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Reactions Involving Superoxide and Normal and Unstable Haemoglobins

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Superoxide ions ($O_2^{-\cdot}$) oxidized oxyhaemoglobin to methaemoglobin and reduced methaemoglobin to oxyhaemoglobin. The reactions of superoxide and H_2O_2 with oxyhaemoglobin or methaemoglobin and their inhibition by superoxide dismutase or catalase were used to detect the formation of superoxide or H_2O_2 on autoxidation of oxyhaemoglobin. The rate of autoxidation was decreased to about 35% in the presence of both enzymes. The copper-catalysed autoxidation of Hb (haemoglobin) was also shown to involve superoxide production. Superoxide was released on autoxidation of three unstable haemoglobins and isolated α and β chains, at rates faster than with Hb A. Reactions of superoxide with Hb Christchurch and Hb Belfast were identical with those with Hb A, and occurred at the same rate. Hb Köln contrasted with the other haemoglobins in that the thiol groups of residue β-93 as well as the haem groups reacted with superoxide. Haemichrome formation from methaemoglobin occurred very rapidly with Hb Christchurch and Hb Belfast, as well as the isolated chains, compared with Hb A. The process did not involve superoxide production or utilization. The relative importance of autoxidation and superoxide production compared with haemichrome formation in the haemolytic process associated with these abnormal haemoglobins and thalassaemia is considered.

The superoxide radical ($O_2^{-\cdot}$) is now known to be a product of an increasingly large number of biological reactions (Fridovich, 1972; Bors *et al.*, 1974). However, the presence of $O_2^{-\cdot}$ *in vivo* is presumably undesirable, since cells that metabolize O_2 require the enzyme superoxide dismutase to break it down. The precise reactions that are damaging to the cell, or whether they involve $O_2^{-\cdot}$ itself or products such as H_2O_2 or hydroxyl radicals, which can be derived from $O_2^{-\cdot}$, are not clear (Bors *et al.*, 1974). One reaction that has been shown to produce $O_2^{-\cdot}$ is the autoxidation of oxyHb† to metHb (Misra & Fridovich, 1972; Wever *et al.*, 1973). Since some 3% of the haemoglobin in an erythrocyte is oxidized per day, and reduced again by methaemoglobin reductase, this constitutes a cycle producing a continuous supply of $O_2^{-\cdot}$. It follows that any situation that increases the turnover of this cycle gives increased production of $O_2^{-\cdot}$ (Carrell *et al.*, 1975). In a normal cell, $O_2^{-\cdot}$ will be broken down by superoxide dismutase, and H_2O_2, which is a product of this reaction, by glutathione peroxidase and catalase. Any $O_2^{-\cdot}$ that bypasses this mechanism should react with other cell constituents, possibly causing irreversible cell damage. In the erythrocyte, which is unable to replace damaged constituents by resynthesis, the escape of even a very small amount of $O_2^{-\cdot}$ could have deleterious effects, and may in fact contribute to the aging and finite lifespan of the cell. However, this mechanism is likely to become more significant if $O_2^{-\cdot}$ is produced in abnormally high amounts, or if any of the protective mechanisms are defective (Carrell *et al.*, 1975).

The broad aim of the present study was to examine the reactions of $O_2^{-\cdot}$ in the erythrocyte, in particular: (1) to confirm that $O_2^{-\cdot}$ is produced during autoxidation of human haemoglobin under near physiological conditions; (2) to demonstrate increased rates of $O_2^{-\cdot}$ production by unstable haemoglobins, free chains and in the presence of catalytic amounts of Cu^{2+} ions; (3) to examine the reactions of $O_2^{-\cdot}$ with haemoglobin, and determine whether they are involved in the denaturation and precipitation processes that with unstable haemoglobins lead to accelerated erythrocyte breakdown.

Formation of $O_2^{-\cdot}$ during haemoglobin autoxidation has been detected by its reaction with adrenaline (Misra & Fridovich, 1972; Brunori *et al.*, 1975) or cytochrome *c* (Wever *et al.*, 1973). In the present study we have used the reactions of $O_2^{-\cdot}$ with haemo-

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† Abbreviations: oxyHb, oxyhaemoglobin; metHb, methaemoglobin; Hb, haemoglobin.

globin itself, namely the oxidation of oxyHb and the reduction of metHb, and their inhibition by superoxide dismutase to demonstrate the reaction. This eliminates complications owing to interaction between haemoglobin and the detecting reagent, which occur with both the previous systems. The results can be considered in terms of the following equations:

$$\text{OxyHb} \longrightarrow \text{metHb} + O_2^{-\cdot} \quad (1)$$

$$\text{OxyHb} + O_2^{-\cdot} + 2H^+ \longrightarrow \text{metHb} + H_2O_2 \quad (2)$$

$$\text{MetHb} + O_2^{-\cdot} \longrightarrow \text{oxyHb} \quad (3)$$

$$2O_2^{-\cdot} + 2H^+ \xrightarrow[\text{dismutase}]{\text{Superoxide}} O_2 + H_2O_2 \quad (4)$$

First, for pure oxyHb, if reaction (1) occurs, the $O_2^{-\cdot}$ formed should produce more metHb by reaction (2). However, in the presence of superoxide dismutase, reaction (4) should prevent reaction (2) and there should be a slower rate of metHb formation. It also follows that since both the dismutation of $O_2^{-\cdot}$ and its reaction with oxyHb produce H_2O_2, which also reacts with oxyHb to give metHb (Lemberg, 1956), addition of catalase should slow the reaction still further. Alternatively, when excess of metHb is present during autoxidation, reaction (3) will be favoured over reaction (2), and any $O_2^{-\cdot}$ produced will be mostly used to re-form oxyHb. When superoxide dismutase is present, reaction (4) will prevent this reverse reaction, and there will be a faster accumulation of metHb.

