The Globin-based Free Radical of Ferryl Hemoglobin Is Detected in Normal Human Blood*

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The reaction between heme proteins and peroxides is known to proceed via formation of two intermediates, a ferryl heme iron, Fe(IV)=O, detected by optical spectroscopy, and a globin-associated free radical Hb(Fe(IV)=O), detected by EPR1 spectroscopy (1–21). These transient species have been implicated in the mechanism of oxidative stress associated with human diseases. In fact, formation of a ferryl form of the heme protein was demonstrated for either ferrous or ferric heme-mediated oxidation of a wide range of biologically important substrates in the presence of lipid hydroperoxides or H₂O₂. These substrates include liposomes (22, 23), low density lipoprotein (24, 25), cholesterol (26), salicylate (26), styrene (27), olefins (28), unsaturated fatty acids (16, 29), and the drug chlorpromazine (13, 30). It is not clear whether the ferryl iron or the free radical plays the critical role in the oxidation. Ferryl species have been demonstrated in rat heart after a period of induced ischemia (31) and in intact red blood cells (10). However, no globin-centered free radicals have been found so far in living systems. It should be noted, however, that a kind of free radical EPR signal has been detected in whole blood (32, 33). This appears unexceptional, since most biological tissues are characterized by a free radical EPR signal (32–38). The problem with interpretation of the signal in blood was in the fact that radicals in most tissues are generally thought to be associated with the respiratory chain in mitochondria (32, 33, 35, 38). There are, however, very few mitochondria in blood, and the nature of the free radicals observed in this tissue is a matter of controversy (32, 33).

Although pro-oxidant activity of the ferryl form of heme and/or of the globin radicals has been discussed for a long time, the actual relevance of the heme protein reaction with peroxides in vivo is not established. The aim of this study was to apply the method of EPR spectroscopy to whole human blood in order to enquire whether those free radicals observed in blood (32, 33) are the radicals formed through the reaction of hemoglobin with hydrogen peroxide. We show in this study that the free radical EPR signal in purified methemoglobin (metHb) oxidized by H₂O₂ and that in frozen human blood are identical.

Furthermore, we show that the intensity of the free radical EPR signal in blood measured at −196 °C varies by a factor of 5 for a series of samples made by freezing from the same blood sample kept in vitro at room temperature. This variation of free radical concentration is clearly not an instrumental error, since the concentration of the Cu(II) ions of ceruloplasmin, measured from another signal within the same EPR spectrum, is bound by only a 5% variation. The effect of “fluctuation” of the concentration of free radicals in frozen blood was found to be associated with reverse fluctuations in the concentration of metHb in the same frozen samples. These opposing fluctuations in metHb and free radical concentrations in frozen blood can be explained in terms of the interaction of metHb with H₂O₂.

Therefore, we conclude that the reaction of metHb with endogenous H₂O₂ takes place in whole blood. We consider this an important issue, since the intermediates of the reaction, namely the globin-based free radical and/or the ferryl form of hemoglobin are shown to be potentially harmful pro-oxidants (13, 16, 22–30).

The methodological conclusion drawn from our results is that freezing biological tissues in liquid nitrogen for EPR measurements is not always as free of drawbacks as generally thought.
The method of freezing has gradually become conventional in the EPR studies of biological systems (32, 33, 37, 39–44), since it has replaced the method of sample lyophilization extensively used before. It was shown that lyophilization, i.e., drying biological tissue in vacuum, results in poorly reproducible EPR spectra (35, 36, 45). Freezing samples has never been reported to have such a drawback. To our knowledge, this study gives the first example of freezing resulting in low reproducibility of the concentration of paramagnetic centers fixed in biological tissue.

**MATERIALS AND METHODS**

**EPR Sample Preparation**—Venous blood was donated by consenting adults and collected under air in glass tubes containing a small amount of sodium citrate used as an anticoagulant. Blood without any anticoagulant was also tested over a limited time scale (1 min) following donation and showed no difference with respect to sodium citrate-treated blood. Dilution by anticoagulant was taken into account in concentration measurements. In some experiments, red blood cells were separated from plasma by centrifugation, 2000 rpm, 20 min.

Human metHb was prepared, and its concentration was determined (in terms of heme iron) as described elsewhere (21). The level of catalase contamination in the preparations (21) was found to be less than 10^{-11} M. The concentration of H_{2}O_{2} (Signal) was calculated by the addition of catalase (4000 units/ml) to solutions in an oxygen electrode and measuring the amount of O_{2} evolved, assuming a stoichiometry of 2O_{2} + 2H_{2}O_{2} → 4H_{2}O + O_{2}.

