Free radical in blood: a measure of haemoglobin autoxidation *in vivo*? †

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The EPR spectrum of whole human or animal blood, measured at 77 K, exhibits a free radical signal identical to that observed when purified methaemoglobin (metHb) reacts with H_2O_2. This signal is usually attributed to a globin-based radical formed as a result of two electron oxidation of metHb. We have recently proposed a mechanism to account for the formation of these globin radicals in blood. H_2O_2 is produced in small amounts *via* dismutation of $O_2^{\bullet -}$ formed during normal autoxidation of haemoglobin and this H_2O_2 then reacts with metHb to form the radical. This mechanism allows us to explain the variability (fluctuations) in metHb and free radical concentrations in apparently identical samples of frozen blood. In the present work we further confirm that the fluctuations are caused by sample freezing. We also show that the range of fluctuations of both metHb and free radical EPR signals in human venous blood decreases with increasing time of incubation at room temperature under aerobic conditions, and the fluctuations are completely absent after three hours. This may be understood as an effect of increasing the oxygenation of the venous blood. When fully oxygenated, haemoglobin autoxidation is suppressed; therefore $O_2^{\bullet -}$ is not produced in significant amounts and H_2O_2 is not formed on sample freezing. To confirm this interpretation we have studied venous and arterial rat blood and found that the free radical concentration is low and does not fluctuate (neither did metHb) in the oxygenated arterial blood, while venous rat blood shows the inverse fluctuations similar to those observed previously in human venous blood. We therefore conclude that the intensity of the free radical and metHb signals in frozen blood samples can be used as a measure of haemoglobin autoxidation and $O_2^{\bullet -}$ production in blood.

Mammalian blood is characterized by the presence of free radicals, which can be detected by the method of electron paramagnetic resonance (EPR) spectroscopy.[1,2] While free radicals in other biological tissues are associated with the mitochondrial respiratory chain,[1–4] the radical observed in blood cannot originate in this way since there are few mitochondria in blood and none in the erythrocytes. In order to understand the nature of this free radical we have compared the EPR signal of frozen human venous blood with the EPR signal of the free radicals formed in the reaction of purified methaemoglobin (metHb) with hydrogen peroxide H_2O_2.[5] The latter signal is usually attributed to a globin based radical formed as a result of two electron oxidation of metHb by H_2O_2 [reaction (1)]. The

$$\underset{\text{metHb}}{HC\underline{\ Fe^{III}}} + H_2O_2 \longrightarrow \underset{\text{ferrylHb}}{{}^{\bullet}C\underline{\ Fe^{IV}}=O} + H_2O + H^+ \qquad (1)$$

two electrons taken by peroxide to form water are thought to come separately from haem and globin resulting in, respectively, two sites of oxidation—the ferryl state of haem ($Fe^{IV}=O$) and a globin based free radical.[6–8] We have demonstrated that the radiospectroscopic properties of the free radicals in blood and in a purified metHb–H_2O_2 system are identical.[5] Furthermore, we have found that the concentrations of the free radicals and metHb fluctuate in an inverse manner (one is higher when the other is lower) in a set of apparently identical frozen blood samples taken from the same liquid venous blood sample.[5] We have proposed a mechanism to explain the formation of globin radicals in blood. This mechanism suggests that the radicals are formed *via* reaction (1) when H_2O_2 is produced in the process of dismutation of $O_2^{\bullet -}$ [reaction (2)]. In turn, the superoxide

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad (2)$$

anion radical $O_2^{\bullet -}$ is formed during normal autoxidation of haemoglobin[5] [reaction (3)]. We also postulated that dismu-

$$Hb^{II} \xrightarrow{O_2\ \ O_2^{\bullet -}} Hb^{III} \qquad (3)$$

tation of $O_2^{\bullet -}$ [reaction (2)] is intensified at the liquid–solid phase separation surfaces during sample freezing. This is due to the concentrating of $O_2^{\bullet -}$ at these interfaces. As a result, high local concentrations of H_2O_2 are produced throughout the sample and the overall H_2O_2 concentration is unique for any given sample; in other words H_2O_2 may vary from sample to sample because the freezing process cannot be controlled. Hydrogen peroxide then reacts with metHb resulting in the observed fluctuating free radical and metHb concentrations; a *decrease* in the average metHb concentration (*via* conversion to ferrylHb) being associated with an *increase* in the free radical concentration.[5]

