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OXIDATION AND CROSS-LINKING OF HUMAN HEMOGLOBINS BY ACTIVATED OXYGEN SPECIES

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The effect of activated oxygen species on human hemoglobins was studied. All radicals induced polymerization in Hb A both intermolecular and by cross-linking of subunits (intramolecular). However, a system producing mainly superoxide ion gave the most important changes. An oxidation step is necessary to produce polymerization since in the case of cyanmet Hb A (where there is no possible oxidation) no polymerization occurs. The effect of O_2^- on blocked SH β 93 Hbs or on the abnormal Hbs tested was practically identical to that on Hb A although their autoxidation rates were modified. Consequently the action of radicals is different from autoxidation processes and the modified residues in the abnormal hemoglobins are not involved in the action of superoxide ion on Hb.

The kinetics of oxidation of Hb by H_2O_2 followed two steps: the first is the oxidation of oxy Hb to ferri Hb and the second is hemichrome formation. This last step is independent of the presence of H_2O_2 since it is not inhibited by catalase. The kinetics of oxidation to ferri Hb were of second order and the rate constant was found to be 16 M^{-1} sec^{-1}.

Key words: Hemoglobin (normal, abnormal, modified), polymerization, cross-links, autoxidation, oxygen radicals.

INTRODUCTION

The reduction of oxygen to water is a fundamental process involving the gain of four electrons, which can be divided into four steps with the sequential formation of the superoxide radical (O_2^-), peroxide ($-O-O-$), the hydroxyl radical ($OH^\cdot$) and water. The term "activated oxygen", therefore, includes all these radicals.

Activated oxygen species and particularly the superoxide radical (O_2^-) are known to be the products of a large number of biological reactions[1,2] such as the oxidation of oxy Hb to met Hb[3]. In this reaction, production of O_2^- is autocatalytic[4]. Since some 3% of the hemoglobin content in erythrocytes is oxidized per day, and reduced again by the methemoglobin reductase system, this constitutes a cycle producing a continuous supply of O_2^-. This implies that any situation increasing the turnover of this cycle

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enhances the production of O_2^{-}[5]. This is the case for unstable hemoglobins. Winterbourn & Carrell[6] have looked for evidence of oxidative processes accompanying unstable hemoglobin precipitation. They have demonstrated that precipitates of normal or unstable hemoglobins were partly constituted of polymers of up to five or six chains. The aim of the present study was to examine the action of activated oxygen species on hemoglobin, in particular on the oxidation process leading to the denaturation of hemoglobin. We have also studied the effect of these species on the cross-linking of the subunits and tried to establish a correlation between oxidation and polymerization.

This study was performed on different forms of normal human hemoglobin A (oxy Hb and cyanmet Hb) on modified Hbs (blocked β SH 93), on some abnormal Hbs (Hb S, Hb C, Hb Créteil and Hb Toulouse) and on isolated chains. In order to make comparisons with the action of activated oxygen species, the autoxidation rates of these hemoglobins have also been determined.

MATERIAL AND METHODS

Preparation of hemoglobins

Venous blood samples were freshly drawn on acid citrate dextrose. The hemolysates were prepared without toluene[7] by centrifugation at 20,000 g and hemoglobin was determined as cyanmet Hb according to Drabkin[7]. Solutions of normal human hemoglobin were purified by column chromatography on DEAE Sephadex in 0.05 M Tris HCl buffer pH 7.85[8]. They were used within 3 days after purification.

Estimation of superoxide dismutase (SOD) was performed according to Puget et al.[9] and showed no contamination of hemoglobin by this enzyme.

Modified hemoglobins were prepared as follows: reaction of β 93 cysteine residues of hemoglobin with iodoacetamide was carried out at pH 7.15 for 3 hours at 25°C with an iodoacetamide/Hb molar ratio of 20[10]. Iodoacetamide (Calbiochem) was freshly recrystallized from petrolum ether. Reaction with cystine was performed under the same conditions.

