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The Reduction of Nitroblue Tetrazolium by Red Blood Cells: a Measure of Red Cell Membrane Antioxidant Capacity and Hemoglobin-membrane Binding Sites

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The reduction of nitroblue tetrazolium (NBT) with intact Red Blood Cells (RBCs) is biphasic with an initial rapid reduction followed by a slower second phase. This biphasic kinetics has been explained with the initial rapid phase attributed to antioxidants in the red cell which reduce membrane bound NBT and the slower phase associated with the reaction of NBT with membrane bound hemoglobin. This model has been confirmed by a utilization of a number of red cell modifications which either increase the red cell antioxidants (vitamin C and vitamin E) or damage the red cell membrane (cumene hydroperoxide and *N*-ethylmaleimide). The utilization of this assay for human blood samples was investigated by studying a series of 20 human subjects ranging between 34 and 87 years of age. It was possible to fit all of these samples with two adjustable parameters which reflect the red cell membrane antioxidant capacity (x) and the hemoglobin membrane interactions (m). The antioxidant capacity shows a significant ($p < .002$; $R = -.67$) decrease with age. This finding is consistent with a decrease in the level of antioxidants in aged subjects. In addition, the number of hemoglobin membrane sites are negatively correlated with the antioxidant capacity ($p < .02$; $R = -.52$) suggesting that the oxidative stress associated with reduced antioxidants results in increased hemoglobin-membrane interactions.

Keywords: Antioxidant capacity, red blood cells, membrane, nitroblue tetrazolium, hemoglobin, antioxidant, vitamin E (α-tocopherol), vitamin C (ascorbic acid)

1. INTRODUCTION

Human erythrocytes are continuously exposed to oxidative stress. During their relatively short life, in which no protein synthesis occurs, these cells, whose principal role is to carry oxygen to the tissues and organs, come in contact with reactive oxygen species from various sources. The normal autoxidation of hemoglobin, which is of particular relevance at reduced oxygen pressure,[1] produces superoxide within the erythrocyte. In addition, erythrocytes are exposed

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to reduced oxygen species generated by the oxidation-reduction of drugs or xenobiotics transported by blood and metabolism in various tissues and organs which release reactive oxygen species into the blood.[2,3]

The high levels of cytoplasmic antioxidants present in erythrocytes including superoxide dismutase, catalase, glutathione and ascorbic acid minimize oxidative damage to the cytoplasmic components of the erythrocyte. Furthermore, within the cytoplasm, enzymes like methemoglobin reductase and diaphorase reverse any hemoglobin oxidation which occurs as a result of the reaction of hemoglobin with oxidants. Membrane associated antioxidants like α-tocopherol minimize oxidative damage to the red cell membrane. Nevertheless, it is more difficult to reverse oxidative damage within the membrane than in the cytoplasm as seen by the accumulation of membrane oxidative products in old red cells.[4]

In this paper, we have used the reduction of nitroblue tetrazolium(NBT) in the presence of erythrocytes to study red cell membrane oxidative processes. NBT is only slightly soluble in aqueous solution and is, therefore, concentrated in the membrane. In an earlier study,[5] we found that NBT, which is known to react with superoxide, also reacts with hemoglobin. In this study, we show that the reduction of NBT associated with the red cell membrane is determined both by the interaction of hemoglobin with the membrane as well as the antioxidants in equilibrium with the membrane. The reduction of NBT by antioxidants reflects the antioxidant capacity of the erythrocyte membrane. The interaction of hemoglobin with the membrane is thought to be associated with erythrocyte membrane damage.

2. MATERIALS AND METHODS

2.1. Chemicals

NBT, dl-α-tocopherol, vitamin C, *N*-ethylmaleimide and glutathione were obtained from Sigma (St Louis, MO), and cumene hydroperoxide (CumOOH) from Aldrich (St Louis, MO).

2.2. Erythrocytes

Red blood cells were obtained from blood samples collected from healthy participants of the Baltimore Longitudinal Study on Aging. The red blood cells were washed thrice in phosphate buffered saline (PBS), pH 7.4, containing 0.1 mM EDTA to remove the buffy coat and plasma.

2.3. Kinetic Reduction of NBT by RBCs

The spectrum of the RBCs without the NBT was acquired in the 640–490 nm region using a Perkin-Elmer Lamda 6 UV-Visible Spectrophotometer in order to determine the concentration of hemoglobin and the initial level of hemoglobin oxidation. A scattering accessory was used to be able to measure spectra of RBCs. NBT was then added to the suspension of cells to a final concentration of 1×10^{-4} M NBT in PBS buffer, pH 7.4. This NBT concentration was four times the concentration of hemoglobin in the RBC sample.[5] Immediately after adding the NBT, spectra were obtained at predetermined times in order to follow the kinetics for the reduction of NBT. Each spectrum was analyzed by fitting it to a linear combination of the parent spectra for oxyhemoglobin, deoxyhemoglobin, pH 7.4-methemoglobin and reduced NBT (Figure 1).

In order to take into account the scattering effect of red cells, the parent spectra used for fitting were generated in red cells for the different hemoglobin components and in the presence of red cell membranes for reduced NBT.[5] The hemoglobin concentration was determined by iron analysis of hemolyzed cells. The concentration of reduced NBT (formazan) was obtained by adding excess potassium superoxide (KO_2) to the NBT. The precipitation of the diformazan[6] was prevented by adding white ghosts within 30 s after the addition of KO_2. The association of

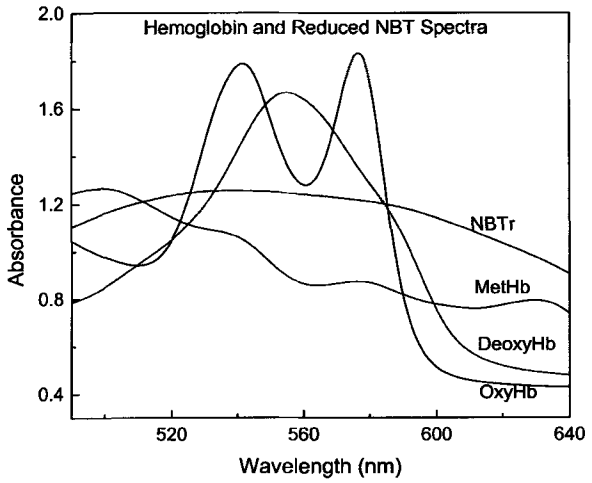

FIGURE 1 Spectra of oxygenated hemoglobin (oxyHb) and deoxygenated hemoglobin (deoxyHb) in the intact RBCs, oxidized hemoglobin (metHb) at pH 7.4, and reduced NBT (NBTr) in the membrane used for the analysis of acquired experimental spectra. The concentration of the hemoglobin components are 9.43×10^{-5} M and of reduced NBT is 5.92×10^{-4} M.

diformazan with membrane presumably prevents the aggregation and precipitation. With a scattering accessory to correct for the scattering of the membrane it was possible to obtain a spectrum of the reduced NBT that was used as one of the parents to fit our experimental data. By using the entire spectra consisting of 300 points between 640 nm and 490 nm for fitting, reliable determinations of small changes in the concentrations of the different species could be quantified as indicated by the standard errors obtained. All preparations and spectrophotometric determinations were carried out at a temperature of 21–22 °C.