In the present work we confirm that the fluctuations in the free radical and metHb concentration in frozen blood is caused by sample freezing in liquid nitrogen. We show that sometimes the concentration of these species can be significantly different in different parts of the same pellet sample. We also show that

† Presented at the 30th International Meeting of the Electron Spin Resonance Group of the RSC, University of Lancaster, 6–10th April 1997.

the range of the fluctuations is dependent on the oxygenation status of blood. When blood is fully oxygenated the fluctuations are absent, which is consistent with our proposed mechanism; complete oxygenation causes decreased Hb autoxidation resulting in a decrease in $O_2^{\bullet -}$ and, in turn, in H_2O_2 production. Therefore the concentration of the free radical formed will be low and the fluctuations, if any, will be within the instrumental error.

Experimental

EPR sample preparation

Human venous blood was collected under air in glass tubes containing a small amount of sodium citrate used as an anticoagulant. The blood was gently stirred every 15 min during incubation under aerobic conditions. 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. Male Sprague–Dawley rats (250–300 g) were anaesthetised with isofluorane (1%) and underwent cannulation of the carotid artery and internal jugular vein with polyethylene tubing. Blood samples were collected and used immediately so that anticoagulant was not necessary. When needed, red blood cells were separated from plasma by centrifugation at 2000 rpm for 10 min.

Human metHb was prepared and its concentration was determined (in terms of haem iron) as described elsewhere.[9,10] EPR samples of blood, metHb–H_2O_2 mixtures and blank samples (distilled water or phosphate buffer) were made in single or in duplicate, using syringes and plastic tubes as described elsewhere.[10] The open ends of the tubes were held in contact with the surface of the liquid nitrogen, thereby sealing them. Once sealed (*ca.* 5 s) the tubes were completely immersed into the liquid nitrogen and the samples rapidly frozen (10–12 s). Alternatively, the samples were immersed gradually (over *ca.* 60 s) into the liquid nitrogen, but this showed no difference in the effects observed. The indicated reaction times are defined as the time of the fast immersion of the plastic tubes into the liquid nitrogen. The frozen tubes were then gradually warmed so that the samples (still frozen) could be carefully pushed out of the plastic tubes into liquid nitrogen with a glass rod.

EPR spectra measurement

The EPR spectra of frozen human blood were measured on a Bruker ER 220D spectrometer equipped with an Aspect 2000 data system. The EPR spectra of rat blood samples were performed using a Varian E109 X-band spectrometer equipped with an Archimedes 440 computer. All EPR measurements were performed at 77 K using a quartz finger Dewar. The EPR spectra of opposite ends of the same sample were measured by mounting the sample in the Dewar in the two possible orientations: either one or the other end of the sample down, the finger of the Dewar being fixed by the cavity collet at such a mark, that the bottom of the sample was levelled with the bottom edge of the working zone of the cavity. Since the height of the working zone was *ca.* 12 mm and the sample length was 30 mm, the top part of the sample did not contribute to the EPR spectrum. Microwave frequency (ν) was 9.266 GHz and the modulation frequency was 100 kHz. The abbreviations for other instrumental parameters used in figure captions: H_0, central field; ΔH, sweep width; GN, signal gain; τ, time constant; A_m, modulation amplitude; ST, sweep time; DP, number of data points; NS, number of scans; P, microwave power. The EPR spectra of the blanks were subtracted from the spectra of the samples. Such a subtraction excluded all background signals, such as any signals of the quartz Dewar, the cavity, or the broad signal caused by oxygen dissolved in liquid nitrogen. The intensities of the EPR signals in blood were measured as described elsewhere.[5]

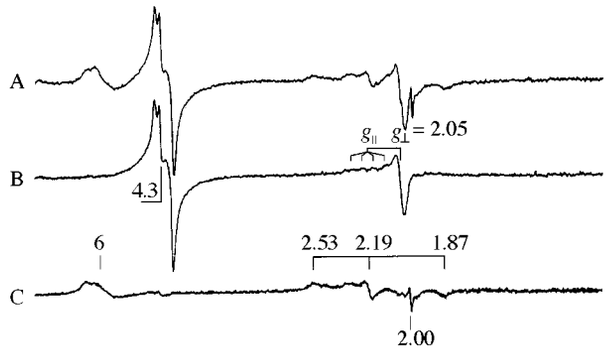

Fig. 1 EPR spectra of rat blood and of blood components. A, whole venous blood; B, plasma; C, cell fraction. The spectra were measured at 77 K. The instrumental parameters: $H_0 = 2550$ G; $\Delta H = 4000$ G; GN = 3.2×10^3; $\tau = 0.128$ s; $A_m = 6.3$ G; ST = 4 min; DP = 2560; NS = 4; $P = 50$ mW; $\nu = 9.266$ GHz.