EPR samples of blood, metHb/H_{2}O_{2} mixtures, and blank samples (distilled water or phosphate buffer) were made singly or in duplicate using syringes and plastic tubes as described elsewhere (21). Either fast (−10 s) or slow (−60 s) freezing of the samples in liquid nitrogen was used and resulted in no difference in the effects observed. (The rate of freezing was varied by the rate of immersion of the plastic tubes with samples into the liquid nitrogen.)

**EPR Spectra Measurement**—The EPR spectra of frozen blood were measured on a Bruker ER 220D EPR spectrometer equipped with an Aspect 2000 data system. An ER 4102 ST Universal X-Band Resonator (TE102 mode) was used. The g-factors of EPR signals were determined by reference to the external magnetic field measured by a Bruker ER 035 Gaussmeter and of the microwave frequency measured by a Systron Donner 6235A frequency counter. Kinetic measurements in the metHb/H_{2}O_{2} system were performed using a Varian E109 X-band spectrometer equipped with an Archimedes 440 computer. All EPR measurements were performed at −196°C using a quartz finger Dewar containing a frozen sample immersed in liquid nitrogen. A sample was fixed inside the finger with a glass rod in order to hold it down and avoid sample dancing due to liquid nitrogen boiling. The sample was long enough that the bottom of the rod was well above the upper edge of the working zone of the microwave. The absence of the liquid nitrogen was a necessary condition for preventing overheating, but it was controlled by making the bubbles small and frequent with proper fixing of the sample with the rod. The microwave power was kept at a 200-milliwatt instrumental setting unless stated otherwise. Modulation frequency was 100 kHz. The time constant was 0.1 s. The EPR spectra of the blanks were subtracted from the spectra of blood and the metHb/H_{2}O_{2} mixtures. Such a subtraction excluded all background signals, such as any signals of the quartz Dewar or the cavity or the broad signal caused by oxygen dissolved in liquid nitrogen.

The EPR signal intensities in blood were measured against fixed standard signals using the D subroutine (dual consideration) of the EP routine (EPR program of Aspect 2000). An EPR spectrum of a blood sample covering both g = 6 and g = 4.3 signals (H_{6} = 1350 G; ΔH = 1000 G) was accumulated at NS = 20. This spectrum was considered as the standard for the metHb and Tf signal determination in a series of blood samples. The EPR spectra of every sample in the series (experiments) were measured at the same conditions but at a lower NS (NS = 2), such a restriction resulting from a large number of samples in a series. A difference spectrum, (experimental spectrum) − k (standard spectrum), was constructed for every sample using the D subroutine. The higher NS in the standard spectrum minimizes the signal/noise ratio in the resultant difference spectrum. The factor k was then varied. The value of k, at which the g = 4.3 signal completely disappeared from the difference spectrum, leaving the g = 6 line only, was taken as a measure of the g = 4.3 signal intensity in the sample. Similarly, the value of k, when the g = 6 signal disappeared, leaving only the g = 4.3 signal, was taken as the g = 6 signal intensity. (If an experimental spectrum had by chance the same partial contribution of g = 6 and g = 4.3 signals as the standard spectrum, then both signals disappeared, of course, at the same value of k, so that the difference spectrum was transformed to a flat line.) An identical procedure was used in determination of the ceruloplasmin (Cp) and free radical signal intensities, with the difference that the spectra were measured at H_{6} = 3300 G and ΔH = 200 G in order to cover the g = 2.05 and g = 2.005 signals.

In the purified metHb experiments the EPR signals of metHb and free radicals were not contaminated with any other EPR lines (after subtraction of the constant base line). Therefore, the D subroutine was not necessary, and the simple peak-to-trough measurement of the signal intensities was appropriate.

Finally, the intensities of the blood EPR signals (g = 6 and g = 2) expressed in units of standard signals (the values of k when a blood sample was used as a standard) were related to the intensities of the signals in the purified metHb/H_{2}O_{2} system expressed in the peak-to-trough units (nm). This was done by using a sample from the metHb/H_{2}O_{2} series as a standard (with signal intensities already measured in nm) when applying the D subroutine to a blood sample (with already known k values). Therefore, a factor transforming k values in the blood series to the nm units used for signal intensities in the metHb/H_{2}O_{2} series was found. This allowed us to determine the concentration of the high spin metHb (in terms of heme iron) and of the free radicals in the frozen samples of blood on the basis of the quantitation procedure developed for the metHb/H_{2}O_{2} system (20). The legitimacy of the procedure for blood was verified by the fact that the line shapes and saturation behavior of the signals were the same in blood and purified metHb.