2.4. Analysis of Kinetic Data

The kinetic data were analyzed using MLAB, a mathematical modeling program originally written by Garry Knott and marketed by Civilized Software, Inc., Bethesda, MD. This program permits you to model complex kinetic reactions by writing the differential equations for the changes in concentration of each chemical species. It is possible to generate the time dependent changes in the concentration of all the species for any set of rate constants after the initial conditions are set. An iterative least square method is used to find the best set of rate constants, which will fit the experimental data.

2.5. Effect of Vitamin E on the Reduction of NBT by RBCs

A stock solution (750 μM) of Vitamin E was dissolved in ethanol. The concentration of the stock solution was determined by absorption at 294 nm where $E^{1\%} = 71$. An aliquot of this solution was transferred to a glass tube with the solution spread out on the surface of the tube. The ethanol was removed by nitrogen gas producing a thin film of vitamin E on the wall of the tube. The red cells in PBS were then added to the tube containing the film of vitamin E and rocked for 30 minutes. The amount of vitamin E remaining on the side of the tube at the end of rocking with RBCs was determined by rinsing with ethanol and taking a fluorescence reading at an emission wavelength of 335 nm and excitation wavelength of 292 nm. The low level of fluorescence in this ethanol solution was used to establish that >99.9% of the vitamin E was incorporated into the red cell membrane by this method. A control mixture of RBCs without vitamin E was rocked under the same condition.

After vitamin E incorporation, NBT was added to the sample. The spectra prior to the addition of NBT and at specified time intervals after the addition of NBT were monitored (see above) to determine the time course for NBT reduction by the vitamin E modified RBCs.

2.6. Effect of Vitamin C on the Reduction of NBT by RBCs

A fresh solution (10 mM) of vitamin C was prepared in PBS buffer, pH 7.4 before starting the experiment. An aliquot of this solution was transferred to the cuvette containing the RBCs in PBS

buffer, pH 7.4 before adding the NBT. The spectra prior to the addition of NBT and at specified time intervals after the addition of NBT were monitored (see above) to determine the time course for NBT reduction by the vitamin C modified RBCs.

2.7. Effect of Cumene Hydroperoxide on the Reduction of NBT by RBCs

A stock solution (20 mM) of CumOOH was prepared in ethanol. Aliquots of the stock solution (1.5–6.0 µl) were added to the suspension of RBCs in PBS buffer, pH 7.4 to make a final concentration of 20, 30, and 40 µM of CumOOH in a total volume of 3 ml. The ethanol volume added never exceeded 0.2% of the total incubation volume. The spectra prior to the addition of NBT and at specified time intervals after the addition of NBT were monitored (see above) to determine the time course for NBT reduction by CumOOH modified RBCs.

2.8. Effect of *N*-Ethylmaleimide (NEM) on the Reduction of NBT by RBCs

Erythrocytes (40% hematocrit) were incubated with NEM at 37 °C for 1 h in PBS buffer, pH 7.4. Incubations were performed at final NEM concentrations of 5, 10, and 50 mM. The cells were subsequently washed in PBS buffer, pH 7.4 to remove excess NEM.[7] The time course for NBT reduction by NEM modified RBCs was followed spectroscopically (see above).

3. RESULTS AND DISCUSSION

3.1. The Reduction of NBT by Hemoglobin in Solution in the Presence of Membranes and in Red Blood Cells

It has been shown that oxidized NBT with no visible absorption reacts with hemoglobin producing oxidized hemoglobin and the two electron reduced NBT or formazan.[5] In the course of this two electron reduction of NBT a reactive tetrazolinyl radical[8] intermediate without any visible spectrum is first produced. The reaction of NBT with hemoglobin can be followed by the spectral changes of hemoglobin and NBT. The oxidation of hemoglobin (Figure 1) results in a decrease in the absorption bands of Fe(II) hemoglobin between 580 nm and 540 nm and increases in the region of 630 nm and 500 nm. This spectral change is superimposed over the increased absorption in this region due to the formation of the two electron reduced formazan (Figure 1).

In comparing the reaction of NBT with purified hemoglobin and with hemoglobin in the presence of red cell ghosts,[9] it was found that, even though red cell ghosts do not reduce NBT, the addition of red cell ghosts to a hemoglobin sample dramatically increases the apparent rate for the reduction of NBT (Figure 2). The results shown in Figure 2 were obtained at low oxygen pressures where the reoxidation of NBT by oxygen (see below) does not take place and,

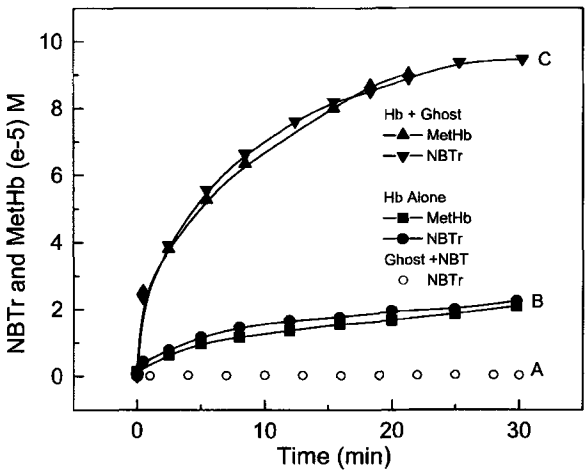

FIGURE 2 Effect of red blood cell ghosts on the reduction of NBT. (A) ghosts in the absence of added hemoglobin; (B) deoxyhemoglobin without added ghosts; and (C) in the presence of both deoxyhemoglobin and ghosts. The increased NBT reduction by hemoglobin in the presence of ghosts was confirmed in three independent experiments. Initial concentrations of deoxyhemoglobin and NBT are 1×10^{-4} M.

therefore, NBT reduction as well as hemoglobin oxidation are both accurate measures of the reactivity. However, the enhanced reactivity in the presence of membranes as determined by increased oxidation of hemoglobin is also observed at atmospheric oxygen pressure (data not shown).

