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055

McCord, J. M., Keele, B. B., Jr., & Fridovich, I. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1024–1027

Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 6960–6962

Rao, P. S. & Hayon, E. (1975) *J. Phys. Chem.* **79**, 397–402

Rotilio, G., Bray, R. C. & Fielden, E. M. (1972) *Biochim. Biophys. Acta* **268**, 605–609

Schuler, R. H. & Allen, A. O. (1956) *J. Chem. Phys.* **24**, 56–59

Stansell, M. J. & Deutsch, H. F. (1966) *Clin. Chim. Acta* **14**, 598–607

Steinman, H. M., Naik, V. R., Abernethy, J. L. & Hill, R. L. (1974) *J. Biol. Chem.* **249**, 7326–7338

Sutton, H. C. & Downes, M. T. (1972) *J. Chem. Soc. Faraday Trans. I* **68**, 1498–1507

Wever, R., Oudega, B. & Van Gelder, B. F. (1973) *Biochim. Biophys. Acta* **302**, 475–478

Winterbourn, C. C., Hawkins, R. E., Brian, M. & Carrell, R. W. (1975) *J. Lab. Clin. Med.* **85**, 337–341

Winterbourn, C. C., McGrath, B. & Carrell, R. W. (1976) *Biochem. J.* **155**, 493–502

Wood, P. M. (1974) *FEBS Lett.* **44**, 22–24

Zehavi, D. & Rabani, J. (1972) *J. Phys. Chem.* **76**, 3703–3709