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Mechanism of autoxidation of oxyhaemoglobin

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Abstract. Oxyhaemoglobins from erythrocytes of different animals including fish, amphibians, reptiles, birds, mammals and human beings have been isolated by ion-exchange chromatography over phosphocellulose and the comparative rates of autoxidation of oxyhaemoglobin studied. The mechanism of autoxidation *in vitro* has been elucidated using toad as well as human oxyhaemoglobin. Autoxidation is markedly inhibited by carbon monoxide as well as by anion ligands, namely, potassium cyanide, sodium azide and potassium thiocyanate. The inhibition by anions is in the same order as their strength as nucleophiles, indicating that it is the oxyhaemoglobin and not the ligand-bound deoxy species which undergoes autoxidation. The structure of oxyhaemoglobin is considered to be mainly $Hb^{3+}O_2^{\cdot-}$ and determination of the rate of autoxidation with or without using superoxide dismutase and catalase indicates that the initial process of autoxidation takes place by dissociation of $Hb^{3+}O_2^{\cdot-}$ to methaemoglobin and superoxide to the extent of 24%. The superoxide thus produced reattacks oxyhaemoglobin to produce further methaemoglobin and hydrogen peroxide. H_2O_2 is a major oxidant of oxyhaemoglobin producing methaemoglobin to the extent of 53%. A tentative mechanism of autoxidation showing the sequence of reactions involving superoxide, H_2O_2 and OH has been presented.

Keywords. Oxyhaemoglobin; methaemoglobin; autoxidation; superoxide; superoxide dismutase; catalase; erythrocytes; animals; phyla.

1. Introduction

Superoxide radical ($O_2^{\cdot-}$) is continuously generated in human erythrocytes (Scarpa *et al* 1984). Several authors have produced evidence that this production of $O_2^{\cdot-}$ can take place during autoxidation of oxyhaemoglobin (HbO_2) to methaemoglobin (MetHb) under certain conditions which can also occur *in vivo* (Misra and Fridovich 1972; Wever *et al* 1973; Wallace *et al* 1974a; Brunori *et al* 1975; Gotoh and Shikama 1976; Lynch *et al* 1976; Winterbourn *et al* 1976). The concentration of MetHb in normal human erythrocytes at any given moment is about 0·5 to 1% (Salvati and Tentori 1981). Apparently, the MetHb content results from an equilibrium between the rate of formation of MetHb and the rate of reduction to haemoglobin (Hb). Experiments on patients with hereditary methaemoglobinemia indicated the rate of MetHb production to be approximately 3% per day (Jaffe and Neumann 1964). While MetHb can lead to haemichrome formation, Heinz body formation and other changes, $O_2^{\cdot-}$ has been implicated in lipid peroxidation resulting in irreversible damage and lysis of red cell membranes (Fridovich 1972, 1979; Kellog and Fridovich 1977). Since autoxidation of HbO_2 has profound influence on the normal

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function and pathophysiology of red cells, the elucidation of the mechanism of autoxidation is extremely important.

The structure of HbO_2 has been considered to be mainly $Hb^{3+}O_2^-$ (Viale *et al* 1964; Weiss 1964; Peisach *et al* 1968; Misra and Fridovich 1972; Wittenberg *et al* 1970; Yamamoto *et al* 1973; Peisach 1975). Using shark HbO2, Misra and Fridovich (1972) observed that direct dissociation of $Hb^{3+}O_2^-$ could account for the slow autoxidation of HbO_2 to MetHb and O_2^- However, the detailed mechanism of autoxidation was not studied, probably because shark HbO_2 was unstable and the rate of autoxidation of shark HbO_2 was too fast (Misra and Fridovich 1972).

On the other hand, using human HbO_2 Wallace *et al* (1982) considered that direct dissociation of HbO_2 to MetHb and O_2^- was not probable. In studies on the effects of strong anionic nucleophiles, not normally found in red cells, namely, potassium cyanide (CN^-), sodium azide (N_3) and potassium thiocyanate (SCN^-), these authors proposed that the anion ligands were promoters of the autoxidation of haemoglobin and that the anions were increasingly effective as promoters in the same order as their strength as nucleophiles. These authors considered that the anion complex of haemoglobin (HbL^-) or a complex of protonated deoxy-heamoglobin species with anion [$Hb(H^+)(L^-)$] reacted with molecular oxygen to produce O_2^- (Wallace *et al* 1982; Watkins *et al* 1985). In other words, these authors proposed that it was the ligand-bound deoxy rather than the oxy species which underwent autoxidation to MetHb. However, Wallace *et al* (1982) did not produce data of control experiments showing the rate of autoxidation of HbO_2 in the absence of any added ligand compared to that obtained in the presence of CN^-, N_3^- or SCN^-. Moreover, the production of O_2^- by the reaction of HbL^- with molecular oxygen was assumed on the basis of simultaneous reduction of ferricytochrome c (cyt c^{3+}) during autoxidation of HbL^- to $MetHbL^-$. On the contrary, we have observed that cyt c^{3+} is directly reduced by HbO_2 at a very fast rate and that about 79% of this reduction of cyt c^{3+} is insensitive to superoxide dismutase (SOD). We have further observed that while CN^- and N^- significantly inhibit the autoxidation of HbO_2, these enhance the reduction of cyt c^{3+}. In earlier studies, Tomoda *et al* (1980) demonstrated that cyt c^{3+} oxidized both human deoxy- and oxyhaemoglobin to MetHb and that the rate of oxidation was directly proportional to the concentration of cyt c^{3+} used. The direct reaction between Hb and cyt c^{3+} has also been reported by others (Brown 1961; Wallace and Caughey 1979). Wu *et al* (1972) have shown that MbO_2 directly reduces cyt c^{3+}.