Results and discussion

Normal mammalian blood is characterised by an EPR spectrum which is probably common to all species. We know that the EPR spectra of human, pig, dog, rat and mouse blood measured at 77 K comprise the same set of EPR signals. Fig. 1 (A) shows a typical EPR spectrum of normal blood measured at 77 K. This spectrum consists of the signals of at least five different paramagnetic centres. The signals with $g = 4.3$ and $g = 2.05$ are characteristics of plasma (Fig. 1, B) while the signals with $g = 6$, $g = 2.00$ and the three component signal with g-factors 2.53, 2.19 and 1.87 are only seen in the cell fraction of blood (Fig. 1, C). The EPR signal with $g = 4.3$ detected in plasma (B) is assigned to transferrin Fe^{III} ions complexed by HCO_3^- ions.[1,2,11,12] The other plasma signal with a strong perpendicular component $g_\perp = 2.05$ and four weak parallel components is a signal of Cu^{II} ions in a tetragonal environment with four strongly bound equatorial ligands and two weakly bound axial ligands.[13] This signal is due to ceruloplasmin (Cp), the major copper containing protein found in blood.[11,14] Both the signal at $g = 6$ and the three component signal (2.53, 2.19, 1.87) are caused by metHb, *i.e.* by the ferric Fe^{III} haem ions: the $g = 6$ signal is caused by the high spin ferric haem[1,2,7,11,15] and the three component signal is caused by the low spin metHb in the O-type form, *i.e.* with OH^- ion in the 6th coordination position.[16]

The signal with $g = 2.00$ is attributed to the presence of free radicals in blood.[1,2] We have previously shown that this free radical EPR signal in frozen human blood has the same radiospectroscopic parameters and properties as the signal of the globin-based free radical, $^{\bullet}Hb(Fe^{IV}{=}O)$, formed in the reaction of purified metHb with H_2O_2.[5] Fig. 2 shows the coincidence of the line shapes and saturation curves of the free radical signals in these two systems. The same g-factor, line shape and saturation behaviour of the EPR signals in blood and in purified metHb mixed with H_2O_2 is a strong indication of the common nature of the free radicals in these two systems.

However, the fact that the EPR signal does not exhibit either a specific hyperfine structure or any anisotropic features but, instead, is a plain singlet, makes unambiguous identification difficult and leaves the faint possibility that the radical in blood is not the same as in purified metHb. In order to strengthen our conclusion that the radicals are indeed the same we have examined the relationship between intensities of the $g = 6$ (metHb) and $g = 2$ (radical) signals in blood.

We have shown that the concentration of the globin based radicals and of metHb exhibited significant variation (fluctuations) in different frozen samples taken from the same liquid blood sample.[5] In any given sample a higher than average concentration of free radicals was associated with a lower than average concentration of metHb and *vice versa*, *i.e.* the fluctu-

Table 1 A non-uniform concentration of the free radicals and metHb throughout the volume of a frozen blood sample

	Paramagnetic centre							
	High spin metHb		Free radicals		Transferrin		Ceruloplasmin	
g-Factor	6		2.005		4.3		2.05	
	Part of the sample measured							
	A	B	A	B	A	B	A	B
EPR signal amplitude/ arbitrary units[a]	0.52	0.62	0.68	0.59	1.07	1.07	1.09	1.10
	0.57	0.62	0.71	0.57	1.09	1.05	1.09	1.02
	0.56	0.64	0.67	0.63	1.08	1.06	1.06	1.08
	0.59	0.63	0.66	0.65	1.07	1.05	1.03	1.04
	0.54	0.61	0.68	0.61	1.07	1.07	1.12	1.05
Mean ± standard deviation	$0.55_6 \pm 0.02_7$	$0.62_4 \pm 0.01_1$	$0.68_0 \pm 0.01_9$	$0.61_0 \pm 0.03_2$	$1.07_6 \pm 0.00_9$	$1.06_0 \pm 0.01_0$	$1.07_8 \pm 0.03_4$	$1.05_8 \pm 0.03_2$
Difference of the means/absolute value	0.06_8		0.07_0		0.01_6		0.02_0	
Confidence level of the two-tails homoscedastic t-test	$p < 0.001$		$p < 0.003$		$p < 0.029$		$p < 0.367$	

[a] The data is presented for two opposite parts (A and B) of a single sample. Five independent EPR measurements were performed when one of the ends of the sample (A) was centred in the cavity, and five measurements for the other end (B).