**Annealing**—The frozen samples were annealed in a unit comprising a transfer Dewar, a nitrogen evaporator, and a temperature controller. After annealing, the samples were recooled to −196°C. The EPR spectra were always measured at this temperature.

**RESULTS**

**EPR Spectrum of Blood at −196°C**—A typical EPR spectrum of normal human blood measured at −196 °C is shown in Fig. 1. This spectrum consists of the signals of at least four different paramagnetic centers. The signal with g = 6 is caused by heme Fe(III) ions in the high spin state (2, 32, 33, 47, 48) and is assigned to metHb. The EPR signal with g = 4.3 is assigned to transferrin Fe(III) ions complexed by HCO_{3} ions (32, 33, 48, 49). The signal with g = 2.05 is the perpendicular component of the anisotropic EPR signal of Cu(II) ions (50). This signal is due to Cp, the major copper-containing protein found in blood (48, 51). A slightly asymmetric singlet with a g-factor of g = 2.005 and the peak-to-trough width of 17 G is observed in the high field.
in terms of heme concentration of high spin metHb and of the radical \( g \) much greater than that of the Cp signal \( g \) around some average values. Fig. 2 shows the data for \( g = 6 \) (A) and for \( g = 2.005 \) (B) plotted together in terms of concentration of high spin metHb and the free radicals, respectively. The variations in metHb and free radical concentration are not time-dependent oscillations in liquid blood but are statistical fluctuations observed in frozen samples.

Plasma does not exhibit any free radical EPR signal (data not shown).

**Fluctuation of metHb and Free Radical Concentration in Frozen Blood**—In a series of samples taken from blood exposed to air in vitro, we have found that the EPR signal intensity of the free radicals and of metHb varies from sample to sample around some average values. Fig. 2A shows the data for the \( g = 6 \) and \( g = 4.3 \) signals (see frame A in Fig. 1) measured for a number of EPR samples consecutively taken from the same liquid blood sample and frozen at various time following donation. For the same set of samples, the intensities of the \( g = 2.005 \) and \( g = 2.05 \) signals were also measured (Fig. 2B). A significantly greater variation in the EPR signal of metHb \( g = 6 \) is seen compared with that of the Tf signal \( g = 4.3 \). Similarly, the variation of the free radical EPR signal \( g = 2.005 \) is much greater than that of the Cp signal \( g = 2.05 \) measured from the same spectrum and with the same absolute error (see Fig. 1B). We used a formalism described in details elsewhere (20) to express the intensities of the \( g = 6 \) and \( g = 2.005 \) signals in terms of heme concentration of high spin metHb and of the free radicals, respectively (Fig. 2C). The average concentration of metHb is approximately 1 order of magnitude greater than the average concentration of the free radicals. When the data for free radicals are magnified by a factor of 8, it is clearly seen that the variations in concentration of these two centers, metHb and radicals, are not independent; they are inversely related, i.e. in a sample with a greater metHb concentration than its mean value, the concentration of free radicals is found to be lower than its mean value (Fig. 2C). Similar variations in metHb and free radical concentrations were found for duplicate samples taken from blood and frozen at the same moment. In fact, any two samples taken randomly from a set of either consecutively or simultaneously frozen samples, which had significantly different metHb concentrations, displayed a difference in the concentrations of free radicals, the higher concentration of metHb being in the sample with the lower concentration of free radicals and vice versa. Moreover, we found that in some cases the concentration of metHb (and of the free radicals) is significantly different in the opposite ends of the same sample, with a higher metHb concentration associated with a lower concentration of the free radicals and vice versa (results not shown).

Thus, these variations of concentration of metHb and free radicals are not time-dependent oscillations in liquid blood but are statistical fluctuations around mean values observed in frozen samples. We therefore conclude that the fluctuations in metHb and free radical concentration are linked and are caused by sample freezing. The effect of fluctuations was observed independently of whether the blood samples were frozen in liquid nitrogen quickly (10 s) or slowly (1 min). The amplitude of the fluctuations in metHb and free radical concentration was found to decrease with time of blood exposure to air. In a 5-h experiment the fluctuations were detectable during the first 3 h after donation, their amplitude becoming indistinguishable from the instrumental error after 4 h (results not shown). After the fluctuations stopped, the radical concentration became approximately equal to the lower limit of the range within which the concentration previously fluctuated. It should be noted that dark venous blood turned bright pink by 4 h of exposure to air, indicating a significant increase in oxygenation.