This phenomenon was attributed to a greater solubility of NBT in the membrane than in aqueous solution.[9] Hemoglobin in close proximity to the membrane, therefore, experiences a much higher local concentration of NBT resulting in an accelerated reaction between hemoglobin and NBT.

For red cells only a small fraction of the hemoglobin is associated with the membrane. However, since most of the added NBT is associated with the membrane and the small fraction of NBT dissolved in the cytoplasm will react very slowly with the cytoplasmic hemoglobin, it can be assumed that the reduction of NBT by red cells takes place almost exclusively on the membrane.

An additional factor necessary to consider in comparing the reaction of NBT with purified hemoglobin and with red cells is the enzymatic reduction of oxidized hemoglobin formed in the red cell. This reaction results in minimal increases in oxidized hemoglobin even with appreciable reduction of NBT. The spectral changes observed are, therefore, dominated by the increased absorption associated with the reduction of NBT (Figure 3). Although these spectral changes are small, reliable changes in reduced NBT are determined by fitting 300 points in the entire 640 nm to 490 nm region (see above). The reliability of this analysis and the ability to clearly distinguish between different samples is indicated by the data shown in Figure 4. In this figure spectral data from 640 nm to 490 nm obtained at a series of times after the addition of NBT at atmospheric oxygen pressure were analyzed as a linear combination of the different spectral components (see above). For each blood sample the data fall along a curve

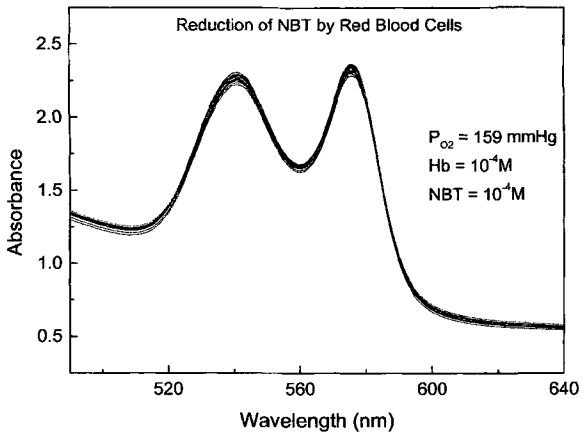

FIGURE 3 Time dependent changes of the spectra of hemoglobin in the red blood cells on reaction with NBT at atmospheric pressure (159 mmHg). Hemoglobin concentration in diluted RBCs was 1×10^{-4} M, and NBT concentration was 1×10^{-4} M.

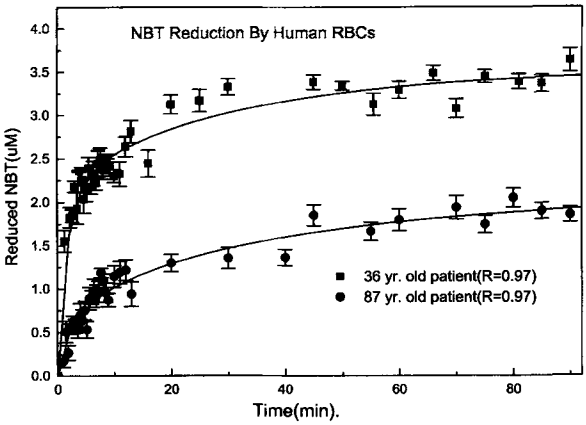

FIGURE 4 Reduction of NBT by RBCs at atmospheric pressure for 36 and 87 year old human subjects. Each point refers to the mean of three separate determinations; vertical bars represent the standard deviation. Concentrations of RBC hemoglobin and NBT are the same as in Figure 3.

defined by our kinetic model (see below) without much scattering, even though the actual concentrations of reduced NBT being formed are relatively low.

In order to establish the nature of the hemoglobin red cell membrane interaction responsible for NBT reduction, we have investigated the effect of adding exogenous hemoglobin to a red cell sample. The observation (Figure 5) that

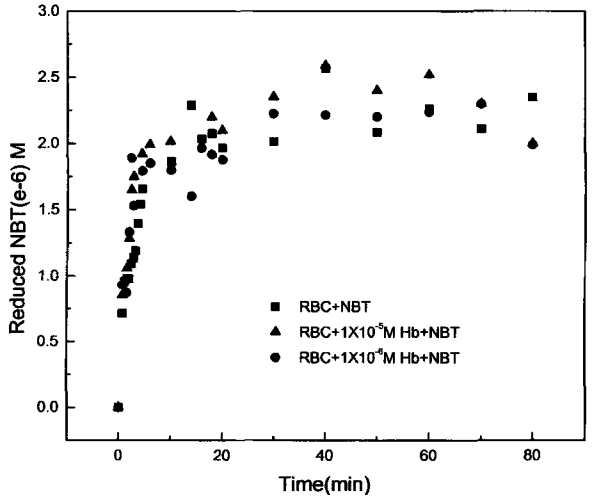

FIGURE 5 The effect of added extracellular hemoglobin on the red blood cell reduction of NBT. (■) RBC + NBT; (▲) RBC + 1×10^{-5} M Hb + NBT; (●) RBC + 1×10^{-6} M Hb + NBT. Concentrations of RBC hemoglobin and NBT are the same as in Figure 3.

extracellular hemoglobin has no observable effect on NBT reduction indicates that NBT reduction is associated with the interactions of hemoglobin with the cytoplasmic surface of the red cell. While no hemoglobin binding sites have been reported on the extracellular side of the red cell, a number of hemoglobin binding sites have been reported for the cytoplasmic surface of the red cell.[10–15] These include the relatively strong binding site on the cytoplasmic end of band 3. These cytoplasmic binding sites for hemoglobin in close proximity to membrane associated NBT are, thus, presumably responsible for the facilitated NBT reduction by hemoglobin in the red cell.