Reaction of hemoglobin with cystamine was carried out at pH 8.6 for 1 hour at 25°C as described by Wegmann & Gilman[11] using cystamine dihydrochloride (Merck) with a molar ratio cystamine/Hb of 20. The percentage of reacted hemoglobin was checked by electrophoresis on cellulose acetate at pH 8.6. Carboxyamidomethyl Hb A and Hb A cysteine are not separated from Hb A by electrophoresis in contrast to Hb cystamine[12]. To overcome this difficulty, the test was made in two steps. The first step was incubation with iodoacetamide or cystine and in the second step, an aliquot of the modified Hb was incubated with cystamine which reacts with the fraction of Hb A SH free thus distinguishing between modified and non modified hemoglobins. Under our experimental conditions, 100% modified Hbs were obtained.

Cyanmet Hb was prepared as follows: Hb A was oxidized by adding an excess of ferricyanide and an excess of cyanide was then added until the spectrum was that of cyanmet Hb. The excess of ferricyanide and cyanide was removed by separation on a Sephadex G25 column equilibrated with buffer B.

Hb Créteil β 89 (F5) Ser → Asn was purified by column chromatography on DEAE cellulose in 0.05 M Tris HCl buffer pH 8.13[13]. Hb Toulouse β 66 (E10) Lys → Glu was isolated by column chromatography on CM cellulose using a 0.01 M phosphate

buffer at pH 6.8[14]. Hb S β 6 (A3) Glu → Val and Hb C β 6 (A3) Glu → Lys were purified by column chromatograph on DEAE Sephadex according to Huisman & Dozy[8]. Isolated chains were prepared according to Geraci et al.[15].

System for production of activated oxygen species

Photoreduction of riboflavin at 365 nm with NADH as hydrogen donor was used[16]. To 1.6 ml of hemoglobin in the appropriate buffer 0.2 ml of 1 mM riboflavin and 0.2 ml of 2 mM NADH were added (both dissolved in the same buffer as Hb) to give a concentration of 0.1 mM riboflavin, 0.2 mM NADH and 4×10^{-5} M hemoglobin in a final volume of 2 ml. The tube was then irradiated with a Mineralight A 100 (365 nm with filter) at 12 cm distance.

Buffers

Production of the different species of activated oxygen occurs in specific buffers[17].

Buffer A 0.149 M NaCl, 0.0088 M Na_2HPO_4, 0.001 M NaH_2PO_4 is used under argon to produce mainly hydroxyl radicals.

Buffer B 0.049 M NaCl, 0.1 M sodium formate, 0.0088 M Na_2HPO_4, 0.001 M NaH_2PO_4 is used under oxygen to produce mainly superoxide radicals†.

Buffer C 0.049 M NaCl, 0.1 M sodium carbonate, 0.0088 M Na_2HPO_4, 0.001 M NaH_2PO_4 is used under argon to produce mainly carbonate radicals.

Buffer D 0.009 M NaCl, 0.07 M sodium formate, 0.07 M sodium carbonate, 0.0088 M Na_2HPO_4, 0.001 M NaH_2PO_4 is used under oxygen to produce mainly superoxide and carbonate radicals.

All buffers were at pH 7.0.

Determination of molecular weights

Molecular weights of polypeptide chains were determined by polyacrylamide gel electrophoresis in the presence of SDS according to Weber & Osborn[18]. The final concentration was 10% for acrylamide and 1% for SDS in 0.1 M phosphate buffer at pH 7.0. Electrophoresis was carried out for about 4 hours at 16 mA/gel. Protein bands were stained by Coomassie brilliant blue (R. 250) during one night. The staining solution was prepared by dissolving Coomassie blue 0.15% (w/v) in a mixture of methanol (50%), water (40%), acetic acid (7%) and glycerol (3%).

The destaining solution was acetic acid 7.5% in water.

Molecular weights were estimated by comparing mobilities with those of standard proteins: myoglobin, M.W. = 17 000; alcohol dehydrogenase, M.W. = 37 000; ovalbumin, M.W. = 43 000 and bovine serum albumin, M.W. = 68 000. The amount of protein in the band was determined by densitometry at 550 nm using a Gilford 250 spectrophotometer.

Molecular weights of hemoglobin species after irradiation were determined using a Sephadex G 200 column in 0.1 M phosphate buffer.

Methemoglobin formation

The spectra of the samples after irradiation were recorded on a Cary 15 spectrophotometer at 25°C using a 1 cm cell pathlength. Percentages of methemoglobin were determined according to Benesch et al.[19].