**Effect of Annealing a Blood Sample**—Freezing a sample of blood at \(-196 \) °C stops the reactions in which metHb and free radicals were involved when the blood was liquid. Such reactions, or some of them, may resume upon heating of the sample at successively increasing temperatures. This procedure is known as stepwise annealing and is widely used in radiation chemistry (39, 41, 52, 53). The result of the annealing experiment is shown in Fig. 3. A frozen sample of blood was annealed, first, at \(-60 \) °C for 30 min. During this period, the sample was repeatedly (at 5, 10, and 20 min) taken from the annealing unit for EPR measurement at \(-196 \) °C and then placed back into the unit for proceeding with the annealing at \(-60 \) °C. The last EPR measurement was made after 30 min of annealing. Then the temperature in the annealing unit was increased to \(-50 \) °C, and a new step of annealing was performed in the same manner. The temperature was successively increased in such a way by 10 °C centigrade steps up to \(-20 \) °C. Then the sample was recooled in a similar way at \(-40 \) °C (the second incubation at \(-40 \) °C, Fig. 3), and the second annealing at \(-20 \) °C followed. The recooling at \(-40 \) °C was performed twice more (the third and the fourth incubation at \(-40 \) °C).

The annealing experiment shows that concentration of metHb in a frozen sample of blood increases with time if the sample is stored at a temperature above \(-40 \) °C. This increase is due to hemoglobin autoxidation. The rate of this process is
strongly dependent on the temperature being highest at $-10^\circ$C, when only 5-min storage was sufficient for a 30-fold increase in metHb concentration compared to the initial value before annealing (Fig. 3). The free radical concentration rises to a maximum and then decreases as the annealing temperature is increased, the maximum concentration being reached at $-40^\circ$C. Repeated recooling of the sample to $-40^\circ$C after annealing at $-20^\circ$C showed significant increase in the free radical concentration with a concomitant decrease in the upward trend of the metHb concentration. For example, when the concentration of metHb is decreased by 3.9 $\mu$M during e.g. second incubation at $-40^\circ$C the increase in the free radical concentration is 0.3 $\mu$M, i.e. $-8\%$ (Fig. 3).

The following conclusions can be drawn from the annealing experiment. Two processes are initiated in a frozen sample of blood in the temperature range between $-60^\circ$C and $-10^\circ$C. The first is Hb autoxidation, which leads to the increase of metHb concentration. The second is a process in which the free radicals are formed while metHb concentration decreases, i.e. the concentration of metHb and radical are inversely related. The time courses depicted in Fig. 3 are therefore complex because of a superposition of these two processes with different temperature dependences.

**Modeling of the Blood Free Radicals**—The radiospectroscopic properties of the free radical signal in blood (the g-factor, signal width, and saturation curve) are similar to those reported for the free radicals observed at $-196^\circ$C in the model reaction of purified metHb with hydrogen peroxide (2, 3, 5, 6, 20, 21). To check the suggestion that the free radicals in the two systems are the same, we have compared the EPR spectra, measured at the same instrumental conditions, of the blood free radicals and of the radicals formed during H$_2$O$_2$-mediated oxidation of metHb. The result of this comparison is shown in Fig. 4.

The fact that the free radical signal increases upon annealing the blood sample at $-40^\circ$C (Fig. 3) was used to obtain an intense signal of free radicals in blood. Two samples of blood, one giving a strong and the other a weak free radical signal were chosen. The sample with the strong signal was then annealed at $-40^\circ$C to make its intensity even stronger (Fig. 4A). The subtraction of the two spectra (the factor of subtraction was chosen 0.76) eliminated the background signal of ceruloplasmin ($g = 2.05$), leaving a pure singlet of the free radicals in blood (Fig. 4C). Free radicals in the purified metHb system were obtained by the addition of H$_2$O$_2$ to metHb (Fig. 4D). The line $g = 2.03$ is due to the parallel component of the EPR signal of peroxyl radicals formed in the reaction (20, 21). The EPR signals of blood free radicals and metHb free radicals are superimposable (Fig. 4E). The saturation curves of the signals in the two systems are also similar (Fig. 5). We suggest therefore that the free radicals formed in blood and in purified metHb after the H$_2$O$_2$ addition are the same.