Materials and Methods

Oxyhaemoglobin was purified from normal erythrocyte haemolysates by column chromatography on DEAE-Sephadex (Huisman & Dozy, 1965). Separations were performed in the cold, and 0.1 mM-EDTA was added to the buffers to decrease autoxidation. No superoxide dismutase or catalase could be detected in the purified haemoglobin. Superoxide dismutase was assayed by the method of Winterbourn *et al.* (1975). Hb Belfast (β-15 Trp → Arg) and Hb Köln (β-98 Val → Met) were also purified by using this system. Hb Christchurch (β-71 Phe → Ser) cannot be separated from Hb A and a purified mixture containing 20% Hb Christchurch was prepared. Free α and β chains were isolated by the method of Geraci *et al.* (1969). Methaemoglobin was prepared from purified oxyhaemoglobin by the addition of a slight excess of potassium ferricyanide, which was subsequently removed by passage through a column of Sephadex G-25.

Superoxide for reaction with haemoglobin was generated either by the reaction of photoreduced riboflavin with O_2 or by the xanthine oxidase-catalysed oxidation of xanthine (Beauchamp & Fridovich, 1971). The riboflavin system was basically as described by Winterbourn *et al.* (1975). Reaction mixtures contained 6.7 mM-EDTA, usually 2 μM-riboflavin, 13 μg of catalase/ml and the desired concentration of oxyHb or metHb in air-saturated phosphate buffer (pH either 7.0 or 7.9 as described for each experiment). The phosphate buffer used in all experiments was prepared from 0.067 M-KH_2PO_4 and -Na_2HPO_4. The xanthine oxidase reaction was also carried out in air-saturated phosphate buffer, containing 1 mM-EDTA, 13 μg of catalase/ml, 1.6 mM-xanthine and 0.003–0.016 unit of xanthine oxidase/ml, at 30°C. Control experiments were carried out in the presence of 2.7 μg of superoxide dismutase/ml. Catalase, superoxide dismutase and xanthine oxidase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The amount of oxidation or reduction of haemoglobin was measured spectrally on a Beckman model 25 spectrophotometer. In some experiments thiol groups were measured by reaction with dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). The reaction of $O_2^{-\cdot}$ with the thiol groups of Hb Köln was also followed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Winterbourn & Carrell (1974).

The rates of autoxidation of the normal and abnormal haemoglobins and the involvement of $O_2^{-\cdot}$ and H_2O_2 in the process were studied by incubating oxyHb solutions at 37°C, in phosphate buffer, pH 7.4, containing 0.1 M-NaCl and 2 mM-EDTA, unless stated otherwise, and recording spectra of the solutions at intervals. Depending on the conditions and rates of autoxidation, spectral changes were followed for 8–47 h. The concentrations of oxyHb, metHb and haemichrome were calculated from the absorbances at 560, 577 and 630 nm and the millimolar extinction coefficients of each of the haemoglobin species at these wavelengths (given in Table 1). Allowance was made for any turbidity caused by precipitation. When the autoxidation of oxyHb was followed in the presence of excess of metHb, spectra were recorded against a metHb blank. With Hb

Table 1. *Millimolar extinction coefficients used for calculating concentrations of oxyhaemoglobin, methaemoglobin and haemichromes*

Values for oxyHb and metHb were taken from Benesch *et al.* (1973). Values were determined for haemichrome prepared by the addition of metHb of known concentration to 8 M-urea.

		MetHb			
λ(nm)	OxyHb	pH 7.0	pH 7.4	pH 7.9	Haemichrome
560	36.5	16.2	18.3	21.7	36.5
577	66	16.2	18.3	23.7	28.6
630	1.0	16.0	15.2	12.8	3.9

Christchurch, both the oxyHb and metHb contained 20% of the abnormal protein. Haemichrome production from metHb was studied at 30°C, and absorbances at 560 and 630nm were used for calculating concentrations.

Results

Reactions of superoxide with haemoglobin

Fig. 1 shows the spectra of solutions of oxyHb and metHb before and after reaction with $O_2^{-\cdot}$ generated from reduced riboflavin at pH7.0. MetHb was progressively reduced to oxyHb (Fig. 1*a*) and oxyHb was oxidized to metHb, although at a slower rate (Fig. 1*b*). Both reactions were largely inhibited by superoxide dismutase. Similar results were obtained at pH7.9, except that both reactions, particularly that of oxyHb, were slower. Essentially the same results were obtained with $O_2^{-\cdot}$ generated from xanthine and xanthine oxidase.

Other potentially vulnerable sites in the haemoglobin molecule for reaction with $O_2^{-\cdot}$ are the thiol groups, particularly those exposed at position β-93. Measurements of these thiol groups in oxyHb before and after 80min reaction with $O_2^{-\cdot}$ generated by riboflavin system at pH7.9 detected no significant oxidation. By comparison, GSH at the same concentration and under the same conditions was 34% oxidized. Over the same period, 5–10% of the haem groups of the haemoglobin were oxidized. Haemoglobin denatured in 0.1% sodium dodecyl sulphate, which also exposes the four buried thiol groups per tetramer, was also examined. Under the same conditions as used for native haemoglobin, 65% of the total thiol groups were oxidized, and oxidation was about three times more rapid than in a solution of GSH with the same thiol-group concentration.