Oxidation by hydrogen peroxide

Hemoglobin solutions were mixed with 0.1 M sodium phosphate buffer at pH 6.5 to obtain a concentration in heme of 60 μM. Changes in the absorbance of the mixture were measured at 578 nm after addition of H_2O_2 to a final concentration of 10^{-4} M at 25°C. 60 μg of catalase (Calbiochem) were added at different times during the kinetics to stop the reaction.

The effect of addition of increasing amounts of hydrogen peroxide between 10^{-5} M and 10^{-4} M was also tested.

Autoxidation

The rate of spontaneous oxidation of hemoglobin at atmospheric pressure was studied by a controlled heating of the pure component in a phosphate buffer (pH 6.5) at 37°C for varying periods of time up to 1 h. The percentage of ferri Hb was then determined as described by Benesch et al.[19].

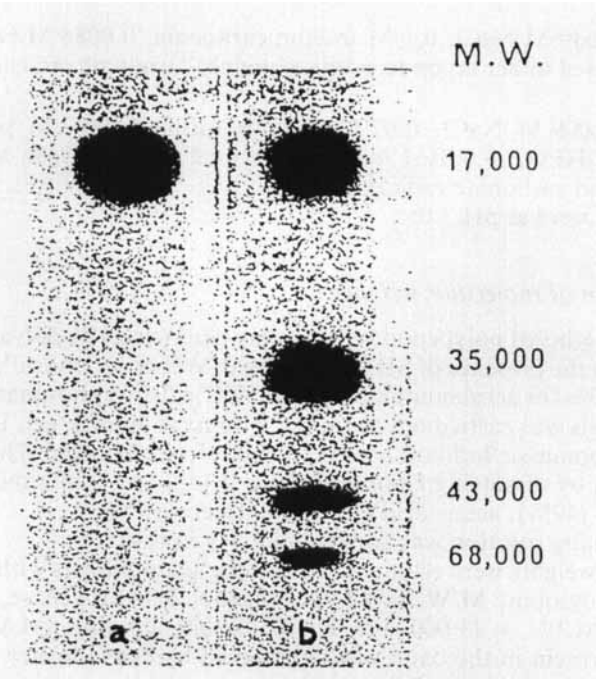

FIGURE 1 Polyacrylamide gel electrophoresis in the presence of SDS stained with Coomassie blue.
a) non treated hemoglobin A.
b) hemoglobin A submitted to the action of superoxide radicals.

RESULTS

Degree of polymerization of hemoglobin submitted to activated oxygen species

Irradiated hemoglobins were examined by polyacrylamide gel electrophoresis in the presence of SDS. Patterns obtained after Coomassie blue staining are shown in Figure 1. Normal hemoglobin (Figure 1a) was dissociated into subunits and ran as a single band with a molecular weight about 17 000. Irradiated hemoglobin gave patterns in which this band was the major component, but a series of bands with increasing molecular weights were also present (Fig. 1b). The relative amounts of the different polymers depended on incubation time (Fig. 2) and the molecular weights of the bands were consistent with their being monomers, dimers, trimers, and tetramers of the polypeptide chains of hemoglobin. The molecular weight of irradiated Hb A was also determined on Sephadex G 200 without SDS. Under these conditions, two peaks were observed, one corresponding to a molecular weight about 70 000 and the other about 120–140 000, This indicates that linkage can occur between tetramers of Hb and not only within the same molecule.