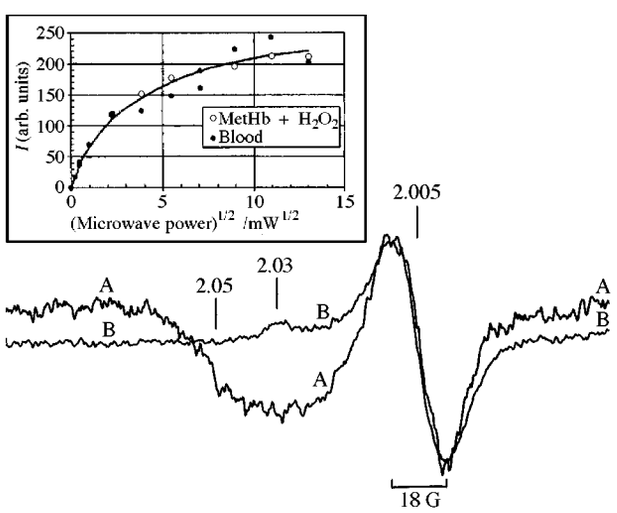

Fig. 2 The EPR signals of free radicals in whole blood and in the metHb–H_2O_2 model as measured at 77 K. A, the EPR spectrum of normal human venous blood frozen 20 min after donation. B, the EPR spectrum of 0.1 mM metHb frozen 1 min after addition of 1 mM H_2O_2. The instrumental parameters: $H_0 = 3320$ G; $\Delta H = 200$ G; GN = 10^4; $\tau = 0.064$ s; $A_m = 4$ G; ST = 50 s; DP = 1280; NS = 6 (A); NS = 1 (B); $P = 30$ mW; $\nu = 9.266$ GHz. *Inset:* saturation behavior of the signals in the two systems. The dependencies for blood and the metHb–H_2O_2 system have been normalized to an equal initial slope.

ations were always of opposite sense. Moreover, we found that in some cases the concentration of metHb (and of the free radicals) was significantly different in the opposite ends of the same pellet sample (Table 1). The part of the sample with lower metHb concentration (A) is characterized by a higher free radical concentration and *vice versa*. In contrast, the concentration of Cp is not significantly different in the different ends of the sample, and although a significant difference is seen in the concentration of transferrin (Tf), the magnitude of this is very small (Table 1). Therefore, we conclude that the fluctuations in the metHb and free radical concentration are inversely related and are caused by the processes involved in freezing the blood.

These fluctuations in the concentration of the free radicals and metHb support the view that the radicals are formed through the reaction of metHb with H_2O_2. An increase in the free radical concentration during blood sample freezing is associated with consumption of metHb (decrease in $g = 6$), exactly in the same way as is observed in the reaction of purified metHb with H_2O_2. Moreover, it is well known from studies of purified metHb that the free radical concentration measured on addition of H_2O_2 to metHb is always between 4–16% of the initial concentration of metHb.[7,8,17,18] This finding is in agreement with our data on the fluctuations—the range of fluctuations of the free radical concentration is approximately eight times less than the range of fluctuations of the metHb concentration,[5] which means that when a blood sample is frozen, a 1 μM increase of the free radical concentration is associated with a 8 μM decrease of the concentration of metHb.

In a separate set of experiments we investigated the dependence of the EPR signals and their fluctuations on the oxygenation state of the blood. Fig. 3A shows that during the first three hours of blood exposure to air the range over which the concentration of metHb varied (fluctuated) is significantly wider than the corresponding range for the concentration of Tf. There were no fluctuations in those samples made at four or five hours, the error in both metHb and Tf concentrations being similar and defined by the small instrumental error. In a similar way, Fig. 3B shows that the range of fluctuations in the concentration of the free radicals is significantly wider than the error in the concentration of Cp during the same initial period of three hours. Fluctuations in the radical concentration ceased after four hours. In addition, the fluctuations in metHb and free radical concentrations are not independent—the higher the free radical concentration, the lower the concentration of metHb (Fig. 4).