3.2. Kinetic Model for the Analysis of the Reduction of NBT by RBCs

In developing a model for the reduction of NBT by red blood cells, the model used for the published work[5] on the reaction of NBT with hemoglobin was used as a starting point. The hemoglobin model indicated that, in addition to the two step reduction of NBT by two hemes, it was necessary to consider the reversible reactions of oxygen with the tetrazolinyl radical and the fully reduced formazan.

$$\text{Reaction 1:}\quad \text{NBTr} + \text{O}_2 \underset{k_{1b}}{\overset{k_1}{\rightleftharpoons}} \text{NBT}^{\bullet} + \text{O}_2^{\bullet -}$$

$$\text{Reaction 2:}\quad \text{NBT}^{\bullet} + \text{O}_2 \underset{k_{2b}}{\overset{k_2}{\rightleftharpoons}} \text{NBT} + \text{O}_2^{\bullet -}$$

Since these reactions produce superoxide, which rapidly dismutates,[16] it was necessary to include the dismutation reaction:

$$\text{Reaction 3:}\quad \text{O}_2^{\bullet -} + \text{O}_2^{\bullet -} + 2\text{H}^+ \xrightarrow{k_3} \text{H}_2\text{O}_2 + \text{O}_2$$

The hemoglobin model[5] was modified to consider the membrane results (Figure 2), which implies that in red cells the reduction of NBT takes place on the membrane (see above). It was, therefore, necessary to include in the red cell model the binding of both reduced and oxidized hemoglobin to the hemoglobin binding sites on the red cell membrane (m).

$$\text{Reaction 4:}\quad \text{Hb} + \text{m} \underset{k_{4b}}{\overset{k_4}{\rightleftharpoons}} \text{Hb-m}$$

$$\text{Reaction 5:}\quad \text{metHb} + \text{m} \underset{k_{5b}}{\overset{k_5}{\rightleftharpoons}} \text{metHb-m}$$

where Hb-m and metHb-m represent reduced hemoglobin and oxidized hemoglobin, respectively, bound to the membrane.

The reaction of NBT with hemoglobin in RBCs was assumed to exclusively involve the hemoglobin bound to the membrane.

$$\text{Reaction 6:}\quad \text{Hb-m} + \text{NBT} \xrightarrow{k_6} \text{NBT}^{\bullet} + \text{metHb-m}$$

$$\text{Reaction 7:}\quad \text{Hb-m} + \text{NBT}^{\bullet} \xrightarrow{k_7} \text{NBTr} + \text{metHb-m}$$

In setting up the model for the red cell, we have assumed the literature value[17] for superoxide dismutation (Reaction 3) previously used in the hemoglobin study. Although Reactions 1, 2, 6 and 7 involving the oxidation of NBT by oxygen and the reduction of NBT by hemoglobin are analogous to those used in the hemoglobin

model, the same rate constants could not be used because for red cells the reaction is assumed to be taking place on the membrane instead of in solution. For the reaction on the membrane, the effective concentrations of the reactants as well as the medium in which the reaction is taking place are different. For reactions 1 and 2 we have assumed that all four rate constants (k_1, k_{1b}, k_2 and k_{2b}) are influenced to the same extent because of the membrane association of NBT. Only one adjustable parameter was, therefore, used in accounting for the reactions of oxygen and superoxide with NBT.

The reaction of red cell hemoglobin with NBT is complicated by the fact that only a small fraction of the total hemoglobin is bound to the membrane at anytime and able to react with NBT. The contribution of hemoglobin to NBT reduction is, therefore, influenced by the concentration of hemoglobin binding sites "m" and the rate constants for the binding of hemoglobin to the membrane (k_4, k_{4b}).

A careful inspection of the red cell data (Figure 4) shows that a model utilizing Reactions 1–7 does not adequately fit the red cell data. For red cells, at atmospheric oxygen pressure there is an initial rapid reduction of NBT, even though no apparent reduction of NBT is observed for hemoglobin in solution at atmospheric oxygen pressures.[5] No NBT reduction for hemoglobin, however, does not indicate that NBT doesn't react with hemoglobin, since enhanced oxidation of hemoglobin is observed under these same conditions.[5] The inability to detect any reduced NBT under these conditions was explained by the reoxidation of NBTr and the tetrazolinyl radical by oxygen (Reactions 1 and 2). The reduction of NBT by red cells at atmospheric oxygen pressure (Figure 4) can in part be explained by the competition between hemoglobin and oxygen for the tetrazolinyl radical. An increased rate for the reaction of hemoglobin bound to the membrane with membrane associated tetrazolium radical would be expected to partially compete with the NBT reoxidation reactions (Reactions 1 and 2) producing some reduced NBT even at atmospheric oxygen pressures. However, the reaction of NBT with membrane bound hemoglobin should be the same for all subjects, and can therefore not explain the variability shown in Figure 4.

In addition to the subject variability, the initial rapid reduction of NBT was less noticeable for samples that were stored, even at 4 °C. This variability is inconsistent with the expectation that NBT reduction should be determined by the concentration of hemoglobin bound to the membrane. The difficulty of obtaining white ghosts with stored blood[18,19] further suggests that storage of blood increases the binding of hemoglobin to the membrane leading to the prediction of an increase in the reduction of NBT instead of the observed decrease.

The reported[20,21] decrease in the concentration of antioxidants during storage of blood implies that the rapid reduction of NBT by red cells may involve a reaction with antioxidants associated with the red cell. Two additional reactions for the reduction of NBT and the tetrazolinyl radical by antioxidants would then be required to explain NBT reduction by red blood cells.

$$\text{Reaction 8:}\quad \mathrm{NBT} + \mathrm{x} \xrightarrow{k_8} \mathrm{NBT}^{\bullet} + \mathrm{ox}$$

$$\text{Reaction 9:}\quad \mathrm{NBT}^{\bullet} + \mathrm{x} \xrightarrow{k_9} \mathrm{NBTr} + \mathrm{ox}$$

where: x represents the antioxidants associated with the red cell and ox is the oxidized antioxidant. The rate constants for these reactions would represent an average for all of the antioxidants present, which can reduce NBT.

Using Reactions 1 to 9, we have been able to fit the data obtained for the reaction of NBT with red cells (Figure 4). For this purpose the program MLAB was used. A differential equation describing the change in concentration with time was written for each reactant (oxidized NBT: NBT, tetrazolinyl radical: $NBT^{\bullet}$, formazan: NBTr, oxygen: O_2, superoxide: $O_2^{\bullet-}$ reduced

TABLE I Rate constants obtained for the kinetic model used to analyze the reduction of NBT by Red Blood Cells