Autoxidation of oxyhaemoglobin A

The effects of catalase and superoxide dismutase on the rate of autoxidation of purified Hb A in the presence of EDTA were examined. As shown in Fig. 2, removal of H_2O_2 by catalase slowed down the reaction to about 60% and the breakdown of $O_2^{-\cdot}$ by superoxide dismutase slowed it down still further, to about 35% of the original rate. Superoxide dismutase alone had only a slight effect. At this pH it was slightly protective, but at pH6.4 it had no initial effect on the rate. As the reaction proceeded and more oxyHb was converted into metHb the relative rate of autoxidation in the presence of superoxide dismutase alone became slightly faster.

In the absence of EDTA, the rate of autoxidation was substantially faster and quite variable. It was inhibited by both superoxide dismutase and catalase. We found a very marked increase in autoxidation rate in the presence of copper. With 28μM-oxyHb, addition of 0.9μM-$CuSO_4$ (i.e. one Cu^{2+} ion per 120 haem groups) increased the rate 4.5 times.

Fe^{2+} or Fe^{3+} ions, added alone or as complexes with ADP (Fe/ADP = 1:20), also stimulated autoxidation, although with less than 1% the efficiency of copper. Equimolar Fe^{2+} or Fe^{3+} ions and haem groups were required to obtain a 40–70% increase in rate,

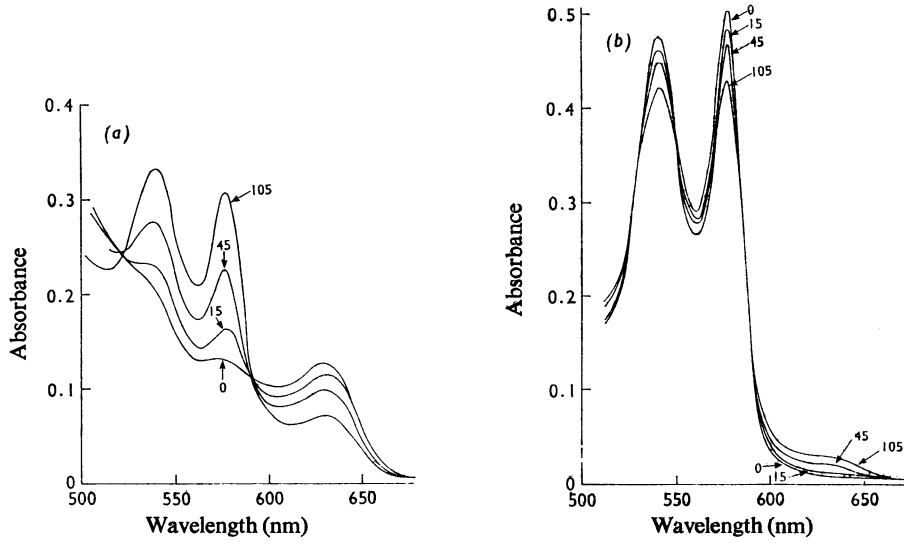

Fig. 1. *Spectra of methaemoglobin (a) and oxyhaemoglobin (b) after exposure to* $O_2^{-\cdot}$

$O_2^{-\cdot}$ was generated by illuminating, with an 18W fluorescent tube, 2μM-riboflavin in O_2-saturated phosphate buffer, pH7.0, containing 6.7mM-EDTA and 13μg of catalase/ml, for 0, 15, 45 and 105min.

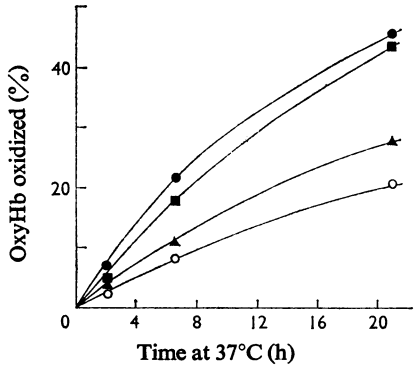

Fig. 2. *Effects of catalase and superoxide dismutase on the rate of autoxidation of oxyhaemoglobin*

Purified oxyHb (8 μM) was incubated at 37°C in 0.067 M-phosphate buffer, pH 7.4, containing 0.11 M-NaCl and 5 mM-EDTA. Catalase (13 μg/ml) and superoxide dismutase (2.7 μg/ml) were added as indicated. ●, Control; ■, +superoxide dismutase; ▲, +catalase; ○, +both enzymes.

with Fe^{2+} being slightly more effective. Co^{2+}, Pb^{2+} and Cd^{2+} ions were also examined and found to have no effect. The copper-stimulated autoxidation involved the production of $O_2^{-\cdot}$ and also H_2O_2. Autoxidation could be inhibited by adding either catalase (40% inhibition) or superoxide dismutase (35% inhibition) and a combination of the two inhibited the reaction by 60–70%. It is probable that the iron effect also involved formation of $O_2^{-\cdot}$ and H_2O_2, as superoxide dismutase and catalase almost eliminated the enhanced autoxidation rate.

The involvement of $O_2^{-\cdot}$ in haemoglobin autoxidation can also be demonstrated by using as a detection system its ability to reduce metHb. This occurs 2–3 times as readily as the oxidation reaction (Sutton *et al.*, 1976). Fig. 3 shows the effect of superoxide dismutase on the rate of oxidation of oxyHb in the presence of catalase and a twofold excess of metHb. The faster rate of metHb accumulation observed when superoxide dismutase was present indicates that the enzyme was preventing the reduction of metHb by $O_2^{-\cdot}$ which must have been produced during the autoxidation.