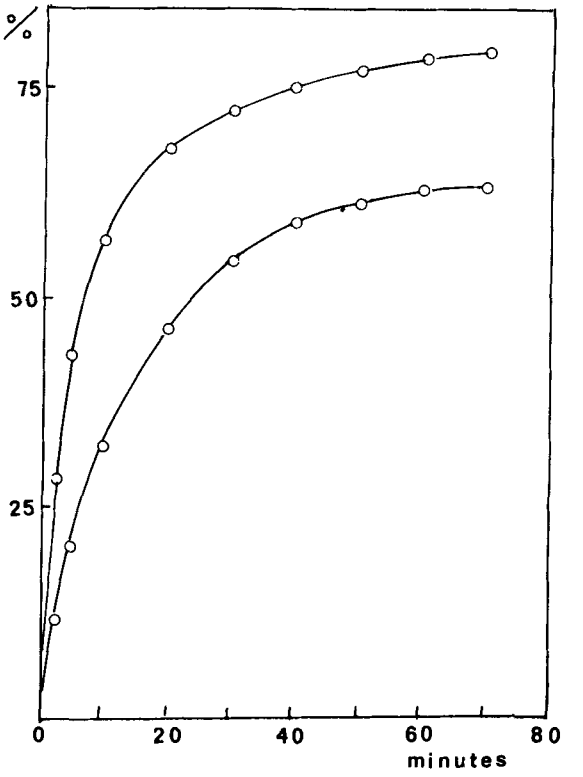

FIGURE 2 Kinetics of oxidation (□—□) and polymerization (O—O) of Hb A by O_2^- (buffer B). Polymers included dimers, trimers and tetramers of chains. Oxidation was measured by spectrophotometry and polymerization by densitometry as indicated in "Methods".

Effect of the different activated oxygen species

Four identical samples of normal human hemoglobin, Hb A, were irradiated for 1 h in the four different buffers in the presence of riboflavin and NADH, as described in "METHODS". Results are presented in Table I. It is clearly shown that hydroxyl radicals gave rise to an important oxidation of hemoglobin but have only a small effect on the polymerization. In contrast superoxide radicals gave rise to significant amounts of both oxidation and polymerization. In view of these results we have chosen buffer B for the following experiments since more important changes occur.

TABLE I
Comparison of the effect of different activated oxygen species on Hb A after 1 h incubation

	Oxidation %	% monomers MW:17 000	% dimers MW:34 000	% trimers MW:43 000	% tetramers MW:68 000
Buffer A $OH^{\cdot}$	75	72	25	3	0
Buffer B $O_2^{-\cdot}$	80	42	34	14	10
Buffer C $CO_3^{-\cdot}$	50	81	17	2	0
Buffer D $CO_3^{-\cdot} + O_2^{-\cdot}$	65	30	32	24	14

Photo-irradiation in D_2O

Since excited singlet oxygen species could play a role in the oxidation of hemoglobin, irradiation in D_2O, which increases the life time of $^1O_2$[20] and hence would increase the rate of oxidation and polymerization, was examined under standard conditions. Singlet oxygen does not appear to play a significant role since protection rather than enhancement was observed: the percentage of polymerization decreased to 40% compared to 60% in presence of O_2^-.

Photo-irradiation of cyanmet Hb

Cyanmet Hb A was tested in the standard system of irradiation. No polymerization was observed. According to the preparative procedure of cyanmet Hb, free cyanide would not be present in the solution. However, we have tested the effect of cyanide on polymerization, and a control oxy Hb A in presence of cyanide showed the same degree of polymerization as without cyanide.

Two hypotheses could explain this results: one is that polymerization requires an oxidation step and follows it. The second is that the conformation of cyanmet Hb does not allow the formation of polymers. In fact cyanmet Hb A is considered as being in an "oxy" distorted state and thus in a conformation identical to that of oxy Hb A[21]. Consequently the first hypothesis is more likely.

TABLE II
Action of superoxide radical on the polymerization of α and β chains

	% monomers	% dimers	% trimers	% tetramers
Hb A	42	34	14	10
α chains	77	20	3	0
β chains	40	42	10	8

Photo-irradiation of isolated chains

To determine whether one of the chains is more sensitive to irradiation than the other, irradiation of isolated native α and β chains was performed. It was not possible to follow the kinetics of oxidation since the chains were oxidized directly to a hemichrome form. However, oxidation does occur since the spectra before and after irradiation are different. The results of the polymerization are shown in Table II. The β chains show the same polymerization as Hb A while α chains give rise to only a small amount of polymers. This is probably because β chains are tetramers whereas α chains are monomers.

Action of thiol blocking agents

The β 93 thiol groups are known to be involved in the oxidation of hemoglobin A only in the presence of Cu^{++}[6]. Therefore we have tested the effect of superoxide radical on Hb A with β SH 93 blocked either by cystamine or by iodoacetamide. There was a small decrease in polymerization of blocked Hbs (50% monomers instead of 42% for Hb A), but the rate of oxidation remained unchanged. This suggests that the β SH 93 groups do not play a significant role neither in the oxidation nor in the polymerization of Hb by superoxide.