It would thus appear that the detailed mechanism of autoxidation of HbO_2 is not clear and remains controversial. We have therefore carried out systematic experiments bearing on the mechanism of autoxidation of HbO_2 and the results are presented in this paper.

2. Materials and methods

2.1 *Chemicals*

Cellulose phosphate P-11 was purchased from Whatman, England. Coomassie brilliant blue R, acrylamide, bovine erythrocyte SOD, epinephrine and

ferricytochrome c were obtained from Sigma Chemical Company, St. Louis, Mo, USA. Catalase (free of SOD) was purchased from the CSIR Centre for Biochemicals, New Delhi; 2-deoxyribose from SRL, India; heparin from Biological E. Ltd. (India) and bisacrylamide from Koch-Light Laboratories, England. Desferrioxamine was a gift from CIBA-GEIGY, Basel, Switzerland. All other chemicals and reagents used were of analytical grade. All solutions were made with double-distilled water.

2.2 *Collection of blood*

Blood from toad (*Bufo melanostictus*), frog (*Rana tigrina*), blood sucker (*Caloter versicolor*), anjani (*Mabuya carinata*), common Indian monitor (*Varanus monitor*), *turtle* (*Lissemys punctata*), rat, guinea pig, rabbit and Indian fruit bat was obtained by cardiac puncture. Chicken and pigeon blood was obtained from the subclavian vein. Fish blood was collected from the caudal vein. The blood of goat and cattle was obtained from jugular vein and collected from the slaughter house. We are indebted to Dr D K Bhattacharyya, Bhoruka Research Centre for Hematology and Blood Transfusion, Calcutta, India for supplies of fresh human blood. Blood samples were collected in heparin (100 units for about 5 ml blood).

2.3 *Haemoglobin*

Red cells were sedimented by centrifugation and washed three times with 0·15 M NaCl. The buffy coat was aspirated with each wash. Packed washed red cells were lysed in 19 volumes of cold distilled water. The ghosts were sedimented by centrifugation at 17,000 *g* for 20 min in a Hitachi automatic high speed refrigerated centrifuge, model SCR 20BA. After adjustment of pH at 6·8, 10 ml of the supernatant was applied to a phosphocellulose column (6 × 1·5 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 6·8. The column was washed four times with equilibrated buffer. The adsorbed Hb was eluted with 0·5 M sodium phosphate buffer, pH 6·8. The Hb fraction was collected in 2 ml of buffer. The recovery of Hb from the haemolysate was 50 to 60%. All steps in the purification of Hb from blood were performed at 0–6° without delay. The eluted Hb solution was free of SOD as assayed by Polyacrylamide gel electrophoresis (Beauchamp and Fridovich 1971) and catalase as assayed by determining the rate of decrease in absorbance at 240 nm of 22·5 mM H_2O_2 (Lynch *et al* 1977). The purity of the Hb was determined by sodium dodecyl sulphate-polyacrylamide discontinuous gel electrophoresis according to the method of Laemmli (1970). Hb eluted from the phosphocellulose column was used without delay and without any chemical treatment for subsequent studies. Hb was estimated by the modified method of Drabkin (Richteric 1969).

2.4 *Determination of the rate of autoxidation of HbO_2 at 37°*

The rate of autoxidation of HbO_2 was determined at 37° by measuring the decrease of the ratio of absorbance at 575:500 nm (Salvati *et al* 1969), increase of absorbance at 630 nm and by recording the change of visible spectra between 700 and 500 nm in a Hitachi model 200–20 spectrophotometer with recorder (model 200) and using a Hitachi temperature-controlled water-circulated cell holder coupled with a LKB

Multi Temp. The reaction mixture (final volume 2 ml) contained 2 μm HbO_2, 50 mM potassium phosphate buffer, pH 6·8, 0·1 mM EDTA, incubated at 37°.

2.5 *Co-oxidation of epinephrine*

Superoxide produced during the autoxidation of HbO_2 was measured by the co-oxidation of epinephrine following the method of Misra and Fridovich (1972). The assay system contained 10 μm HbO_2, 50 mM aerated potassium phosphate buffer, pH 6·8, 0·1 mM EDTA and 0·6 mM epinephrine. The formation of adrenochrome was followed at 480 nm at 37°C using a temperature-controlled water-circulated cell holder.

Measurement of the production of OH·

Hydroxyl radical formed during the autoxidation of HbO_2 was measured by the method of deoxyribose degradation as described by Puppo and Halliwell (1988).

3. Results

3.1 *Contents of oxy and met forms of haemoglobin isolated from different animals*

Table 1 gives comparative values of the oxy and met forms of Hb isolated from fish, toad, frog, blood sucker, turtle, anjani, common Indian monitor, chicken, pigeon, Indian fruit bat, rat, rabbit, guinea pig, goat, cattle and human being. The results show that Hb isolated from different animals except fish contains 90 to 99% HbO_2. Hb isolated from fish contained only 79% HbO_2 and 21 % MetHb. That fish HbO_2 loses it stability after purification has also been reported by Riggs (1981).

3.2 *Rate of autoxidation of HbO_2 from different animals*

Comparison of the rates of autoxidation of HbO_2 at 37°C from different animals indicate that the rate is very high in the case of fish and blood sucker. Table 2 shows the approximate pseudo-first-order rate constants observed during the autoxidation of HbO_2 of different animal species. The constants were determined by the method of Satoh and Shikama (1981).

3.3 *Co-oxidation of epinephrine*

Figure 1a shows the spectral changes obtained during autoxidation of toad HbO_2 in the presence of epinephrine. The figure indicates, that autoxidation of HbO_2 to MetHb (increase of A_{630}) is accompanied by oxidation of epinephrine to adrenochrome (increase of A_{480}). The inset of figure la shows that the amount of adrenochrome formed (ε_{480} = 4 mM^{-1} cm^{-1}) and MetHb produced at any time during the autoxidation is in the molar ratio of approximately 1. The co-oxidation of epinephrine was almost completely inhibited by SOD and catalase (figure 1b). The possibility that the co-oxidation of epinephrine was mediated by any free iron was eliminated because addition of 20 μm desferrioxamine did not inhibit the co-oxidation of epinephrine.