Rate constants		Reactions
k_1	$20.06 \pm 0.62\,M^{-1}min^{-1}$	Oxidation of reduced NBT (Formazan) by O_2
k_{1b}	$(1.05 \pm 0.22) \times 10^{10}\,M^{-1}min^{-1}$	Reaction of superoxide ion with tetrazolinyl radical
k_2	$(1.14 \pm 0.34) \times 10^{8}\,M^{-1}min^{-1}$	Oxidation of reduced tetrazolinyl radical
k_{2b}	$(7.77 \pm 0.46) \times 10^{5}\,M^{-1}min^{-1}$	Reaction of superoxide ion with NBT
k_3	$(9.0 \pm 1.8) \times 10^{10}\,M^{-1}min^{-1}$	Dismutation of superoxide ions (ref. 31)
k_4	$(2.11 \pm 0.13) \times 10^{6}\,M^{-1}min^{-1}$	Binding reaction of hemoglobin to membrane
k_{4b}	$(1.64 \pm 0.14) \times 10^{-8}\,min^{-1}$	Dissociation reaction of hemoglobin-membrane
k_5	$(4.94 \pm 0.16) \times 10^{8}\,M^{-1}min^{-1}$	Binding reaction of methemoglobin to membrane
k_{5b}	$3.07 \pm 0.10\,min^{-1}$	Dissociation reaction of methemoglobin-membrane
k_6	$1278.28 \pm 0.45\,M^{-1}min^{-1}$	Reaction of NBT with hemoglobin bound to membrane sites
k_7	$(1.31 \pm 0.12) \times 10^{10}\,M^{-1}min^{-1}$	Tetrazolinyl radical with Hb on membrane sites
k_8	$1267.81 \pm 0.11\,M^{-1}min^{-1}$	Reaction of NBT with antioxidants
k_9	$(1.81 \pm 0.24) \times 10^{8}\,M^{-1}min^{-1}$	Reaction of tetrazolinyl radical with antioxidants

hemoglobin: Hb, oxidized hemoglobin: metHb, membrane hemoglobin binding sites: m, reduced hemoglobin bound to the membrane: Hb-m, oxidized hemoglobin bound to membrane: metHb-m, reduced antioxidant: x and oxidized antioxidant: ox). Equation (1) shows the differential equation used for one of these species, oxidized NBT.

$$d[NBT]/dt = k_2^*[NBT^\bullet]^*[O_2] - k_{2b}^*[NBT]^*[O_2^{\bullet -}] - k_6^*[NBT]^*[Hb\text{-}m] - k_8^*[NBT]^*[x] \quad (1)$$

The whole series of differential equations can be used to generate curves for the time dependent change in concentration of these species. Furthermore, it is possible to perform a least square fit to the experimental curve by fitting the constants.

In order to obtain reliable values for the rate constants to be used for the red cells the four rate constants for Reactions 1 and 2 obtained in the hemoglobin reaction[5] involving the reactions of NBT with oxygen were all corrected by a single fitting parameter which reflected the different reactivities in the membrane (see above). The rate constant for superoxide dismutation was taken from the hemoglobin data. To further constrain the rate constants for Reactions 4–9 a set of rate constants were obtained which gave a satisfactory fit to a large number of samples with different initial rates of reduction (see below). The only parameters permitted to vary were the concentration of antioxidants, "x", and the concentration of hemoglobin membrane binding sites, "m". The rate constants obtained are given in Table I.

Although both hemoglobin and the antioxidants react with NBT they produce distinct changes in the time course for the reduction of NBT as shown in simulated Figures 6, 7 and 8. Figure 6 indicates the effect of increasing the concentration of antioxidants, "x". In this figure, the reaction of hemoglobin with NBT was eliminated by setting $m = 0$. This demonstrates that the initial rapid reduction of NBT (Figure 4) is

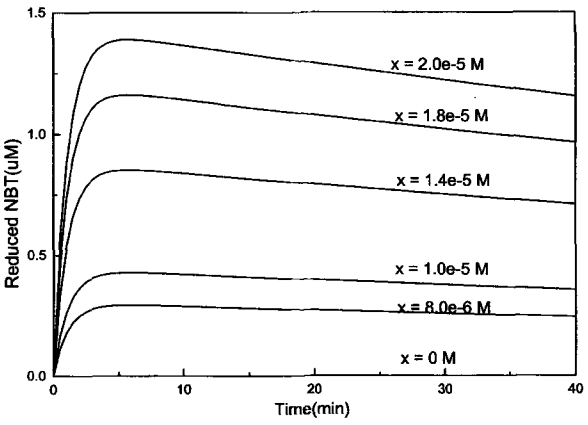

FIGURE 6 The contribution of the level of antioxidants, "x", to the kinetics of the reduction of NBT by RBCs at atmospheric pressure. For these simulations, the rate constants in Table I were used with $m = 0$, and "x" varied as shown.

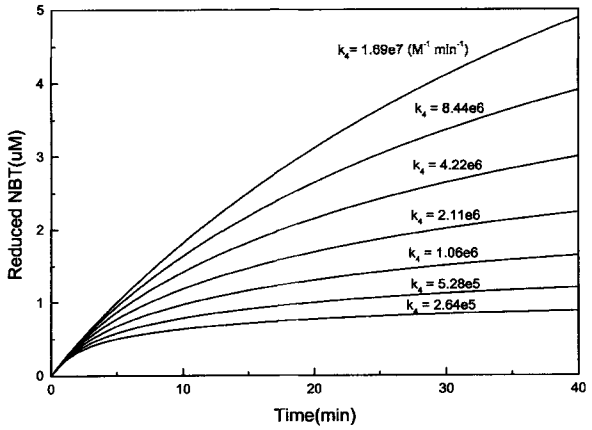

FIGURE 7 The effect of changing the rate constant for hemoglobin binding to membrane (k_4) on the reduction of NBT by RBCs. For these simulations, the rate constants in Table I were used except for k_4 that was varied as shown; $x = 0$, and $m = 1 \times 10^{-6}$ M.

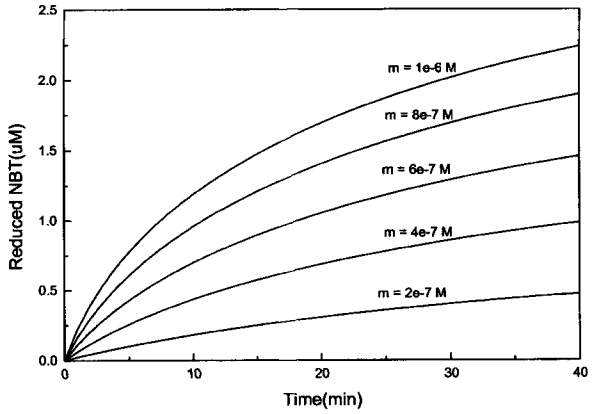

FIGURE 8 The effect of changing the concentration of heme-membrane binding sites, "m", on the reduction of NBT by RBCs at atmospheric pressure. The rate constants in Table I were used and "m" varied as shown.

attributed to the reaction of antioxidants. The actual slope of this initial reaction depends on the rate constants for the reaction of NBT with the antioxidants. At later times, because of the depletion of the antioxidants, a slow reversal takes place involving the reoxidation of NBTr.