Autoxidation of unstable haemoglobins

The autoxidation of three unstable haemoglobins, Hb Christchurch, Hb Belfast and Hb Köln, was investigated. Hb Belfast was examined after separation from Hb A and other erythrocyte proteins. Hb A and Hb Christchurch cannot be separated, and a purified mixture containing 20% Hb Christchurch was used. Hb Köln lacking one or two β-chain haem groups can be separated from Hb A, and the preparations we examined contained 2.1–2.5 haem groups per tetramer. Any haem groups in excess of two were always present in the ferric form. It seems probable that these were the residual β-chain haem groups, and that we studied autoxidation of the α chains. We also examined the major haemoglobin fraction from cells containing Hb Köln, which, although being predominantly Hb A, would contain any Hb Köln with both β-chain haem groups present.

Each of the three unstable haemoglobins produced $O_2^{-\cdot}$ and H_2O_2 during autoxidation, as shown by the inhibitory effects of catalase and superoxide dismutase. Catalase alone decreased the rate of autoxidation of Hb Christchurch to about 60%, and catalase plus superoxide dismutase decreased it to about 35–40% (Fig. 4). Superoxide dismutase alone caused slight inhibition. The same pattern of inhibition was observed with Hb Belfast. The behaviour of haem-depleted Hb Köln was basically similar, although the amount of inhibition by catalase and superoxide dismutase was more variable, and sometimes less than with the other haemoglobins. Production of $O_2^{-\cdot}$ during the autoxidation of Hb Christchurch was also demonstrated by carrying out the reaction in the presence of a threefold excess of metHb plus catalase. As with Hb A, superoxide dismutase prevented the reduction of metHb by $O_2^{-\cdot}$, and increased the rate of autoxidation.

Rates of autoxidation of the unstable haemoglobins

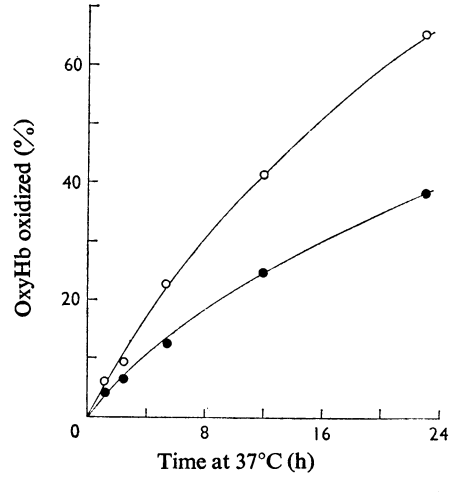

Fig. 3. *Effect of superoxide dismutase on the autoxidation of oxyhaemoglobin in the presence of excess of methaemoglobin*

Solutions containing 6.5 μM-oxyHb, 17 μM-metHb, 0.2 M-NaCl, 5 mM-EDTA and 5 μg of catalase/ml in 0.067 M-phosphate buffer, pH 7.0, were incubated with (○) and without (●) 4.1 μg of superoxide dismutase/ml.

relative to Hb A are shown in Table 2. Hb Christchurch autoxidized most readily, with the presence of 20% of the abnormal haemoglobin causing a 54% increase in overall rate. This suggests that pure Hb Christchurch would autoxidize approximately 3.4 times as rapidly as Hb A. Pure Hb Belfast, however, autoxidized only about 1.4–1.6 times as rapidly as did Hb A. The rates of oxidation of both Hb Christchurch and Hb Belfast relative to Hb A were similar in the presence or absence of catalase and superoxide dismutase. The absence of the enzymes therefore in effect amplified the differences.

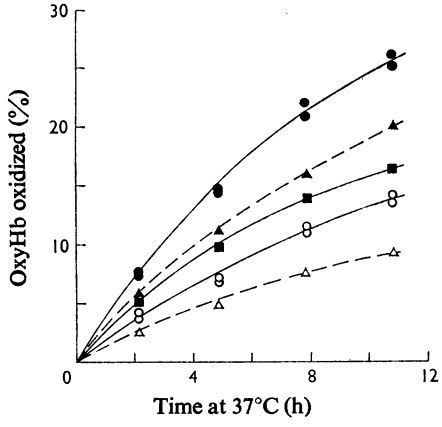

Fig. 4. *Rate of autoxidation of Hb Christchurch*

A purified mixture of Hb Christchurch (20%) and Hb A was incubated in 0.067M-phosphate buffer, pH7.4, containing 0.09M-NaCl and 2mM-EDTA. The total haemoglobin concentration was 8μM. With no enzymes (●), with 13μg of catalase/ml (■), with 13μg of catalase and 2.7μg of superoxide dismutase/ml (○). Points on each curve are from two experiments. Rates of autoxidation of Hb A under identical conditions are shown for comparison. Hb A with no enzymes (▲) and with catalase and superoxide dismutase (△).

As shown in Table 2, autoxidation of the α chains in haem-depleted Hb Köln proceeded only slightly faster than in Hb A, in either the presence or the absence of catalase and superoxide dismutase. Rates were more variable than with the other haemoglobins, especially in the absence of catalase and superoxide dismutase, and in one case, Hb Köln autoxidized slightly more slowly than did Hb A. The main haemoglobin fraction from Hb Köln cells, which consisted of Hb A and 6% Hb Köln with all haem groups present, autoxidized slightly faster than did Hb A, suggesting a substantially increased rate for the abnormal β chains.