3.4 *Effects of SOD, catalase, thiourea and mannitol*

SOD inhibits about 31 % of the autoxidation of toad HbO_2 indicating that $O_2^{\cdot-}$ is

Table 1. Per cent HbO_2 and MetHb from different animals.

Animal	HbO_2 (%)	MetHb (%)
Fish		
*Labeo rohita** (6)	79	21
Amphibians		
Toad (*Bufo melanostictus*) (18)	97	3
Frog (*Rana tigrina*) (12)	97	3
Reptiles		
Blood sucker (*Caloter versicolor*) (12)	97	3
Anjani (*Mabuya carinata*) (8)	98	2
Turtle (*Lissemys punctata*) (4)	90	10
Common Indian monitor (*Varanus monitor*) (2)	99	1
Birds		
Pigeon (2)	93	7
Chicken (4)	97	3
Flying mammal		
Indian fruit bat (4)	91	9
Mammals		
Rabbit (2)	99	1
Rat (12)	97	3
Guinea pig (6)	99	1
Goat (4)	99	1
Cattle (2)	99	1
Human (6)	99	1

Two ml of the assay mixture contained 2 μM HbO_2, 0·1 mM EDTA, 50 mM potassium phosphate buffer, pH 6·8.
Numbers in parentheses represent the number of animals used.
The data are average values of HbO_2 and MetHb.
*Similar results were obtained with *Catla catla, Labeo calbasu* and *Cirrhina mrigala.*

not only produced but also involved in the further oxidation of Hb to MetHb. The autoxidation is also inhibited about 53% by catalase indicating that H_2O_2 is produced during autoxidation and it is a major oxidant of HbO_2. Figure 2 shows that the low concentration of H_2O_2 oxidizes HbO_2 to MetHb at a fast rate. The amount of H_2O_2 used (2 nmol) was approximately three times that produced (0·64 nmol) during the autoxidation under the experimental conditions used. The amount of H_2O_2 produced was calculated on the basis of catalase inhibition. The oxidation of HbO_2 by added H_2O_2 was completely inhibited by catalase. When SOD and catalase were present together, the oxidation of HbO_2 was inhibited by about 76%. This would indicate that about 24% of the MetHb formed was probably due to dissociation of $Hb^{3+}O_2^-$ The autoxidation of HbO_2 was also inhibited 50% by thiourea and 23% by mannitol, the scavengers of $OH^{\cdot}$, indicating that OH was also involved in the autoxidation. That $OH^{\cdot}$ is produced during autoxidation of HbO_2 has been demonstrated by the method of deoxyribose degradation (Puppo and Halliwell 1988) as shown in table 3. The deoxyribose degradation was inhibited about 98% by thiourea and 71 % by mannitol.

Table 2. Approximate pseudo-first-order rate constant observed during autoxidation of HbO_2 of different animals.

Animals	$K_{app} \times 10^3$ (min^{-1})
Blood sucker	25·0
Fish	19·0
Guinea pig	14·0
Toad	7·0
Frog	6·5
Goat	6·0
Common Indian monitor	4·5
Rat	2·0
Anjani	1·5
Chicken	1·5
Rabbit	1·5
Cattle	1·0
Human	0·7

Condition: 2 μM HbO_2 in 0·1 M potassium phosphate buffer, pH 6·8, 0·1 mM EDTA at 37°. The K_{app} (average) was calculated by the method of Satoh and Shikama(1981).

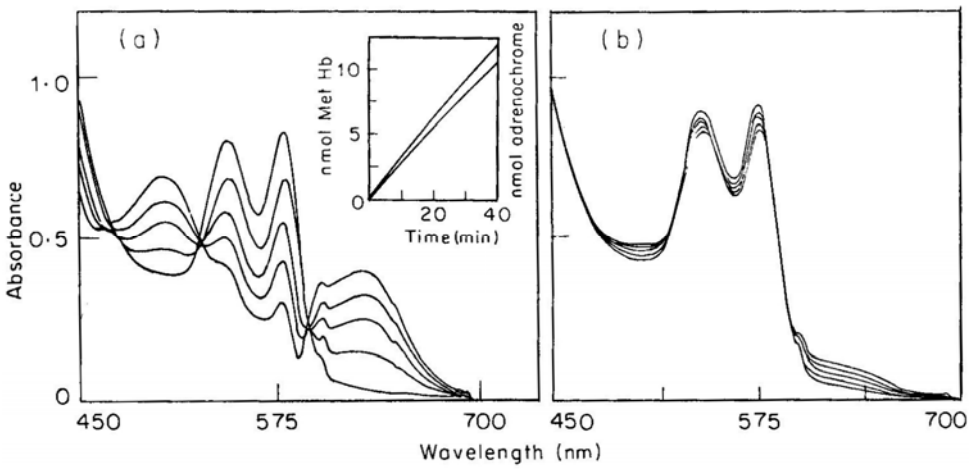

Figure 1. Autoxidation of HbO_2 in the presence of epinephrine.

(a) Control, containing aerated 50 mM potassium phosphate buffer, pH 6·8, 0·1 mM EDTA, 10 μM HbO_2, 0·6 mM epinephrine; **(b)** with 20 μg SOD plus 80 μg catalase. Spectra were obtained at 0, 10, 20, 30 and 40 min elapsed time. *Inset*: (1) nmol of adrenochrome formed (ε_{480} = $4mM^{-1}$ cm^{-1}) and (2) nmol of MetHb produced during the co-oxidation of epinephrine.