The cessation of fluctuations after three hours of exposure of blood to air can be understood by our proposed mechanism of free radical formation. Venous blood incubated at room temperature and aerated by gentle stirring turns bright pink after three hours indicating that the blood has been almost fully oxygenated by that time and thus nearly all haemoglobin has been transformed into the oxy-form. It is known that the haemproteins autoxidation rate[19,20] and concomitant superoxide production[21] both decrease on full oxygenation of the protein. Therefore, once full (or nearly full) oxygenation has been

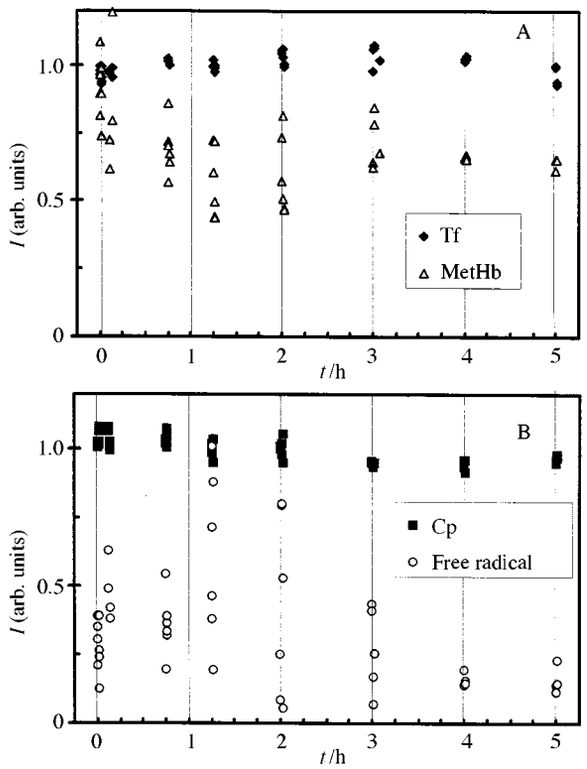

Fig. 3 The intensities of the EPR signals of Tf and high spin metHb (A) and of Cp and the free radicals (B) in the frozen samples of human venous blood. On the *x*-axis is the time of blood exposure to air prior to sample freezing. There are eight groups of samples made over a five hour period, samples in each group frozen within as short period as possible. The EPR spectra which covered the metHb and Tf signals were measured at $H_0 = 1350$ G; $\Delta H = 1000$; $A_m = 5$ G. The spectra from which the free radical and Cp signals were determined were measured at: $H_0 = 3300$ G; $\Delta H = 200$ G; $A_m = 4$ G. Other instrumental parameters were: $P = 50$ mW; $\nu = 9.266$ GHz; $G = 3.2 \times 10^3$; $\tau = 0.128$ s; ST = 50 s; DP = 1280; NS = 2. The amplitudes of the metHb and free radical signals for the first group of samples are shown in more detail in Fig. 4.

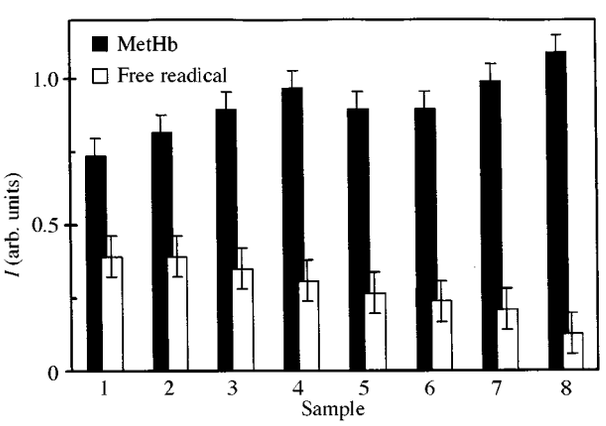

Fig. 4 The EPR signal intensities for the high spin metHb and the free radical in the group of samples frozen straight after the beginning of donation (the first group of samples in Fig. 3). The eight samples of the group are arranged in the order of descending free radical signal. Correspondingly, the signal of metHb is ascending with only one exception (sample 4) which is within the instrumental error.

achieved (after four hours in our experiment) the rate of Hb autoxidation becomes very small resulting in such low $O_2^{\bullet -}$ concentrations in the liquid blood that freezing does not cause significant additional H_2O_2 formation. Under such conditions the H_2O_2 concentration does not fluctuate significantly in frozen samples and therefore no detectable fluctuations in metHb and free radical concentration are observed.