**Kinetic Behavior of metHb and Free Radical in the Model Reaction**—The concentrations of high spin metHb and free radicals were monitored during the course of the reaction between metHb and H$_2$O$_2$ (Fig. 6). Three different initial molar ratios metHb/H$_2$O$_2$ (1:1, 1:3, and 1:10) were used. It can be seen in Fig. 6 that there are no fluctuations in the free radical and metHb concentrations in the model reaction, since values for duplicate samples were always very close ($<5\%$). Within 15 s after mixing, the concentration of metHb decreased by $-75\%$.
due to oxidation to the ferryl species (20, 21). Simultaneous to this change the radicals were formed (Fig. 6). We shall refer to this fast process as stage 1. There follows a period of relatively slow change in the concentration of both species (stage 2), which can turn into virtual steady state in the case of a significant initial excess of $\text{H}_2\text{O}_2$ over metHb (20, 21). The duration of this period is proportional to the initial $\text{H}_2\text{O}_2$ concentration and corresponds to the time taken for full $\text{H}_2\text{O}_2$ decomposition (Fig. 6). The concentration of $\text{H}_2\text{O}_2$ was monitored in parallel experiments by measurement of O$_2$ evolution after the addition of catalase to the reaction mixture at different times. The time taken for complete consumption of $\text{H}_2\text{O}_2$ measured in this way corresponded to the duration of stage 2 measured by EPR spectroscopy (results not shown). Upon complete exhaustion of $\text{H}_2\text{O}_2$, the radical began to decay, and the metHb concentration began to increase (stage 3). This decay of the free radicals is a first order reaction (20) and is believed to be an autoreduction process involving modification of the heme protein (2).

It should be emphasized that the observed concentration of free radicals is low ($\leq 10\%$) compared with the amount of metHb transformed to the ferryl form (this is considered to be a characteristic feature of the reaction of metHb (or metMb) with $\text{H}_2\text{O}_2$) (2–4, 17). Also, the mean concentration of the radicals in stage 2, i.e., during the steady state or the period of slow change, decreases with increasing initial $\text{H}_2\text{O}_2$ concentration (Fig. 6).

**DISCUSSION**

The technique of freezing biological samples in liquid nitrogen for EPR measurements has become conventional. An advantage of the technique, as compared with the alternative method of sample lyophilization, is good reproducibility of results. This study, however, shows that when aliquots from the same blood sample are frozen ($\approx 196^\circ \text{C}$), significant variations in EPR signal intensities of metHb and free radicals are found, while signals for Tf and Cp are reproducible (Fig. 2). It follows, therefore, that freezing blood in liquid nitrogen does not fix free radicals and metHb in the concentrations present in liquid blood prior to freezing but instead results in fluctuating concentrations of these centers. The fact that the concentrations of metHb and free radicals in blood are inversely related (Figs. 2 and 3) suggests that these two species are involved either in a single redox reaction or in a system of interrelated redox reactions.

It was shown as early as 1956 that free radicals are formed when metHb reacts with hydrogen peroxide (1). The reaction is fast (within seconds) and results in metHb oxidation at two sites; heme is oxidized to the ferryl form Fe(IV)$^5\text{O}$, and a free radical is formed on the globin $\text{Hb}-(\text{Fe(IV)}^5\text{O})$. The nature of the protein-based radical is still under discussion. Formation of free radicals was demonstrated for $\text{H}_2\text{O}_2$ oxidation of both metHb and metMb using fast freezing techniques (2–4, 6, 16, 17, 20, 21) as well as in the liquid phase either directly (5, 7, 9, 12, 15) or by spin trapping (11, 14–16, 18). Many investigators agree that at least two different kinds of radicals are formed on the protein (12, 16–18, 20). Formation of peroxyl radicals has been proven for both metHb and metMb treated with $\text{H}_2\text{O}_2$ (16), this kind of radical constituted only a fraction of the total concentration of radicals in the metHb system (20). The peroxyl radical in metMb was recently shown to be derived from oxygen addition to the Trp-14 radical (18, 19), although there is also evidence...
that tyrosine radicals are formed with metMb (7–9, 12) and metHb (15).

Our study provides strong evidence that the free radicals observed in frozen blood samples and the free radicals resulting from oxidation of purified metHb by hydrogen peroxide are the same. The parameters of the EPR signals of blood free radicals and the globin-based radicals coincide (Fig. 4). Both EPR signals have similar saturation curves (Fig. 5). The fluctuations in metHb and the free radical concentrations in frozen blood samples are inverse (Fig. 2C), implying that an excess of free radicals is formed when the metHb drops, as is the case in the reaction of purified metHb with H$_2$O$_2$ (Fig. 6).

The globin-centered free radicals decay rapidly in model systems (1–5, 15). On the other hand, it is possible to observe similar radicals in blood at a virtually constant (within the fluctuations) level during several hours. We propose that the reactions shown in Scheme 1 are responsible for the observed concentrations of metHb and the globin-centered free radical in blood. (For clarity of presentation, the reactions in the scheme are not balanced and indicate only the pathways of the species interaction and transformation.)