3.5 *Effect of pH*

It has been observed that the rate of autoxidation is increased as the pH is decreased. The approximate rate constants at different pH are given in table 4. Similar observations were made with human HbO_2. Under the conditions used for toad HbO_2, the percentage of MetHb formed from human HbO_2 was 6 at pH 6·8

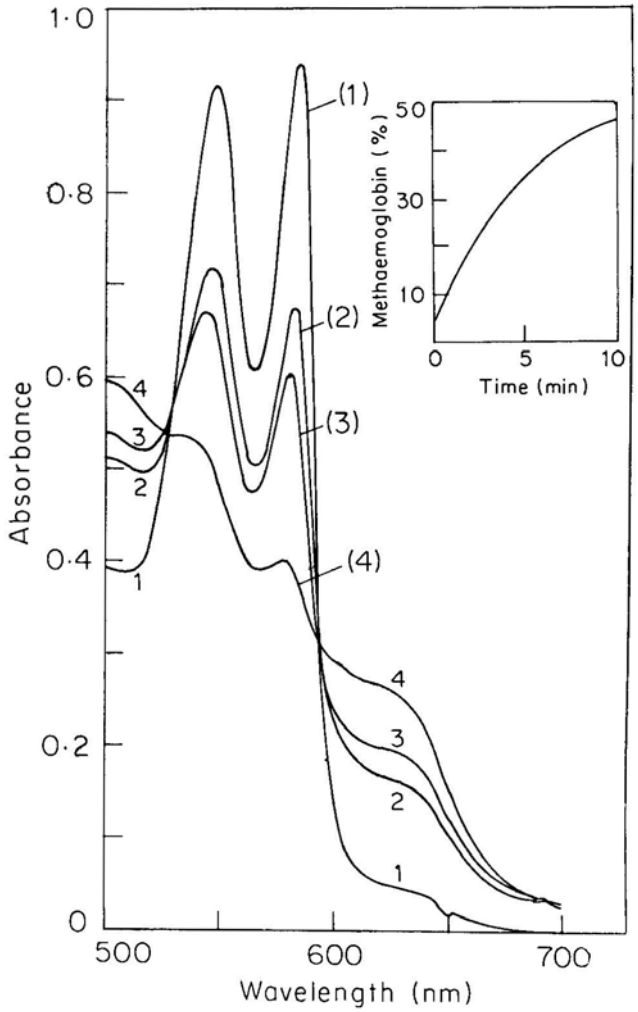

Figure 2. Spectral changes obtained during the oxidation of toad HbO in the presence of hydrogen peroxide.

Spectra at different time intervals are: (1) 0 min (control without H_2O_2), (2) 4 min, (3) 10 min, after addition of 2 μM H_2O_2. *Inset:* Increase of % MetHb against time. Graph 4 represents the spectrum obtained after 7 min incubation with 10 μM H_2O_2. The MetHb formed with 10 μM H_2O_2 was 74%. Other conditions are given under 'materials and methods'.

and 10 at pH 6·0 in 40 min. The increased rate of autoxidation was probably due to the increased rate of dissociation of $Hb^{3+}O_2^{\overline{\cdot}}$, because the rate of formation of MetHb in the presence of SOD and catalase increased as the pH was decreased (figure 3). The approximate rate constant $K \times 10^3$ (min^{-1}) observed in the presence of SOD and catalase were at pH 7·4, 0·5; pH 6·8, 1·7; pH 6·0, 2·4 and pH 5·5, 4·4. Nevertheless, the inhibition of autoxidation by SOD plus catalase was similar at different pH. The per cent inhibition was 76 at pH 6·8, 68 at pH 6·0 and 73 at pH 5·5.

Table 3. Hydroxyl radical formation during toad HbO_2 autoxidation as determined by deoxyribose degradation.

Reagent added to reaction mixture	A_{532}	Inhibition of deoxyribose degradation (%)
None (complete reaction mixture)	0·048	0
Thiourea (10 mM)	0·001	98
Mannitol (40 mM)	0·014	71

Reaction mixture was incubated at 37° for 90 min. It contained the following reagents at the final concentrations stated: HbO_2 (50 μM), deoxyribose (5·6 mM) and KH_2PO_4/K_2HPO_4 buffer, pH 6·8 (50 mM in phosphate). After incubation colour was developed, extracted into butan-1-01 and measured at 532 nm as described (O'Connell *et al* 1986). Scavengers were added to the reaction mixture to give the final concentrations stated. Absorbances were read against respective blanks which contained everything but not incubated.

Table 4. Effects of pH, enzymes and oxyradical scavengers on the observed first order rate constant (K_{app}) of the autoxidation of toad HbO_2.

Variable factors	$K_{app} \times 10^3$ (min^{-1})
pH	
6·8	7·0
6·0	10·0
5·5	18·0
Enzymes[a]	
SOD	5·0
CAT	3·6
SOD + CAT	1·7
Hydroxyl radical scavengers	
Thiourea	3·9
Mannitol	5·8
Ligands[a]	
CO	0·1
CN^-	0·3
N_3^-	1·4
SCN^-	3·6

Reaction conditions: 2 μM HbO_2, 37°, 50 mM potassium phosphate buffer, pH 6·8 and 6·0, 50 mM sodium acetate buffer, pH 5·5, 20 unit SOD, 80 µg catalase, 20 mM mannitol, 10 mM thiourea, 2 mM CN^-, 2 mM N_3^- and 2 mM SCN^-.
[a]All at pH 6·8.