As is described in the following section, the thiol groups of residue β-93 of Hb Köln, unlike those of Hb A and other haemoglobins examined, are susceptible to attack by $O_2^{-\cdot}$, and they react more readily with H_2O_2 (C. C. Winterbourn & R. W. Carrell, unpublished work). Some evidence for thiol oxidation by the $O_2^{-\cdot}$ and H_2O_2 produced during autoxidation of Hb Köln was also obtained. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate performed on purified Hb Köln showed that autoxidation of approx. 40% of the haem groups was accompanied by a slight increase in β-chain dimers, and there was a corresponding small decrease in measurable thiol groups (10–15%). These changes were not seen in the presence of catalase and superoxide dismutase, or with Hb A.

In the absence of EDTA, oxidation of Hb Belfast was considerably increased, and the addition of Cu^{2+}

Table 2. *Relative rates of autoxidation of Hb A and the unstable haemoglobins*

Each haemoglobin solution was incubated at 37°C in pH7.4 phosphate buffer (see the text) containing 5mM-EDTA and 0.1M-NaCl. Rates were determined from logarithmic plots of percentage of oxyHb remaining versus time, which were linear.

Haemoglobin	Relative rate of oxidation of oxyHb: No enzymes added	Relative rate of oxidation of oxyHb: With superoxide dismutase+catalase
Hb A	1.00	1.00
20% Hb Christchurch, 80% Hb A*	1.54	1.42
Hb Belfast	1.60	1.42
Haem-depleted Hb Köln†	0.9–1.25	1.25–1.5
Major Hb fraction from Köln cells (approx 6% Köln)	1.2	1.3–1.4
α-chains	1.8	2.0
α-chains+*p*-hydroxymercuribenzoate	10	
β chains	2.6	2.8
β-chains+*p*-hydroxymercuribenzoate	2.4	

* Logarithmic plots were biphasic, representing initial oxidation of the abnormal haemoglobin and Hb A, followed by Hb A. Initial rates are quoted.

† Range obtained for four preparations from three donors.

Table 3. *Effect of EDTA and Cu^{2+} on the autoxidation of Hb A and Hb Belfast*

Results were calculated from a logarithmic plot of percentage of oxyHb remaining versus time. Solutions were incubated at 37°C and pH 7.4.

	OxyHb oxidized (%/h)		
	5mM-EDTA	No EDTA	2μM-$CuSO_4$
Hb A (7μM)	1.7	6.3	15
Hb Belfast (7μM)	2.6	7.1	15

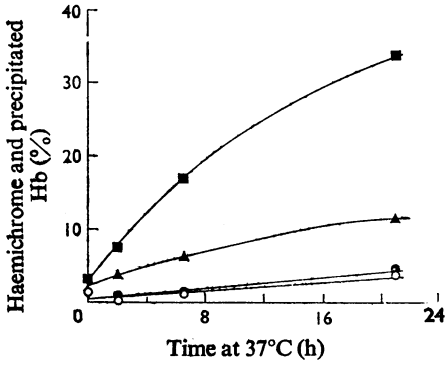

Fig. 5. *Accumulation of soluble haemichromes and precipitated haemoglobin during incubation of oxyHb A and oxyHb Belfast*

Solutions of each haemoglobin (8μM) were incubated with and without catalase (13μg/ml) and superoxide dismutase (2.7μg/ml) in 0.067M-phosphate buffer, pH 7.4, containing 0.11M-NaCl and 5mM-EDTA. OxyHb Belfast with (▲) and without (■) enzymes; oxyHb A with (●) and without (○) enzymes.

ions increased the rate still further (Table 3). However, the effect of Cu^{2+} on Hb Belfast was almost identical with that on Hb A, and both autoxidized at almost the same rate. In the absence of EDTA, catalase and superoxide dismutase inhibited autoxidation of both Hb A and Hb Belfast by about 64%.

As well as differences in rate of autoxidation, the unstable haemoglobins were converted from metHb into haemichromes much more readily than was Hb A. Over a period of 24h at 37°C, when 40% of oxyHb A was converted into metHb, there was no spectral evidence for soluble haemichrome formation and no precipitation. However, spectra of Hb Christchurch and Hb Belfast showed a build-up of soluble haemichromes and there was slow precipitation (Fig. 5). Haemichrome formation from haem-depleted Hb Köln, although detectable, was much less evident and 24h at 37°C produced no precipitation. Haemichrome formation and precipitation of Hb Belfast and Hb Christchurch were much less in the presence of superoxide dismutase and catalase (Fig. 5). This can be attributed entirely to the lower rate of metHb formation in the presence of the enzymes, and does not imply involvement of $O_2^{-\cdot}$ or H_2O_2 in haemichrome formation. For both unstable haemoglobins, plots of the amount of haemichrome produced relative to the amount of metHb produced was the same in the presence and the absence of superoxide dismutase and catalase. If $O_2^{-\cdot}$ or H_2O_2 was involved, the enzymes would be expected to decrease the ratio of haemichrome/methaemoglobin formed.

The rate of haemichrome formation from metHb Belfast was unaffected by superoxide dismutase or catalase, but it was markedly suppressed by the addition of EDTA. Addition of Cu^{2+} ions increased the rate. This mechanism was also unaffected by the addition of superoxide dismutase or catalase. EDTA similarly suppressed haemichrome formation from metHb Christchurch, and the addition of 2–10μM-Cu^{2+} ions progressively increased the rate.