The effect of changing the level of antioxidants in Figure 6 can be compared with the effect of increasing the binding constant for hemoglobin to the membrane (Figure 7) and the effect of increasing the value of "m", the concentration of membrane sites for hemoglobin (Figure 8). Both of these effects produce a more gradual increase in the reduction of NBT, but cannot generate the initial rapid reduction observed in red cell samples. This is true even though the rate constants for the reaction of hemoglobin with NBT and NBT$^{\bullet}$ (1.28×10^3 and 1.31×10^{10}) are actually somewhat greater than those for the reactions of NBT with antioxidants (1.27×10^3 and 1.81×10^8). This phenomenon is explained by the limited number of hemoglobin membrane binding sites. Unlike the reaction with antioxidants, only a small fraction of the hemoglobin is bound to the membrane and able to react with NBT at any time.

A comparison of the simulated curves in Figures 7 and 8 distinguish between the effect of increasing hemoglobin binding (Figure 7) and increasing the concentration of binding sites (Figure 8). Increasing this rate constant (k_4) for a given value of "m" affects the slope for the slower second phase of the reaction. The increased slope is indicative of increased efficiency of the recycling of hemoglobin through the "m" binding sites. The increase in "m" (Figure 8) produces a parallel increase in the magnitude of the second phase for NBT reduction as expected for an increase in the concentration of sites involved in the recycling process. The difference in the kinetics associated with the level of antioxidants and the hemoglobin-membrane interactions makes it possible to obtain information from the kinetics regarding both processes associated with NBT reduction (see below).

3.3. Red Cell Modifications Used to Confirm the Contribution of Antioxidants and Membrane Interactions to Red Cell Induced Reduction of NBT

In the analysis of the reduction of primarily membrane associated NBT by RBCs, we have postulated two processes which are responsible for the reduction. These are (1) the antioxidants

present in the cell which are able to directly reduce NBT and (2) hemoglobin which binds to the membrane. In order to demonstrate that these interactions do actually alter the reaction of NBT with red cells, we have changed the cellular concentrations of antioxidants by incorporating more antioxidants (vitamins C and E) into the red cell. At the same time red cell modifications (using cumene hydroperoxide and *N*-ethylmaleimide) have been performed which as a result of oxidative damage[6,22,23] alter the properties of the red cell membrane. The interaction of hemoglobin with band 3 of the membrane is controlled by the conformation of band 3, as well as interactions involving a number of other membrane proteins. Oxidative damage to any of these membrane proteins will influence the binding of hemoglobin to the membrane and, thereby, the reduction of NBT.

Prior to actually performing the necessary modifications of the red cell, it was necessary to determine whether the reagents used directly reduced NBT. Figure 9 shows that ascorbic acid and α-tocopherol reduce NBT, while glutathione, *N*-ethylmaleimide and cumene hydroperoxide do not.

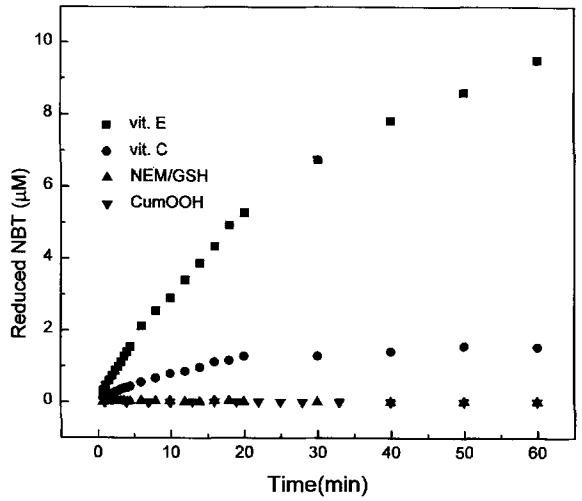

FIGURE 9 Reduction of NBT by vitamins C and E, glutathione, *N*-ethylmaleimide and cumene hydroperoxide in the absence of red blood cells.

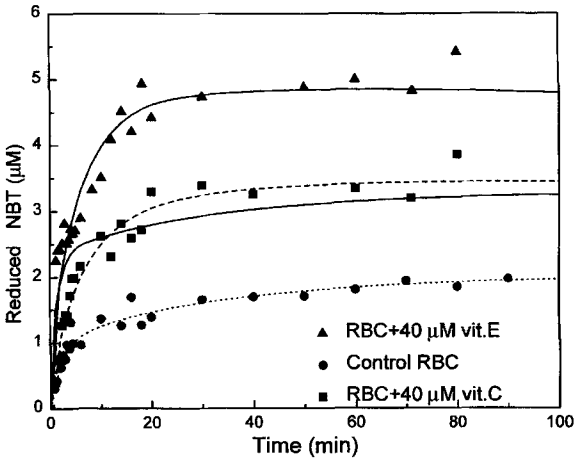

FIGURE 10 Effect of added vitamins C and E on the reduction of NBT by RBCs. (●) control RBCs, (■) control RBCs + 40 μM vitamin C; (▲) Control RBCs + 40 μM vitamin E. ..... Control RBCs data fit with model using rate constants of Table I. —— Vitamins C and E data fit with model setting x = 43.27 μM, the rate constants for the reaction of the antioxidant with NBT (k_8, k_9) were varied (see Table II) and --- vitamin C data fit with model varying only x, k_8, k_9 (see Table II for R^2 values).

Since vitamins C and E directly reduce NBT, the addition of these vitamins to red blood cells are expected to increase the reduction of NBT. Figure 10 shows the effect of increasing the concentration of these two antioxidants by 40 μM. The expected increase in the reduction of NBT is clearly discerned.

Since vitamin E is membrane soluble, all of the added vitamin E is expected to react with NBT predicting an increase in "x" of 40 μM. The results for vitamin E in Figure 10 have been analyzed by increasing the value of "x" by 40 μM. In order to fit this data, the only parameters adjusted (Table II) were the rate constants for the reaction of the antioxidants with NBT (k_8) and the tetrazolinyl radical (k_9). These constants, which represent the average rate constant for all the antioxidants present, are expected to change when additional vitamin E is added.