3.6 *Effect of CN^-, N_3^-, SCN^- and CO*

Figure 4a shows the spectral changes obtained during the autoxidation of toad HbO_2 in the absence of any added ligand. Figure 4b-e shows that autoxidation is inhibited by CO as well as by anion ligands, namely, CN^-, N_3^- and SCN^- respectively. Further, the inhibition by the anion ligands is in the same order as their strength as nucleophiles. This would indicate that it is the oxy species, that is

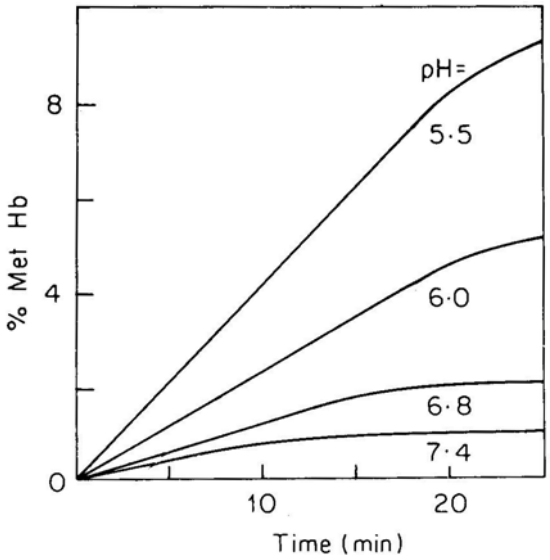

Figure 3. Rate of autoxidation of toad HbO_2 at different pH in the presence of SOD and catalase.

Two ml reaction mixture contained 2 μM HbO_2, 01 mM EDTA, SOD (20 μg) and catalase (80 μg); pH 7·4, 6·8 and 6·0 were 50 mM potassium phosphate buffer and pH 5·5 was 50 mM sodium acetate buffer.

HbO_2, and not the ligand-bound deoxy species as presumed by Wallace *et al* (1982), which undergoes autoxidation. A similar result was obtained with human HbO_2. Although the rate of autoxidation of human HbO_2 was very slow, nevertheless, CN^- and N_3^- significantly inhibited autoxidation. In the absence of any ligand the percentage of MetHb formed was 7, whereas in the presence of CN^- and N_3^- MetHb% were 2 and 4 respectively. Wallace *et al* (1982) and Watkins *et al* (1985) assumed that protonated anion-ligands were effective as promoters of autoxidation of HbO_2. However, the marked inhibitory effect of CN^- was also observed when the pH was decreased (figure 5a, b).

3.7 *MetHb formation and ferricytochrome c reduction*

Figure 6a shows that the addition of ferricytochrome c (cyt c^{3+}) to a solution of toad HbO_2 results in the reduction of cyt c^{3+} to cyt c^{2+} as evidenced by the increase of A_{550}. The reduction of cyt c^{3+} is accompanied by oxidation of HbO_2 to MetHb as indicated by the increase of A_{630} and decrease of A_{575}. Figure 6a further shows that the initial rate of cyt c^{3+} reduction is very fast. In a reaction period of 10 min, about 78% of the cyt c^{3+} reduction takes place within the first 3·5 min. There is no stoichiometry between cyt c^{3+} reduction and MetHb formation. During the initial period of 1·5 min, the amount of cyt c^{3+} reduced is 1·14 nmoi (ε_{550} = 21 mM^{-1} cm^{-1}) whereas the MetHb formed is only 0·1 nmol indicating a molar ratio of 11·4. The observed molar ratios in 2·5 min, 3·5 min and 4·5 min are 8, 7·3 and 6·5 respectively (inset of figure 6a). This would indicate that cyt c^{3+} reduction is not directly related to heme iron oxidation. This has been further

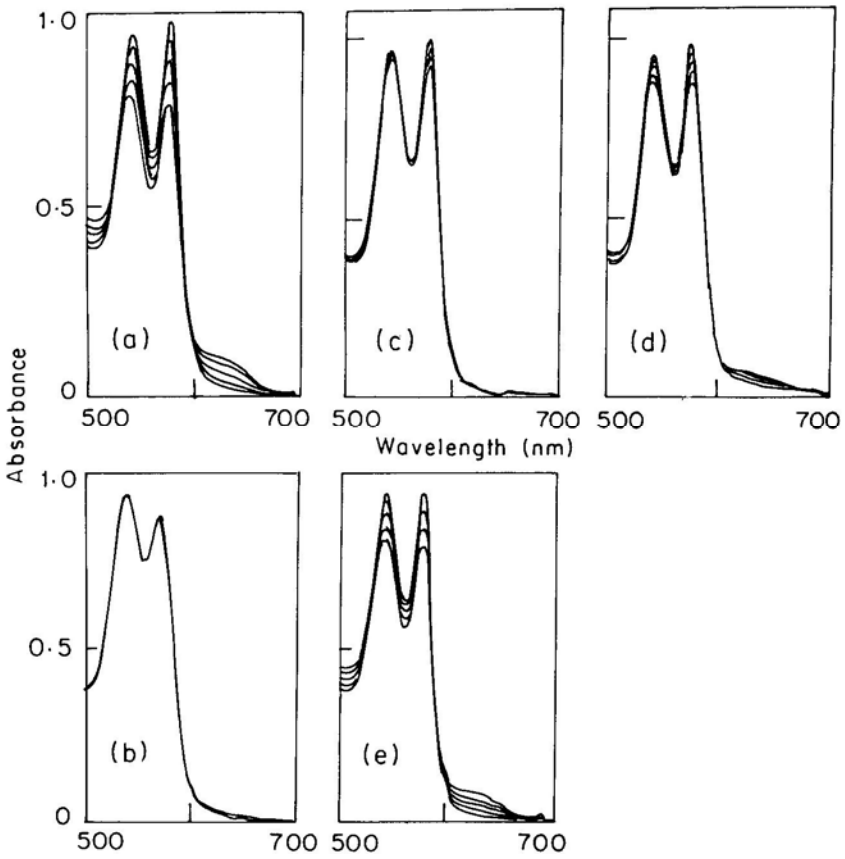

Figure 4. Spectral changes observed during the autoxidation of toad HbO_2 in the absence and presence of different ligands. The reaction mixture contained 50 mM potassium phosphate buffer, pH 6·8, 0·1 mM EDTA, 2 μM HbO_2. (**a**) Control; (**b**) 2 μM HbCO; (**c**) 2 mM cyanide; (**d**) 2 mM azide; (**e**) 2 mM thiocyanate. Spectra were obtained at 0, 10, 20, 30 and 40 min elapsed time.