It was also of interest to study the autoxidation rates of these blocked hemoglobins to compare the results with those of the action of superoxide rdical. Therefore the autoxidation rates of various blocked β 93 hemoglobins were examined and the results are shown in Fig. 3. Carboxyamidomethyl Hb A has the same autoxidation rate as Hb A in contrast with Hb A — cystamine and Hb A — cysteine which have an increased autoxidation rate. Consequently, the autoxidation rate depends on the blocking reagent. This result would indicate that this reaction is different from the oxidation with superoxide ion for which no difference was observed between Hb A cystamine and carboxyamidomethyl Hb A. However the charge of the reagent is not responsible for the difference since Hb A — cysteine and carboxyamidomethyl Hb A which both have the same pl as Hb A show different autoxidation rates.

Variant hemoglobins

Studies on the effect of activated oxygen species on blocked β SH 93 Hbs have demonstrated that this residue was not implicated. Some abnormal hemoglobins were used to test other residues particularly in the β chain. These hemoglobins were chosen for their modified autoxidation rates, either increased or decreased. Hb S (β 6 Glu → Val)

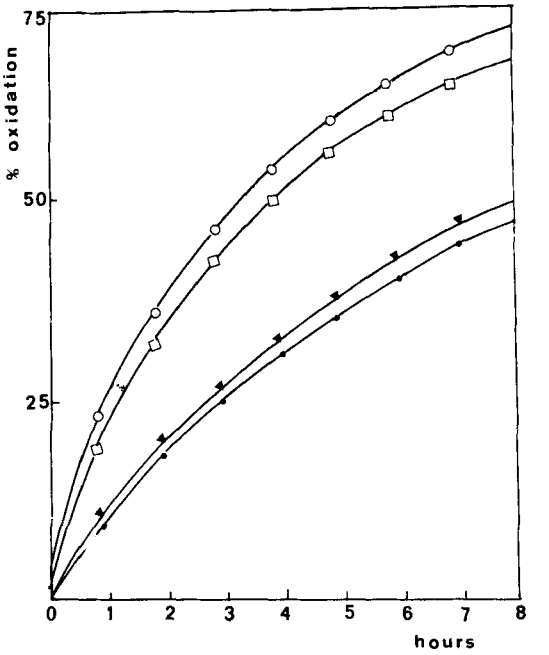

FIGURE 3 Autoxidation rated of Hb A (●—●), carboxyamidomethyl Hb A (△—△), Hb A cystamine (O—O) and Hb A-cysteine (□—□) at 37°C in 0.1 M sodium phosphate buffer pH 6.5.

and Hb C (β 6 Glu → Lys) are mutated at the same site. However their autoxidation rates are very different. Hb S autoxidizes more slowly than Hb A while Hb C has an increased autoxidation rate. It is difficult to imply conformation as an explanation of this difference, since the two hemoglobins are identical in their oxyform. However in view of these results, the β 6 residue seems to be in a critical position for the oxidation of human hemoglobin. Two other hemoglobins were also studied: Hb Créteil β 89 Ser → Asn[13] presented a decreased autoxidation as compared to Hb A. It seems that the oxy conformation is responsible for the decreased autoxidation rate as has been demonstrated for Hb Syracuse[22].

Hb Toulouse β 66 Lys → Glu[23] has a markedly increased autoxidation rate. The mutation is responsible for a modification in the charge inside the heme pocket which induces a more acidic environment for the iron. This explains the increased oxidation rate of Hb Toulouse.

These abnormal hemoglobins were tested for their reaction with O_2^-. Oxidations of Hb S and Hb C by the action of superoxide were similar to that of Hb A while the formation of polymers was slightly decreased in the two cases. In the case of Hb Créteil the polymerization was also reduced in contrast to its oxidation which remains the same as that of Hb A. Polymerization and oxidation of Hb Toulouse were identical to that of Hb A (Table III).