Autoxidation of isolated α and β chains

Isolated α and β chains also autoxidized more rapidly than did Hb A (Table 2). The β chains autoxidized slightly more rapidly than the α chains. As with Hb A, catalase decreased the rates to between 50 and 60%, and superoxide dismutase as well decreased them further to 35–40%. Autoxidation of both chains was pH-dependent, occurring twice as rapidly at pH 5 as at pH 7.4.

Preparation of α and β chains involves treating the thiol groups with *p*-hydroxymercuribenzoate, and frequently these chains are studied without removing this. Whereas *p*-hydroxymercuribenzoate had little effect on the rate of autoxidation of the β chains, with the α chains it increased the rate about five times (Table 2).

Methaemoglobin was produced only transiently during autoxidation of isolated α and β chains. Spectra showed only small amounts of metHb, and a build up of soluble haemichrome, which slowly precipitated.

Reactions of unstable haemoglobins with superoxide

As with Hb A, $O_2^{-\cdot}$ generated from xanthine oxidase or reduced riboflavin oxidized oxyHb Belfast and oxyHb Christchurch to metHb, and reduced the metHb to oxyHb. No other reactions at the haem group could be detected. Rates of reaction of $O_2^{-\cdot}$ with both unstable oxyhaemoglobins and methaemoglobins were the same as with oxyHb A and metHb A.

Superoxide generated from xanthine oxidase caused no detectable oxidation of cysteine residue β-93 in Hb Belfast or Hb Christchurch, but with Hb

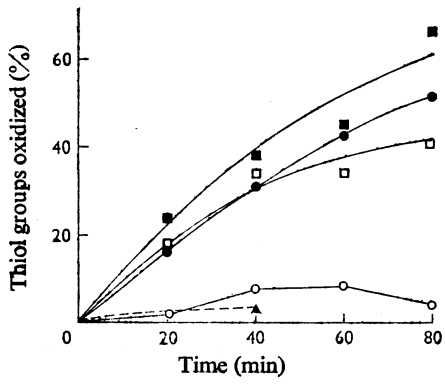

Fig. 6. *Reaction of $O_2^{-\cdot}$ with the available thiol groups of Hb Köln, Hb A and GSH*

Reaction mixtures containing xanthine (1.7mM), xanthine oxidase (0.012 unit/ml), EDTA (2mM), catalase (50μg/ml) and each thiol in 0.067M-phosphate buffer, pH7.8, were incubated at 30°C. Samples were removed at intervals, cooled on ice and treated with dithiobis-(2-nitrobenzoic acid). ●, GSH (15μM); ○, Hb A (11μM-thiol groups); ■, Hb Köln (10μM-thiol groups); □, Hb Köln (18μM-thiol groups); ▲, Hb Köln+superoxide dismutase (2.7μg/ml).

Köln, these residues were oxidized at a rate comparable with that of GSH (Fig. 6). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed on the products with Hb Köln. This showed that in addition to the normal band corresponding to single haemoglobin chains there was a band with double this molecular weight. This band was eliminated if the sample was treated with dithiothreitol, and it most likely consisted of β-chain dimers disulphide-bonded through cysteine-93.

Discussion

By using the reactions of $O_2^{-\cdot}$ with haemoglobin itself and their ability to be inhibited by superoxide dismutase we have obtained further evidence that the autoxidation of haemoglobin produces $O_2^{-\cdot}$. This method has the advantage of eliminating reactions of haemoglobin with the detection system, and any suspicion that this might somehow be contributing to the production of $O_2^{-\cdot}$. It also allows slower rates of autoxidation to be followed, and we have been able to show $O_2^{-\cdot}$ production under near-physiological conditions and pH. These experiments have stressed that the autoxidation of pure haemoglobin is considerably slowed by the addition of catalase and superoxide dismutase, and therefore that the $O_2^{-\cdot}$ and H_2O_2 produced during autoxidation do react with further oxyHb to give metHb. The results suggest the mechanism represented by eqns. (5)–(7), in which one initial autoxidation step results in the oxidation of four haem groups.

$$Fe^{(II)}O_2 \rightarrow Fe^{(III)} + O_2^{-\cdot} \quad (5)$$

$$Fe^{(II)}O_2 + O_2^{-\cdot} + 2H^+ \rightarrow Fe^{(III)} + O_2 + H_2O_2 \quad (6)$$

$$2Fe^{(II)}O_2 + H_2O_2 + 2H^+ \rightarrow 2Fe^{(III)} + 2O_2 + 2H_2O \quad (7)$$

$$4Fe^{(II)}O_2 + 4H^+ \rightarrow 4Fe^{(III)} + 3O_2 + 2H_2O \quad (8)$$

The stoicheiometry of eqn. (8) has been observed experimentally for myoglobin oxidation (Brown & Mebine, 1969). It should be noted, however, that dismutation of $O_2^{-\cdot}$ can compete with its reaction with oxyHb, and give the same stoicheiometry. With this mechanism, metHb formation should theoretically be decreased to 50% by catalase, and to 25% by catalase and superoxide dismutase. Our values of about 60 and 35% are a little higher, but in reasonable agreement. Any side reactions of $O_2^{-\cdot}$ or H_2O_2 (such as spontaneous dismutation or reactions with metHb) or direct oxidation of the haemoglobin by other than O_2 would give less than the theoretical inhibition.