evidenced by the fact that in the presence of CN^-, oxidation of heme iron is. markedly inhibited whereas the reduction of cyt c^{3+} is rather enhanced (figure 6b). A similar observation has been made with (figure 6c). Moreover, the reduction of cyt c^{3+} by HbO_2 is only 21% inhibited by SOD indicating that 79% cyt c^{3+} reduction is not related to production. During a reaction period of 10 min, the amount of cyt c^{3+} reduced in the absence of SOD is 3·79 nmol, and that in the presence of 20 units of SOD, 3 nmol. Human HbO_2 also reduces cyt c^{3+}, but the rate of reduction is very slow compared to that observed with toad HbO_2. In 20 min and under the conditions used for toad HbO_2, the amount of cyt c^{3+} reduced by human HbO_2 was 0·95 nmol, whereas the amount of MetHb formed was 0·2 nmol, indicating a molar ratio of 4·5. The reduction of cyt c^{3+} by human HbO_2 was only 20% inhibited by SOD, confirming our results obtained with toad HbO_2, that cyt c^{3+} reduction by HbO_2 was neither directly related to heme iron oxidation, nor did it account for the O_2^- production.

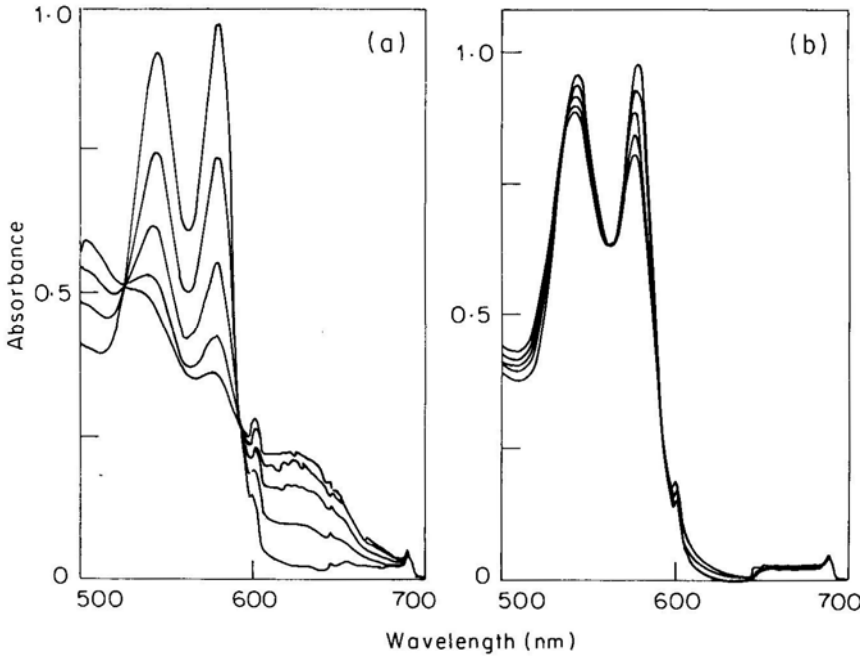

Figure 5. Spectral changes observed during the autoxidation of toad HbO_2 in the absence and presence of cyanide.

(**a**) 50 mM sodium acetate, pH 5·5, 0·1 mM EDTA, *2 μM* HbO_2; (**b**) same as in figure 4c plus 2 mM cyanide. Spectra were obtained at 0, 10, 20, 30 and 40 min elapsed time.

4. Discussion

Elucidation of the detailed mechanism of autoxidation of HbO_2 needs a sample of HbO_2, the rate of autoxidation of which should be neither too fast like fish HbO_2 as used by Misra and Fridovich (1972) nor too slow like human HbO_2 as used by Wallace *et al* (1982). In this regard, toad HbO_2 appears to be a suitable sample for studying the sequence of reactions involved in the process of autoxidation. Perutz (1979) indicated that though haemoglobins of different species may vary in amino acid sequence depending on the position of the species on the evolutionary tree (Romero–Herrera 1973; Goodman 1975), the three-dimensional structure and function of the different haemoglobins are similar. Therefore, results obtained with toad HbO_2 may represent a general mechanism of autoxidation of HbO_2. We have also verified some of the results using human HbO_2.

Using the technique of co-oxidation of epinephrine for the estimation of $O_2^{\cdot-}$ we have observed that during autoxidation of HbO_2, the adrenochrome formed and MetHb produced is in the molar ratio of 1. That the initial step of autoxidation is dissociation of $Hb^{3+}O_2^{\cdot-}$ into MetHb and $O_2^{\cdot-}$ is also apparent from kinetic studies. Since epinephrine is an acceptor of $O_2^{\cdot-}$ the $O_2^{\cdot-}$ produced by the dissociation of $Hb^{3+}O_2^{\cdot-}$ may be considered to be fully consumed by epinephrine. The rate of formation of MetHb in the presence of epinephrine would represent the rate of dissociation of $Hb^{3+}O_2^{\cdot-}$ in the presence of an acceptor of $O_2^{\cdot-}$ When SOD and catalase are added along with epinephrine, epinephrine can no longer act as an