Generally, the balance between Hb and metHb can be described in terms of two reactions, Hb autoxidation to metHb (reaction 1) and metHb reduction by enzyme methemoglobin reductase (reaction 2). Autoxidation of Hb is associated with formation of the superoxide anion radical O$_2^-$ (reaction 1), which either dissociates from heme in oxyhemoglobin after intramolecular electron transfer from Fe(II) to oxygen (54) or can be formed, as has been shown for myoglobin (55), in a bimolecular reaction between molecular oxygen and a weakly bound complex between water and deoxyhemoglobin. It has been shown recently that superoxide production correlates well with the known bell-shaped dependence of Hb autoxidation on oxygen partial pressure (56). Superoxide radicals dismutate, producing hydrogen peroxide (reaction 3). The steady state concentration of H$_2$O$_2$ in bovine red blood cell under physiological conditions, i.e. in the presence of H$_2$O$_2$ decomposing enzymes catalase and glutathione peroxidase, has been reported to be 2 $\times$ 10$^{-16}$ M (57). Kinetic comparison of H$_2$O$_2$ production and oxyhemoglobin autoxidation indicated that reactions 1 and 3 probably represent the main route of H$_2$O$_2$ generation in red blood cells (57).

Once formed, we suggest, hydrogen peroxide in blood reacts with different forms of hemoglobin in the ways known from biochemical model studies. Our data on the kinetic behavior of metHb and free radicals in the model reactions (Fig. 6) show that the free radical concentration in stage 2, i.e. in the stage of slow changing/steady state concentration, is greater when the molar excess of H$_2$O$_2$ is lower. This can only be understood if both the rate of production and the rate of decay of the radical are H$_2$O$_2$ concentration-dependent (reactions 4 and 5, respectively, of Scheme 1) and the decay process is favored at a high excess of H$_2$O$_2$. We have attempted to simulate the time courses in Fig. 6, employing a mechanism similar to that shown in Scheme 1 except that it incorporates the reactions between metHb and H$_2$O$_2$ only, and the ferrous state was omitted. This simulation does not perfectly fit the experimental data, especially in the initial stage of the reaction (first 2 min). However, it does reproduce several essential features, namely 1) relatively low radical yield compared with initial protein concentration (often reported in the literature (2–4, 17)); 2) the presence of the steady state/slow changing stage followed by free radical decay and ferryl reduction to the metHb; 3) longer steady state at higher initial H$_2$O$_2$ concentration; 4) the lower concentration of the free radical in the steady state at higher initial [H$_2$O$_2$]. We were only able to simulate these important characteristics seen in Fig. 6 by assuming a [H$_2$O$_2$]-dependent decay pathway for the radicals. No steady state could be achieved if only two reactions, a very fast radical formation (stage 1 is less than 15 s; see Fig. 6) and relatively slow radical decay (t$_{1/2}$ = 8–10 min in stage 3; Fig. 6) were used.

Hydrogen peroxide can also react directly with deoxyhemoglobin (10, 54), resulting in the ferryl form (reaction 6), which either comproportionates with oxyhemoglobin (10) (reaction 7) or undergoes reduction to metHb via a first order reaction (reaction 8) (21, 58). The autocatalysis of the free radicals, which causes radical decay after H$_2$O$_2$ is exhausted (Fig. 6, stage 3) is depicted in Scheme 1 as a first order reaction (reaction 9).

For blood in vitro, reactions 1–9 lead to a steady state concentration of every species shown in Scheme 1, or rather a quasi-steady state, because the concentrations change due to slow oxygenation of blood (during hours). Reaction 1 slows upon increasing oxygen concentration in blood (54–56, 58), while reaction 2 changes little (presumably). Thus, oxygenation results in a gradual decrease in the mean metHb concentration (Fig. 2). Nevertheless, one may consider that within a short time interval (~10 min) reactions 1–9 generate a steady state concentration of every species. We propose that freezing blood perturbs this steady state (see below) and that the extent of this perturbation is different for every sample frozen, causing fluctuations in the concentration of metHb and free radicals observed at $-196^\circ C$.

In contrast to blood, we found no fluctuations in metHb and free radical concentrations in the frozen samples of reaction mixture containing purified metHb and H$_2$O$_2$. To account for this we propose that it is the concentration of H$_2$O$_2$ that fluctuates in blood as a result of freezing in liquid nitrogen. We suggest that the process of freezing blood creates nonequilibrium high concentrations of H$_2$O$_2$ as a result of intensified dismutation of superoxide radicals due to phase transitions. A very similar effect of intensified disproportionation of ascorbic acid radicals upon freezing solutions of ascorbic acid in water is documented (39). When ascorbic acid is autoxidized in water, ascorbic acid free radicals may easily be observed by EPR spectroscopy. If, however, such a solution is frozen in liquid nitrogen (~15 s), ascorbic acid radicals disproportionate, and no EPR signal can be detected in the frozen sample. Alternatively, if autoxidation proceeds in a water-glycerol (1:1) solution, ascorbic acid free radicals can be detected at $-196^\circ C$ as well as at room temperature because cooling of such a solution down to $-196^\circ C$ does not result in phase separation, and the radicals do not vanish due to intensified disproportionation.