Although superoxide dismutase had an additional inhibitory effect of about 25% in the presence of catalase, alone it had considerably less effect. We are not able to explain this unexpected finding, but note that Fee & Teitelbaum (1972) observed a similar effect of superoxide dismutase on the dialuric acid (2,4,5,6-tetrahydroxypyrimidine)-induced lysis of erythrocyte membranes.

As with normal haemoglobin, the unstable haemoglobins that we examined, and isolated α and β chains, all produced $O_2^{-\cdot}$ and H_2O_2 on autoxidation. Our results for the isolated chains are essentially similar to those of Brunori *et al.* (1975), who studied much faster rates of autoxidation of chains with *p*-hydroxymercuribenzoate attached, at low pH. Autoxidation rates and hence $O_2^{-\cdot}$ production were higher for the abnormal haemoglobins than for Hb A. For Hb Belfast, which has a relatively minor structural change, and Hb Christchurch, which has a major structural change, the increases in rate correlated quite well with the degree of instability. The situation with Hb Köln is more complex, as this haemoglobin appears to exist in the cell as a mixture of molecules having variable numbers of β-chain haem groups. Hb Köln is a moderately unstable haemoglobin, yet the purified protein autoxidized only slightly faster than Hb A and more slowly than Hb Belfast. However, purified Hb Köln essentially contains only α-chain haem groups, and this rate represents autoxidation of these. Other evidence indicates that the abnormal β chains autoxidize much more rapidly. In purified Hb Köln, which contained more than two haem groups per tetramer, the excess of haem groups, presumably on the β chains, were present almost entirely in the ferric form. Also, a mixture

of Hb A and approx. 6% Hb Köln with β haem groups present autoxidized significantly faster than did Hb A. The increase in autoxidation rate, by about 2 times for isolated α chains and 2.5 times for β chains, is perhaps less than expected, especially for the α chains, which are considered extremely unstable. This suggests that the prime cause of the much greater instability is the conversion of metHb into haemichrome, which occurs very rapidly with isolated α chains, more slowly with β chains, and extremely slowly with Hb A, rather than the increased rate of metHb formation.

Another situation in which there is accelerated haemoglobin autoxidation is in the presence of catalytic amounts of Cu^{2+} ions. This is well documented and it has been suggested that it is the main mechanism operating in the erythrocyte (Rifkind, 1974). Our studies show that the Cu^{2+}-catalysed oxidation also produces $O_2^{-\cdot}$ and H_2O_2, and that the rate of oxidation can be decreased to about 30% by the addition of superoxide dismutase and catalase. High erythrocyte copper content must therefore be considered as a source of increased production of oxygen radicals as well as metHb. Some stimulation of autoxidation by iron was observed, probably involving $O_2^{-\cdot}$ production, but it was many times less efficient than with Cu^{2+}. However, very high erythrocyte iron concentrations can occur and may give rise to increased oxidation. It is noteworthy that although the spontaneous autoxidation of Hb Belfast is slightly faster than Hb A, the Cu^{2+}-catalysed rates are approximately equal. The theoretical reason for increased oxidation of unstable haemoglobins is that the mutations alter the protein structure about the haem group, facilitating entry of water and small anions, and exit of $O_2^{-\cdot}$. If Cu^{2+} assisted in this process, it would be expected to augment the faster rate of oxidation of Hb Belfast. Failure to observe this suggests that the Cu^{2+} ions act through another mechanism, possibly involving direct oxidation of the haem iron, as has been proposed by Rifkind (1974).

In the erythrocyte with a normal enzyme complement, of the reactions described in eqns. (5)–(7) only the first step should occur, and the spontaneous autoxidation rate would be decreased to nearly one-quarter of that in a pure haemoglobin solution. $O_2^{-\cdot}$ should be broken down by superoxide dismutase, and H_2O_2 by catalase and glutathione peroxidase. This could be considered as one important role for these enzymes, as their absence would result in a fourfold increase in the NADH needed from glycolysis to reduce the additional metHb formed. Haemoglobin autoxidation results in the production of H_2O_2, regardless of whether or not the $O_2^{-\cdot}$ is broken down enzymically, and under normal conditions in the erythrocyte where there is no exposure to external oxidants, it is likely that breakdown of this H_2O_2 by the glutathione system is the major contributor to NADPH oxidation and cause of hexose monophosphate shunt activity. It follows that any condition that causes accelerated haemoglobin autoxidation, such as the unstable haemoglobins and the isolated chains found in thalassaemia, or an increase in erythrocyte Cu^{2+} concentration, will result in increased activity of both energy cycles. If autoxidation were sufficiently increased, some $O_2^{-\cdot}$ may by-pass enzymic breakdown and at least one effect would be a still further increase in energy metabolism. Another effect of increased $O_2^{-\cdot}$ (and H_2O_2) production could be an overloading of the reductive mechanisms of the cell. With the unstable haemoglobins, and the free chains that would be present in the thalassaemias, the amount of increase should be relatively small, and it seems likely that unless there is additional stress (e.g. oxidant drug administration), the cells should be able to accommodate the increased load. However, it is probable that elevated Cu^{2+} concentrations could sufficiently increase production of $O_2^{-\cdot}$ and H_2O_2 to be a threat to the cell.