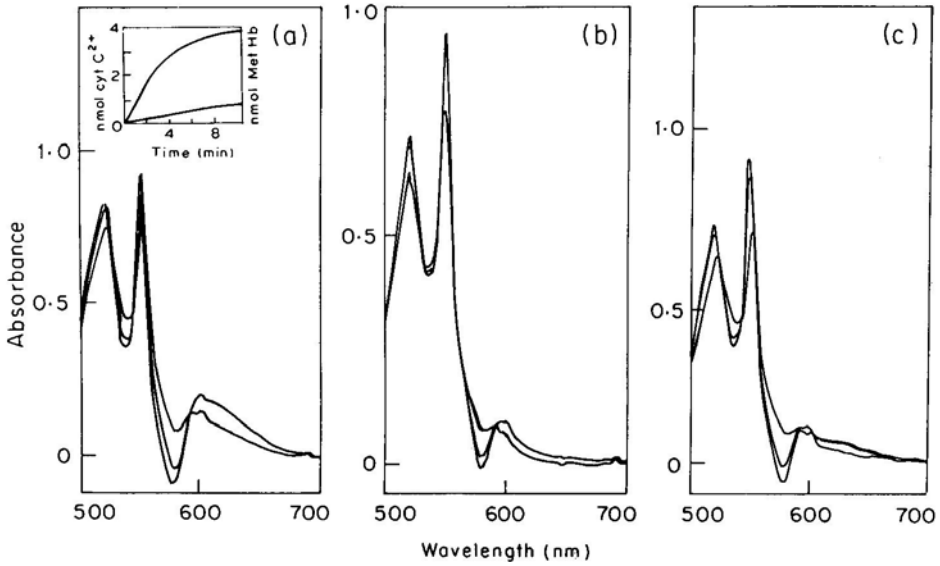

Figure 6. Spectral changes observed when toad HbO_2 was allowed to autoxidize in the presence of cyt c^{3+} with cyanide and azide.

(**a**) Control, containing 50 mM potassium phosphate buffer, pH 6·8, 2 μM HbO_2, 10 μM cyt c^{3+}; (**b**) in the presence of 2 mM cyanide; (**c**) in the presence of 2 mM azide. Spectra were obtained at 1·5, 6 and 11 min elapsed time. *Inset:* (1) nmol cyt c^{2+} formed (ε_{550} = 21 mM^{-1} cm^{-1}) and (2) nmol MetHb produced during the autoxidation of HbO_2 in the presence of cyt c^{3+}.

acceptor of $O_2^{\cdot-}$ and the rate of formation of MetHb represents the actual rate of dissociation of $Hb^{3+}O_2^{\cdot-}$ It is expected that the rate of dissociation should increase markedly in the presence of a acceptor. In fact, the results show that when SOD and catalase are present along with epinephrine, only 1·6 nmol of MetHb are produced in 40 min. Whereas, when SOD and catalase are omitted, the amount of MetHb formed is 11·2 nmol. In other words, the rate of dissociation of $Hb^{3+}O_2^{\cdot-}$ increased about 7 times in the presence of epinephrine, an acceptor of $O_2^{\cdot-}$. Comparing the rate constant of the reaction observed in the presence of epinephrine along with SOD and catalase [K_{app}= 2·1 × 10^{-3} $(min)^{-1}$] and that in the absence of SOD and catalase [*K*app = 17 × 10^{-3} $(min)^{-1}$], it would appear that the rate of dissociation of $Hb^{3+}O_2^{\cdot-}$ is increased about 8 times when an acceptor of $O_2^{\cdot-}$ is present in the reaction mixture. Apparently, the rate of dissociation of $Hb^{3+}O_2^{\cdot-}$ is also increased as the pH is decreased. As mentioned before, the production of MetHb during autoxidation of HbO_2 in the presence of SOD and catalase would represent the actual dissociation of $Hb^{3+}O_2^{\cdot-}$.

The O_2 produced during autoxidation of HbO_2 could not be measured by the method of cyt c^{3+} reduction because cyt c^{3+} is directly reduced by HbO_2. Stoichiometric studies indicate that the initial rate of cyt c^{3+} reduction is very fast compared to that of MetHb formation. The slow rate of heme iron oxidation compared to the fast rate of cyt c^{3+} reduction may be explained by the consideration that cyt c^{3+} cannot abstract an electron directly from the heme iron and the reaction apparently takes place on the outer surface of the haemoglobin

molecule as indicated by Wallace *et al* (1982). The oxidation of Fe^{2+} to Fe^{3+} probably takes place by charge transfer in a relay system, the mechanism of which is yet to be known. That cyt c^{3+} reduction is not directly related to heme iron oxidation has been further proved by the fact that in the presence of CN^-, oxidation of heme iron is markedly inhibited, whereas the reduction of cyt c^{3+} is enhanced.

That it is the oxy form of haemoglobin and not the deoxy form which undergoes autoxidation is proved by the fact that anion ligands like CN^-, and N_3^- SCN^- markedly inhibit the autoxidation of HbO_2 and that inhibition by ligands is in the same order as their strength as nucleophiles.

Several studies have implicated the involvement of $O_2^{\cdot-}$ and H_2O_2 on the autoxidation of HbO_2 (Wallace *et al* 1974a,b; Wallace and Caughey 1979; Winterbourn *et al* 1981). In an attempt to elucidate the mechanism of autoxidation of HbO_2, Watkins *et al* (1985) studied the effects of SOD, catalase and OH· scavenger at different pH. However, the data obtained were inconclusive and no stoichiometric involvement of $O_2^{\cdot-}$, $H2O_2$ and OH · could be ascertained. Also, Watkins *et al* (1985) did not demonstrate the actual formation of OH· during autoxidation of HbO_2. The varied effect of SOD and catalase observed by Watkins *et al* (1985) is probably due to the fact that autoxidation of human HbO_2 is a very slow process and the authors have carried out the incubation for a prolonged period of 20 h at 37·5°, which affects the catalytic activities of SOD and catalase. Regarding the mechanism of $O_2^{\cdot-}$ production during autoxidation of HbO_2, Watkins *et al* (1985) assumed that a complex of protonated deoxyhaemoglobin species with anions reacted with molecular oxygen to produce $O_2^{\cdot-}$. As indicated before, we have observed that it is the oxy species and not the ligand bound deoxy form which undergoes autoxidation and the initial step of autoxidation is dissociation of $Hb^{3+}O_2^{\cdot-}$ to MetHb and $O_2^{\cdot-}$.