![Scheme 1: Globin-based Free Radical in Human Blood](image-url)
Therefore, we suggest that O$_2^\cdot$ behaves similarly, being effectively concentrated at the phase separation surfaces as blood freezes. High local concentration causes enhanced disproportionation and formation of a relatively high concentration of H$_2$O$_2$. The macroscopic distribution of phase separation surfaces in the sample volume, and their propagation within the time taken for complete freezing can never be the same in different samples. Therefore, the concentration of H$_2$O$_2$ fixed eventually in a blood sample will fluctuate. The higher the O$_2$ concentration before freezing, the wider will be the range of possible H$_2$O$_2$ concentrations and, consequently, the wider the range of the metHb and free radical fluctuation observed.

This mechanism also allows us to explain why the fluctuations decrease with time. The hemoglobin/myoglobin autoxidation rate decreases upon increasing O$_2$ pressure, indicating that the deoxy heme fraction of the protein facilitates the process of oxidation (54–56). Blood oxygenation, therefore, results in a decrease in the steady state concentration of metHb (reaction 1 slows compared with reaction 2; see Scheme 1), which can be seen as gradual decrease of mean intensity of the g = 6 signal (Fig. 2A). Since one molecule of superoxide radical is formed per one ferrous heme oxidized to the ferric form (55), superoxide production during Hb autoxidation in blood (reaction 1) also decreases at a higher blood oxygenation level (56). Four hours of blood oxygenation in our experiment resulted therefore in such low O$_2^\cdot$ concentrations in liquid blood that freezing did not cause significant additional H$_2$O$_2$ formation. Under such conditions, the H$_2$O$_2$ concentration does not fluctuate significantly in frozen samples, therefore causing no detectable fluctuations in metHb and free radical concentration.

The result of the annealing experiment (Fig. 3) can also be understood within the mechanism shown in Scheme 1. We postulate that under our experimental conditions a small fraction of the H$_2$O$_2$ is left unreacted in blood by the end of the freezing time. This low concentration of H$_2$O$_2$ reacts with metHb (reaction 4) when a frozen blood sample is incubated at an elevated temperature. It is known that small molecules, like water and molecular oxygen, gain mobility at $-90$ °C and become free to interact with macroradicals in frozen water matrices during stepwise annealing of irradiated samples (39, 52, 53). It is reasonable, therefore, to suppose that the peroxide molecules are mobile and can react with metHb, creating the radicals when frozen matrix is kept at $-60$ °C (Fig. 3). However, due to low concentration, as we suggest, H$_2$O$_2$ does not induce the radical decay, since it is only in the presence of an excess of H$_2$O$_2$ over metHb that H$_2$O$_2$ interacts with the radicals (reaction 5), resulting in their decay, as was shown in the experiment with purified metHb (Fig. 6). Under such conditions the concentration of the radicals formed in a blood sample at $-60$ °C will be nearly stoichiometric with H$_2$O$_2$ and with the concentration of metHb oxidized to the ferryl form. The growth of the radical upon annealing at $-60$ °C is $-1 \mu$m (Fig. 3). We expect therefore that concomitant loss of metHb in the course of reaction 4 will not be greater than $1 \mu$m, which is just below our detection limit ($-2 \mu$m), and that is why we do not observe it (Fig. 3).

Elevating the annealing temperature above $-50$ °C causes hemoglobin autoxidation in frozen blood sample (reaction 1); the higher the temperature, the steeper the increase of metHb concentration (Fig. 3). The reverse process of metHb enzymatic reduction (reaction 2) is probably blocked at such temperatures, since it needs two high molecular weight species to meet each other through diffusion. As a result of Hb autoxidation, the H$_2$O$_2$ formed (reactions 1 and 3) causes both radical formation and decay (reactions 4 and 5). The balance between reaction 4 on one hand and reactions 3 and 5 on the other depends on temperature. As temperature increases from $-50$ °C to $-20$ °C, the rate of radical formation (reaction 4) increases more slowly than the rate of radical decay (reaction 5 and 9), so that observed concentration of free radicals has a maximum at a temperature of $-40$ °C (Fig. 3). Interestingly, a very similar incubation temperature of $-38$ °C has recently been used to demonstrate superoxide radical production in partially oxygenated human Hb samples (56). Superoxide radicals were directly observed in Hb samples at liquid helium temperatures, and their signal intensity depended on the oxygenation status of Hb in the same fashion as did the rate of autoxidation (56).