Oxidation of haemoglobin by H_2O_2 gives, in addition to metHb, a series of irreversibly denatured haemoglobin derivatives (Lemberg, 1956). Reactions of haemoglobin with $O_2^{-\cdot}$ have not previously been documented. With $O_2^{-\cdot}$ generated from either xanthine oxidase or reduced riboflavin, the only reactions with normal haemoglobin that we have detected are the oxidation of oxyHb to metHb and the reduction of metHb, with the latter being 2–3 times as rapid (Sutton *et al.*, 1976). However, in the erythrocyte with normally very low metHb concentration, the oxidation reaction should predominate. Both reaction rates, particularly the oxidation, appeared to increase when the pH is decreased from 7.9 to 7.0. The reduction of metHb by $O_2^{-\cdot}$ can be regarded as the reverse of the autoxidation reaction, and it shows some similarity to the reaction of $O_2^{-\cdot}$ with peroxidase (Wittenberg *et al.*, 1967; Rotilio *et al.*, 1975). No further reactions of $O_2^{-\cdot}$ with the haem group were evident; in particular there was no formation of haemichromes or precipitation. We could also measure no oxidation of the cysteine residues at position β-93 of Hb A under conditions when oxidation of GSH was observed. However, the thiol groups of denatured haemoglobin were much more susceptible to oxidation than was GSH.

The reactions of $O_2^{-\cdot}$ with haemoglobin therefore give products that are normally encountered and can be handled by the erythrocyte, and not irreversibly denatured derivatives. Since haemoglobin is by far the major constituent of the erythrocyte, it must be regarded as one of the prime targets for reaction with any $O_2^{-\cdot}$ which might escape breakdown by superoxide dismutase. Comparison of the rates of reaction of $O_2^{-\cdot}$ with oxyHb and superoxide dismutase shows that in a normal erythrocyte, about 1% of the $O_2^{-\cdot}$ generated could be expected to react with

haemoglobin in this way (Sutton *et al.*, 1976). In this case at least, we find no evidence for resultant deleterious effects to the cell. However, it may be as a precursor of H_2O_2 or hydroxyl radicals as a product of $O_2^{-\cdot}$ and H_2O_2 (Bors *et al.*, 1974) that excess of superoxide poses a greater threat to the cell.

The unstable haemoglobins, Christchurch and Belfast, were similar to Hb A in their reactions with $O_2^{-\cdot}$. The three methaemoglobins and oxyhaemoglobins all reacted at the same rates. The absence of any haemichrome formation or precipitation is noteworthy, as Brunori *et al.* (1975) have reported that superoxide dismutase and catalase protect isolated α and β chains against these transformations. However, our results suggest that their findings can be explained by the enzymes decreasing the rate of oxidation to metHb. Since the unstable haemoglobins can be considered to be partially denatured, we might expect their thiol groups at residue β-93 to be more reactive towards $O_2^{-\cdot}$. This was true of haem-depleted Hb Köln, the thiol groups of which reacted at about the same rate as GSH. Increased reactivity of the thiol groups of Hb Köln has been observed (Jacob & Winterhalter, 1970), and we have also found that they react faster with H_2O_2 than does Hb A (C. C. Winterbourn & R. W. Carrell, unpublished work). The reaction appears to produce inter-β-chain disulphide bonds, presumably between different molecules. $O_2^{-\cdot}$ and H_2O_2 produced in cells containing Hb Köln could therefore undergo these reactions in addition to those encountered in normal cells, and we found some evidence suggesting that this occurs to a small extent during autoxidation in the absence of catalase and superoxide dismutase. In contrast with Hb Köln, we could detect no reaction of the thiol groups at position β-93 of Hb Belfast or Hb Christchurch with $O_2^{-\cdot}$. Although it was only possible to analyse a mixture containing 20% Hb Christchurch, reactivity of the order of Hb Köln would have produced detectable changes in thiol-group concentration. This is therefore another property that differs between some unstable haemoglobins (probably those that lose haem groups) and others (probably those that do not).

Probably a more striking defect in the unstable haemoglobins than their increased susceptibility to autoxidation is the instability of the methaemoglobins, and ease with which they are converted into haemichromes and precipitate. Although metHb A is quite stable at 37°C for several days, metHb Belfast and metHb Christchurch are converted into haemichromes over a few hours. Isolated chains are even more rapidly converted from metHb into haemichromes (Rachmilewitz *et al.*, 1971; Brunori *et al.*, 1975). Haemichrome formation from the α chains of haem-depleted Hb Köln was not nearly so pronounced. This implies that under these conditions any dissociation into free α and β chains, which has been proposed as a step in the denaturation of Hb Köln (Jacob, 1970), must also be slow. Our results suggest that the abnormal β chains may form haemichromes more readily. This transformation may be one step on the way to haem loss (Rachmilewitz, 1974). Our results show that the rate of haemichrome formation during autoxidation of Hb Belfast and Hb Christchurch depends only on the amount of metHb produced and therefore that $O_2^{-\cdot}$ or H_2O_2 is not involved in the process. However, other factors appear to influence haemichrome formation. It is considerably slowed down in the presence of EDTA, with at least part of this effect being attributable to the presence of Cu^{2+} ions.

There has been some speculation as to the relative importance of the two processes of haemichrome formation and precipitation of the protein and of production of oxygen radicals to the haemolysis associated with thalassaemia and the unstable haemoglobins (Carrell *et al.*, 1975). Our results suggest that of the two, the increased ease of haemichrome formation with resultant precipitation is much more pronounced, and seems more likely to be the major cause of cell breakdown. However, the additional reductive requirements of these cells should make them more prone to oxidant injury, and the alternative mechanism may predominate under oxidant stress.

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