Sutton *et al* (1976) and Lynch *et al* (1977) have shown that $O_2^{\cdot-}$ oxidizes HbO_2 to MetHb with production of H_2O_2. Demma and Salhany (1977) demonstrated that HbO_2 dissociated into MetHb and $O_2^{\cdot-}$ when flashed with low intensity white light. The authors observed that $O_2^{\cdot-}$ produced by the dissociation reattacked a fresh molecule of HbO_2 to produce MetHb and H_2O_2 and they observed 23% inhibition of oxidation by SOD. We have also observed that autoxidation is 31 % inhibited by SOD indicating that $O_2^{\cdot-}$ produced by the dissociation of $Hb^{3+}O_2^{\cdot-}$ reattacks HbO_2. Autoxidation of HbO_2 is also 53% inhibited by catalase confirming the observations of others (Sutton *et al* 1976; Demma and Salhany 1977; Lynch *et al* 1977; Watkins *et al* 1985) that H_2O_2 is produced by the reaction of $O_2^{\cdot-}$ with HbO_2 and that H_2O_2 further oxidizes HbO_2 to MetHb. Consideration of the rate constant (K_{app} = 1·6 × 107 M^{-1} s^{-1}) of the reaction of toad HbO_2 with $O_2^{\cdot-}$ (Mal *et al* 1991) and that (K = l·0 × 105 M^{-1} s^{-1}) of spontaneous dismutation of $O_2^{\cdot-}$ (McCord *et al* 1977) would indicate that the source of H_2O_2 is rather a product of the reaction of HbO_2 with $O_2^{\cdot-}$ than spontaneous dismutation of $O_2^{\cdot-}$ Autoxidation is also 50% inhibited by thiourea and 23% by mannitol indicating that OH· is also involved in autoxidation. The large extent of inhibition by thiourea is probably due to the fact that thiourea is not a specific inhibitor of OH· (Cederbaum *et al* 1979; Wasil *et al* 1987). HbO_2 is considered to be a biological Fenton reagent (Sadrzadeh *et al* 1984; Puppo and Halliwell 1988), so OH· is apparently formed by the reaction of HbO_2 with H_2O_2. Using the deoxyribose degradation method (Puppo and

Halliwell 1988), we have demonstrated that OH· is actually produced during autoxidation of HbO_2.

Based on the aforesaid results, the mechanism of autoxidation of HbO_2 may be represented by the following sequence of reactions.

$$HbO_2 \rightleftharpoons Hb^{3+}O_2^{\cdot-}, \tag{1}$$

$$Hb^{3+}O_2^{\cdot-} \rightarrow MetHb + O_2^{\cdot-}, \tag{2}$$

$$HbO_2 + O_2^{\cdot-} + 2H^+ \rightarrow MetHb + H_2O_2 + O_2, \tag{3}$$

$$HbO_2 + H_2O_2 \rightarrow MetHb + OH\cdot + OH^- + O_2, \tag{4}$$

$$HbO_2 + OH\cdot \rightarrow MetHb + OH^- + O_2. \tag{5}$$

The overall reaction may be represented as

$$4HbO_2 + 2H^+ \rightarrow 4MetHb + 2OH^- + 3O_2. \tag{6}$$

The aforesaid mechanism of autoxidation is also supported by the observed inhibition of autoxidation by the anion ligand (L^-) namely, CN^-, and SCN^-. The anion ligands convert HbO_2 to HbL^- according to their strength as nucleophiles and consequently the formation of $Hb^{3+}O_2^{\cdot-}$ and its subsequent dissociation to MetHb and $O_2^{\cdot-}$ is inhibited. Since formation of $O_2^{\cdot-}$ is the rate-limiting step of autoxidation, the overall process is inhibited in the presence of anion ligands. A similar explanation may be given for HbCO.

We have observed that human erythrocytes contain per g Hb enough of SOD (1000 units), ascorbic acid (132 nmol) which is a chemical scavenger of $O_2^{\cdot-}$ (Nandi and Chatterjee 1987) and catalase (100×10^3 units). These scavengers of reactive oxygen species will prevent reattack of HbO_2 by $O_2^{\cdot-}$ and H_2O_2. Therefore, the dissociation of Hb3 + $O_2^{\cdot-}$ probably represents the main mechanism of the formation of MetHb and $O_2^{\cdot-}$ *in vivo*. Our results indicate that this dissociation accounts for the *in vitro* formation of about 2·55% MetHb per hour. If the results obtained *in vitro* are extrapolated to *in vivo* conditions, the rate of formation of $O_2^{\cdot-}$ in human erythrocytes may be roughly calculated as 6·5 nmol per g HbO2 per min. Taking the average concentration of HbO2 as 300 g per 1000 ml packed cell, the rate of $O_2^{\cdot-}$ formation becomes $3{\cdot}25 \times 10^{-8}$ M s^{-1}. This is nearer the value (2×10^{-8} M s^{-1}) calculated by Scarpa *et al* (1985) in the erythrocytes of healthy individuals. It remains a question if in the presence of scavengers of reactive oxygen species, this amount of $O_2^{\cdot-}$ can produce any oxidative damage to the erythrocytes. However, no defence is perfect and some fraction of generated $O_2^{\cdot-}$ may always escape. If they do so, they will produce injury to the erythrocyte membrane. This may be one of the causes of aging in erythrocytes.

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