Measurement of the oxidation of hemoglobin by H_2O_2

The kinetics of oxidation of human Hb A by hydrogen peroxide were measured at

TABLE III
Action of superoxide radicals on abnormal hemoglobins

	% oxidation	% monomers	% dimers	% trimers	% tetramers
Hb A	80	42	34	14	10
Hb S	80	55	32	9	4
Hb C	75	60	25	10	5
Hb Créteil	82	53	28	12	7
Hb Toulouse	85	45	32	15	8

578 nm as shown in Fig. 4. The spectrum at the end of the reaction is that of a mixture of methemoglobin and hemichrome. Addition of catalase at the same time as H_2O_2 inhibited completely the reaction. In contrast, if catalase was added a short time after the beginning of the reaction, the oxidation was not stopped. This indicates that a step is independent of the presence of hydrogen peroxide. In view of the spectra, the first step is the oxidation to ferri Hb and the second is hemichrome formation. This step does not require H_2O_2 once the reaction is initiated.

This is the first direct evidence that the formation of hemichrome is independent of the presence of an oxidant.

To investigate the reaction mode of hemoglobin with H_2O_2, the effect of different concentrations of H_2O_2 was studied. When the concentration of H_2O_2 was changed from 50 to 500 μM, the initial rate was dependent on H_2O_2 concentration. The reaction proceeded as a bimolecular process with respect to Hb and H_2O_2. The rate constant was calculated to be 16 M^{-1} sec^{-1} for Hb A.

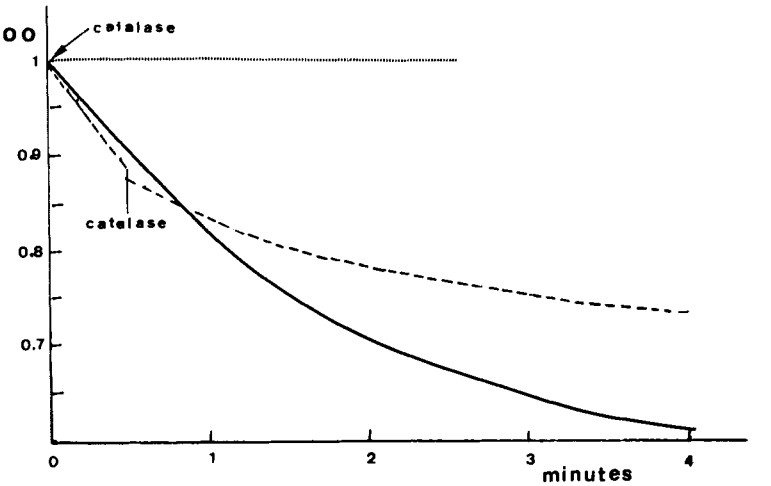

FIGURE 4 Kinetics of oxidation of Hb A by hydrogen peroxide (100 μM). Experiments were carried out in the absence of catalase (——) or in the presence of catalase at the beginning of the reaction (. . . . .) and 30 sec. after the beginning of the reaction (-----). Changes in absorbance at 578 nm.

DISCUSSION

Photoreduction of riboflavin at 365 nm in the presence of a hydrogen donor has been extensively used as a source of O_2^- resulting from the one electron reduction of oxygen by reduced flavin. It is also possible to produce other radicals such as CO_3^- and $^{\cdot}OH$ by changing the ionic composition of the buffer. We have prefered this system to $^{\cdot}\gamma$ irradiation of the samples since it is easier. Both systems give the same results, 45 mn of γ irradiation (600 rads/min) leading to the same polymerization and oxidation as 1 h. of photoreduction. The effects of radicals produced by the photoreducing system were studied on human hemoglobin. All the radicals produced appeared to oxidize normal hemoglobin but not in equal amounts. The plateau is reached in about 30 mn in all cases but the maximum of oxidation varies between 50 and 80% according to the radical used (Table I). The system generating mainly superoxide ion gives most oxidation and for this reason has been used preferentially. To determine whether certain amino acid residues are directly involved in the mechanism of oxidation we have used modified and variant hemoglobins. The modified Hbs were blocked at β SH 93 group with cystamine or iodoacetamide. In both cases the maximum of oxidation was identical to that of Hb A. It is possible that in the case of Hb-cystamine, the blocking agent was removed during irradiation since the linkage is not covalent and this would explain the similar results for Hb A and Hb A cystamine. However iodoacetamide is irreversibly bound to Hb and the same result being obtained for both modified hemoglobins it can be concluded that the β 93 cysteinyl residues do not play a significant role in oxidation of hemoglobin by superoxide ion. This result is in accord with that obtained by Winterbourn & Carrel[6]. Measurement of the reactivity of cysteine β 93 after the action of O_2^- on hemoglobin, showed no oxidation of these residues.