The reduction of NBT by ascorbic acid in the red cell has previously[24] been attributed to the involvement of ascorbate as the electron donor for a transmembrane oxidoreductase present in the red cell membrane. NBT was assumed to be

TABLE II Effect of vitamins C and E on the reduction of NBT by RBCs

Sample	k_8 ($M^{-1}min^{-1}$)	k_9 ($M^{-1}min^{-1}$)	R^2	x(μM)
Control	1267.81 ± 0.11	$(1.81 \pm 0.24) \times 10^8$	0.96	3.27 ± 0.28
†Control + 40 μM Vit. C	2692.91 ± 295.60	$(2.09 \pm 0.53) \times 10^5$	0.56	43.27 ± 0.28
†Control + 40 μM Vit. E	507.13 ± 35.00	$(9.72 \pm 1.59) \times 10^6$	0.92	43.27 ± 0.28
*Control + 40 μM Vit. C	364.08 ± 52.00	$(1.04 \pm 0.50) \times 10^7$	0.94	16.41 ± 0.27

† Vitamins C and E modified RBCs data fitted by assuming x equals control conc. + added vitamin conc. (43.27 μM), adjusting only k_8 and k_9.
* Vitamin C data fitted by adjusting k_8, k_9 and x.

the electron acceptor for this reaction. This process would explain the reduction of membrane bound NBT by cytoplasmic ascorbic acid. An additional mechanism for the reaction of vitamin C with membrane bound NBT could involve the cycling between vitamin C and vitamin E,[25,26] by which vitamin C re-oxidizes vitamin E. This process maintains the cellular membrane antioxidant capacity and would further reduce NBT. Both of these mechanisms do not necessarily predict a quantitative oxidation of vitamin C by membrane bound NBT. The contribution of only a fraction of the added vitamin C to the value of "x" is consistent with the smaller increase in NBT reduction for vitamin C (Figure 10). The analysis of the vitamin C data by adjusting both the value of "x" as well as k_8 and k_9 is shown by the dashed line through the vitamin C data in Figure 10. In this case (Table II), the value of x increased by only 13.14 μM which corresponds to 32.9% of the added vitamin C. As indicated by the solid line through the vitamin C data line in Figure 10, it was possible to fit the vitamin C data (although the fit was clearly not as good) increasing the value of x by 40 μM as done for vitamin E, and only adjusting the values for k_8 and k_9.

The reduction of stearic acid nitroxide radicals incorporated into red cell membranes[27] by red cell antioxidants has previously been suggested as a method to evaluate the ability of red cell antioxidants to protect the red cell membrane. Surprisingly, even though the nitroxide is associated with the membrane, vitamin E, associated with the membrane had almost no effect, while the dominant effect was attributed to cytoplasmic vitamin C. The difference between our method and the nitroxide method[27] can be explained by the difference in the kinetics for the reactions of NBT and the spin label. Unlike the spin label, which only undergoes one reduction step, the subsequent reduction of the tetrazolinyl radical to the formazan, k_9, is orders of magnitude higher than the initial reduction, k_8. This amplifies the effect of slowly reacting antioxidants. Thus, the NBT method is able to detect antioxidants which could not be detected by the spin label method.

Notwithstanding the very rapid reaction of the tetrazolinyl radical with antioxidants, our NBT based assay requires that the antioxidant is either associated with the membrane and/or interacts, even transiently, with the membrane. Antioxidants which function exclusively in the cytoplasm should not reduce NBT. These antioxidants as well as enzymes with specific functionality which will not reduce NBT do not contribute to the antioxidant capacity measured by the NBT assay. Glutathione a cytoplasmic antioxidant present at high concentrations also does not react with NBT (Figure 9) even when added in solution, and can therefore not contribute to the NBT determined measure of antioxidants.

NEM, a reagent which readily reacts with sulfhydryl groups, does not directly react with NBT (Figure 9). Its reaction with cytoplasmic sulfhydryl groups including those on glutathione is not expected to influence the reduction of NBT

by red cells. However, NEM also reacts with sulfhydryl groups on membrane proteins and has been implicated as providing oxidative stress to the red cell membrane.[7,28] As shown in Figure 2, red cell ghosts in the absence of hemoglobin do not reduce NBT. The same result shown in Figure 2 was observed even if the ghost sulfhydral groups were blocked by NEM (data not shown). Oxidative damage to the red cell membrane, which alters hemoglobin binding explains the effect of NEM on NBT reduction shown in Table III. It has no effect on the antioxidant capacity, "x", but does produce an increase in both "m" and k_4.

The effect of membrane damage on hemoglobin-membrane interactions was confirmed by reacting the red cell with cumene hydroperoxide, which reacts exclusively with the membrane and is known to damage[22] the membrane. Cumene hydroperoxide does not directly react with NBT (Figure 9). Therefore, the enhanced NBT reduction by cumene hydroperoxide should be attributed to enhanced binding of hemoglobin to the membrane. This prediction is consistent with the results shown in Table IV where no change in "x" was observed, but increases in both "m" and k_4 were observed.

3.4. Relationship of x and m to Red Cell Membrane Properties

Our analysis indicates that the kinetics for the reduction of NBT by red blood cells provides information regarding (1) the antioxidants available to protect the red cell membrane from oxidative stress and (2) the interactions between hemoglobin and the red cell membrane. The model described above was used to fit the NBT reduction by RBCs for a number of different subjects. In fitting the data, all the rate constants (Table I) were kept the same. The only variable fitting parameters were "x", which is a measure of the red cell membrane antioxidant capacity and "m", which reflects the interaction of hemoglobin with the membrane.

From the analysis of the kinetics of NBT reduction in RBC samples from these different subjects, the range of values for "x" are between 0.61 and 10.97 μM (Figure 11). These values should reflect the total antioxidant capacity of the red cell membrane, which should include both membrane associated antioxidants like vitamin E as well as cytoplasmic antioxidants, which interact with the membrane and/or membrane antioxidants, like vitamin C. There is

TABLE III Effect of *N*-ethylmaleimide (NEM) on the reduction of NBT by RBCs

		NEM		
	Control RBC	5 mM	10 mM	50 mM
x(10^{-6}M)	1.46 ± 0.11	1.46 ± 0.11	1.46 ± 0.11	1.46 ± 0.11
k_4 (10^6M^{-1} min^{-1})	2.11 ± 0.13	2.18 ± 0.17	2.67 ± 0.29	15.53 ± 3.06
m(10^{-6} M)	0.42 ± 0.02	0.44 ± 0.02	0.56 ± 0.02	1.50 ± 0.05
R^2	0.92	0.80	0.92	0.88

TABLE IV Effect of cumene hydroperoxide on the reduction of NBT by RBCs

		CumOOH		
	Control RBCs	20 μM	30 μM	40 μM
x(10^{-6}M)	7.07 ± 0.52	7.07 ± 0.52	7.07 ± 0.52	7.07 ± 0.52
k_4 (10^{-6}M^{-1}min^{-1})	2.11 ± 0.13	2.93 ± 0.88	8.60 ± 1.60	14.09 ± 1.71
m (10^{-6}M)	0.51 ± 0.07	0.64 ± 0.11	0.69 ± 0.09	0.79 ± 0.06
R^2	0.92	0.85	0.97	0.94

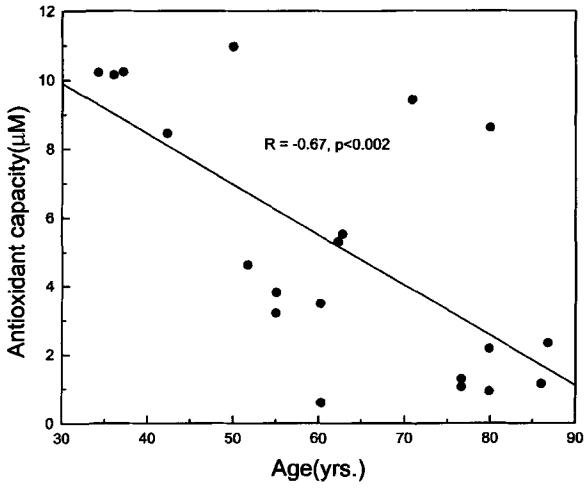

FIGURE 11 Correlation of membrane antioxidant capacity, "x", (for a 1×10^{-4} M heme suspension) with age.