When the sample is cooled to $-196$ °C after annealing at $-20$ °C, the concentration of H$_2$O$_2$ produced by autoxidation is high enough to cause a measurable loss of metHb on a following incubation at $-40$ °C via reaction 4. However, the increase of the free radical concentration at this second incubation at $-40$ °C is not as big as at $-60$ °C (Fig. 3) because it is determined now by both H$_2$O$_2$-dependent formation (reaction 4) and H$_2$O$_2$-dependent decay (reaction 5) of the radicals.

Finally, there are no fluctuations in metHb and free radical concentration in the annealing experiment (the curves in Fig. 3 are smooth within each temperature section) because the sample has been kept solid and has not been subjected to a phase transition.

We conclude, therefore, that in spite of the presence of catalase and glutathione peroxidase, there is a steady state concentration of H$_2$O$_2$ in whole human blood, which leads to metHb oxidation to the ferryl form and concomitant production of globin-centered free radical. The concentration of the radicals in whole blood is $-1 \mu$m and depends on the oxygenation status of the blood. As stressed above, the pro-oxidant activity of the ferryl form of heme and/or of the globin radical has been extensively studied in model systems. This allowed many to speculate as to whether these species have relevance to the processes in vivo. Although ferryl hemoglobin formation has been demonstrated in intact red blood cells (10), the present study is the first, to the best of our knowledge, to show globin-based radical formation in whole blood. Our results imply that hemoglobin interaction with endogenously formed hydrogen peroxide does take place in normal human blood.

REFERENCES

1. Gibson, J. F., and Ingram, D. J. E. (1956) Nature 178, 761–762.
2. King, N. K., and Winfield, M. E. (1963) J. Biol. Chem. 238, 1550–1558.
3. King, N. K., Looney, F. D., and Winfield, M. E. (1967) Biochim. Biophys. Acta 133, 65–82.
4. Yoshinori, T., and Schleyer, H. (1967) J. Biol. Chem. 242, 1974–1979.
5. Shiga, T., and Imaizumi, K. (1975) Arch. Biochem. Biophys. 167, 469–479.
6. Tomoda, A., Sugimoto, K., Suhara, M., Takeshita, M., and Yoneyama, Y. (1979) Biochem. J. 171, 329–335.
7. Harada, K., and Yamaizaki, I. (1987) Biochem. 101, 283–286.
8. Tew, D., and Ortiz de Montellano, P. R. (1988) J. Biol. Chem. 263, 17860–17866.
9. Miki, H., Harada, K., Yamazaki, I., Tamura, M., and Watanabe, H. (1989) Arch. Biochem. Biophys. 275, 354–362.
10. Giulivi, C., and Davies, K. J. A. (1990) J. Biol. Chem. 265, 19453–19460.
11. Xu, Y., Asghar, A., Gray, J. I., Pearson, A. M., Haug, A., Grauke, A. E. (1990) J. Agric. Food Chem. 38, 1494–1497.
12. Davies, M. J. (1991) Biochim. Biophys. Acta 101, 86–90.
13. Kelder, P. P., Fischer, M. J. E., deMol, N. J., and Janssen, L. H. M. (1991) Arch. Biochem. Biophys. 294, 313–319.
14. Kelman, D. J., and Mason, R. P. (1992) Free Rad. Res. Commun. 16, 27–33.
15. McArthur, K. M., and Davies, M. J. (1995) Biochim. Biophys. Acta 1292, 171–181.
16. Kelman, D. J., DeGray, J. A., and Mason, R. P. (1994) J. Biol. Chem. 269, 7458–7463.
17. Cooper, C. E., Green, E. S. R., Rice-Evans, C. A., Davies, M. J., and Wrigglesworth, J. M. (1994) Free Radical Res. Commun. 20, 219–227.
18. Gunther, M. R., Kelman, D. J., Corbett, J. T., and Mason, R. P. (1995) J. Biol. Chem. 270, 16075–16081.
19. Gunther, M. R., DeGray, J. A., Tschirret-Guth, R., and Mason, R. P. (1995) Oxygen '95: Annual Meeting of the Oxygen Society, Pasadena, CA, November 16–20, 1995, Abstr. A-31, The Oxygen Society, San Francisco.
20. Svitkinensko, D. A., Patel, R. P., and Wilson, M. T. (1996) Free Radical Res. 24,