Some β variant hemoglobins were also tested since the β chain was shown to be more sensitive to oxidation by O_2^- than α chains (Table II). In all cases the rate and the maximum of oxidation were identical with that of Hb A. This also indicates that the substituted residues in abnormal hemoglobins are not implicated in the oxidation mechanism by radicals. These hemoglobins were chosen because of their increased or decreased autoxidation properties. Modified autoxidation rates of an abnormal hemoglobin can be explained in different ways. The most general case is that of abnormal hemoglobins with the mutation inside the heme pocket. In this case it is generally admitted that the increased autoxidation rate is induced by a perturbation of the hydrophobic environment of the heme[24]. Hb Toulouse, for example, belongs to this group. However in some cases, such an explanation is not possible. Certain hemoglobins have a high oxygen affinity for which it has been postulated that the oxy conformation was responsible for the decreased autoxidation rates[22]. Hb Créteil belongs to this group. It is more difficult to explain the results obtained for Hb S and Hb C. The β 6 position is far from the heme and at the surface of the molecule and moreover the two hemoglobins exhibit very different autoxidation rates, one being increased (Hb C) and the other decreased (Hb S). Another mechanism could imply a facilitated electron transfer from the iron to unknown amino acid residues in the case of an increased oxidation rate or an inhibited electron transfer in the case of a decreased oxidation rate. In fact we have observed important differences of autoxidation rates of blocked or abnormal hemoglobins as compared with Hb A but they do not correspond to differences in the action of superoxide radicals. This is a first indication of different mechanisms for autoxidation and oxidation by radicals. It

seems that autoxidation is very sensitive to variations in the protein environment of the heme whereas radicals act directly on heme iron without intermediate oxidation.

Oxidation of heme iron is not the only phenomenon induced by free radicals since cross-linkage of hemoglobin subunits is also observed. Kinetic studies show that the two processes, polymerization and oxidation, are concomitant. The oxidation step is apparently a prerequisite for polymerization, since in the case of cyanmethemoglobin (no oxidation) there is no polymerization. Like oxidation, polymerization of blocked β SH 93 hemoglobins was the same as that of normal hemoglobin. Consequently the β SH 93 groups are not involved either in oxidation or in polymerization induced by free-radicals. To confirm this finding, structural studies are currently in progress to determine the amino acid residues implicated in the cross-link of the subunits. Preliminary experiments have shown that the β SH 93 groups are not engaged in the cross-link. In their study of hemoglobin oxidation by superoxide ion, Winterbourn et al.[25] did not examine polymerization of hemoglobin subunits. However they have shown[6] that precipitates of unstable hemoglobins are cross-linked and that this cross-link was partly due to β SH 93 groups. This suggests that the process of polymerization induced by superoxide radicals is different from that involved in precipitates of unstable hemoglobins. Two other distinctions can be made between the two processes.

1. Polymers remains soluble for oxidation by radicals whereas they are only found in the precipitated form of unstable hemoglobins.
2. It is generally admitted that unstable hemoglobins pass by hemichrome before precipitation and precipitate as this form. In contrast, we have observed no formation of hemichrome during oxidation of normal hemoglobin by radicals.

It appears that human hemoglobin can be oxidized in different ways which may interact. Oxidation by hydrogen peroxide proceeds in two steps, the first oxidation to ferri Hb and the second hemichrome formation, this last being independent of H_2O_2 once the reaction is initiated. This process is different from that observed for isolated subunits. The chains are directly oxidized to the hemichrome state[26] whereas Hb A is first oxidized to the ferri form. This difference is also observed on oxidation by superoxide ion, the isolated chains being directly oxidized to the hemichrome form. This indicates that association of α and β chains to form tetramers acts as a protective mechanism against the attack of oxidants present in red blood cells such as hydrogen peroxide or superoxide ion, a protection which is deficient in diseases such as β-thalassemia.

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