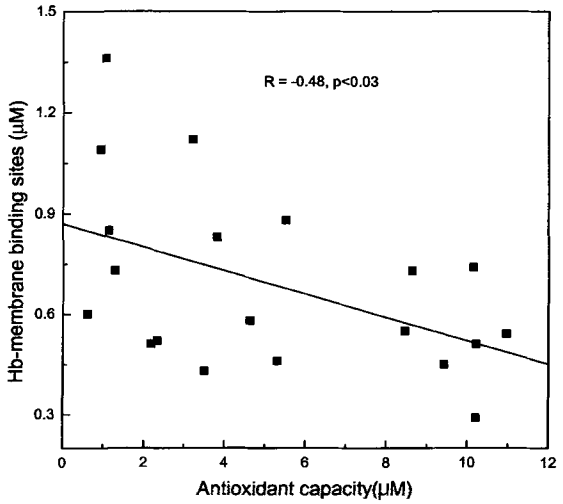

FIGURE 12 Negative correlation of membrane antioxidant capacity (for a 1×10^{-4} M heme suspension) with concentration of hemoglobin-membrane binding sites, "m".

isolated data on the level of certain antioxidants in the red cell,[29–33] and in one study[33] they determined the antioxidant chain breaking activity in red cell membrane and plasma lipid extracts. This lipid extract contain only the lipid soluble antioxidants which were shown to predominantly involve vitamin E. However, the antioxidant activity, measured in plasma by this method corresponded to only a fraction of the total plasma antioxidant activity[34] and was much less than the red cell value measured by our method (see below). Since no data is available which estimates the total antioxidant capacity in the red cell, we have compared the total antioxidant capacity determined in our study with the total antioxidant capacity reported for plasma. In order to make this comparison, it is necessary to correct our values of antioxidant capacity determined for a 1×10^{-4} M heme suspension to the ~20 mM heme concentration present in red cells.[35] This correction results in a 0.1–1.68 mM concentration of antioxidants in the red cell after normalizing for the hemoglobin concentration present in whole blood, which is within the range of the values of total antioxidant activity determined for the plasma by the "total radical-trapping antioxidant parameter (TRAP) analysis".[34,36]

From the analysis of the kinetics of NBT reduction in RBC samples the range of values for "m" was 0.29–1.36 μM (Figure 12). This parameter should reflect the concentration of hemoglobin binding sites on the membrane which result in facilitated reduction of NBT dissolved in the red cell membrane. The strongest binding sites for hemoglobin to the membrane are on band 3.[12–14] There are, however, a greater number of weaker hemoglobin binding sites on the membrane,[15] which can also contribute to the facilitated NBT reduction. We have previously[37] determined the stoichiometry for the interaction of hemoglobin with NBT incorporated into red cell membranes by studying the enhanced rate of NBT reduction (see Figure 2), which depends on the hemoglobin/membrane ratio. On the basis of these earlier studies it was determined that there are $\sim 7 \times 10^6$ hemoglobin tetramer sites which interact with membrane bound NBT per red cell. In order to relate the binding sites of hemoglobin to the membrane to our determination of "m" for a 1×10^{-4} M heme concentration, it is necessary to first estimate the number of cells present in our experiment in terms of the mean cell hemoglobin

(MCH) of ~30.[38] On the basis of this estimation an estimated value of "m" equal to 0.62 μM is predicted. This value is within the range found in the human subjects studied (Figure 12).

3.5. Aging Effect on x and m

A number of red cell properties including cell volume[39] and deformability[40] have been shown to change during subject aging. It has been suggested that many of these changes are associated with enhanced oxidative stress experienced by the red cells in older subjects. This increased oxidative stress is thought to be associated with increased radical formation and/or decreased antioxidant levels.[41] We have studied 20 different subjects ranging from 34 to 87 years of age. Our analysis indicates that the kinetics for the reduction of NBT by red blood cells provides information regarding (1) the antioxidants available to protect the red cell membrane from oxidative stress and (2) the interactions between hemoglobin and the red cell membrane. Figure 11 shows that as a function of age the value of "x", the measure of the antioxidants available to protect the red cell membrane, significantly decreased ($R = -.67$; $p < .002$). This reflects a loss in the antioxidant capacity of the red cell membrane during aging. Despite results, which suggest that antioxidant levels in the plasma generally increase with age,[42] these results suggest that the red cell membrane should be more susceptible to oxidative damage in older subjects. This finding is however, consistent with the reported decrease[42] in total plasma antioxidant capacity in male subjects past 74 years of age.

These results indicate that the red cell membrane in the aged has a lower antioxidant capacity. A possible relationship between this lower antioxidant capacity and red cell oxidative damage is shown in Figure 12. In this figure it is shown that the antioxidant capacity "x" is negatively correlated with the concentration of hemoglobin membrane binding sites "m". This data suggests that an increase in the concentration of hemoglobin membrane interactions may be associated with increased oxidative damage resulting from a lower level of membrane antioxidants.

It has been reported[43] that hemoglobin membrane interactions are regulated by the conformation of band 3, which is influenced by interactions with ankyrin, band 4.1 and glycophorin. Membrane damage, which influences these interactions, could increase hemoglobin binding. This hypothesis is supported by reports[44] that hemoglobin membrane interactions increase in old cells that have undergone oxidative damage. Furthermore, hemoglobin binding to the membrane provides a mechanism for oxidants generated during hemoglobin oxidation to escape the red cell protective processes.[1,37] This possibility suggests a synergistic relationship, whereby oxidative stress damages the membrane causing enhanced binding of hemoglobin to the membrane. This enhanced binding results in non-neutralized reactive oxygen species which further damage the red cell membrane amplifying red cell oxidative damage.

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