To confirm this explanation we compared the EPR spectra of deoxygenated venous blood with the spectra of arterial, *i.e.*

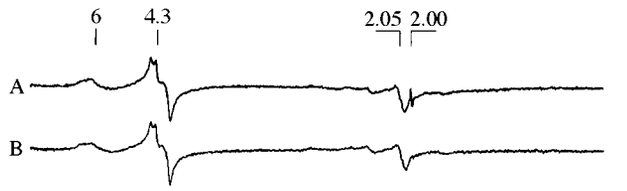

Fig. 5 The EPR spectra of rat venous (A) and arterial (B) blood measured at 77 K. The free radical signal ($g = 2$) varies significantly in venous blood, being not the same even in duplicate samples made of a common blood aliquot. The free radical signal is low and does not show any significant fluctuations in arterial blood. The spectra were measured at 77 K. The instrumental parameters: $H_0 = 2550$ G; $\Delta H = 4000$ G; GN = 3.2×10^3; $\tau = 0.128$ s; $A_m = 6.3$ G; ST = 4 min; DP = 2560; NS = 2; $P = 50$ mW; $\nu = 9.266$ GHz.

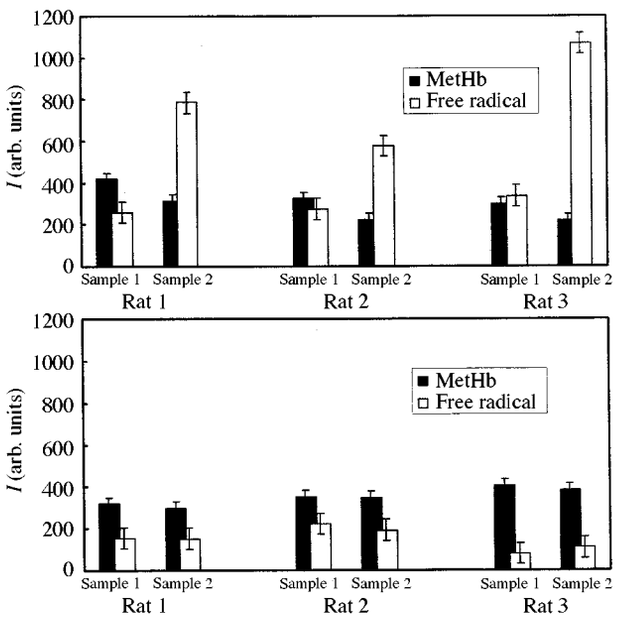

Fig. 6 The intensities of the EPR signals of the high spin metHb and free radical in duplicate samples of venous and arterial blood. The concentrations of metHb and free radical fluctuate in an inverse manner in the venous blood, while the difference in the signals intensities in duplicate samples of arterial blood is within the instrumental error.

oxygenated, blood (Fig. 5). We found that both free radical and metHb concentrations fluctuate in the rat venous blood, while the EPR signals from these species are insignificantly different in oxygenated arterial blood. Fig. 6 shows the intensities of the metHb and free radical EPR signals in venous and arterial blood. The concentrations of these species fluctuate in the frozen samples of venous blood, significant differences being observed between duplicate samples taken from the same animal and frozen at the same moment. These fluctuations are inverse, for any pair of duplicate samples the free radical concentration is higher in the sample with the lower concentration of metHb (Fig. 6, top). In contrast to venous blood, arterial blood shows a very low level of free radicals (Fig. 5) and no significant fluctuations in the metHb or free radical concentration (Fig. 6, bottom). This result confirms that oxygenation of blood results in a decrease of the average free radical concentration and, therefore, in cessation of any detectable fluctuations.

We therefore conclude that the intensity of the free radical and metHb signals can be used as a measure of haemoglobin autoxidation in blood just prior to freezing. The fluctuations in the free radical concentration and the inversely related fluctuations in the concentration of metHb imply that autoxidation of Hb is proceeding, the extent of the process being reflected in the range of the fluctuations. The absence of the fluctuations and a very low level of the free radicals in frozen blood imply that autoxidation is suppressed. These results are of medical

interest as, in principle, they yield a method whereby the autoxidation rates and superoxide production may be accurately estimated *in vivo* by examination of the radical and metHb EPR signals in frozen blood.

Acknowledgements

D. A. S., M. T. W. and C. E. C. thank the Wellcome Trust and N. A. D. thanks the Research Promotion Fund, University of Essex, for financial support.

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Paper 7/02483E
Received 11*th April* 1997
Accepted 10*th July* 1997