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Spin Label Studies of Radical Reduction in Normal and Sickle Erythrocyte Membranes Associated with Antioxidants

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SPIN LABEL STUDIES OF RADICAL REDUCTION IN NORMAL AND SICKLE ERYTHROCYTE MEMBRANES ASSOCIATED WITH ANTIOXIDANTS

by

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To My Parents and Children

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LIST OF ABBREVIATIONS

5-DSA	5-doxylstearic acid
16-DSA	16-doxylstearic acid
AA	ascorbate, ascorbate acid, vitamin C
ADSOL	adenine saline solution
AFR	ascorbate free radical, ascorbyl radical
AO	ascorbate oxidase
Avg	average
BSA	bovine serum albumin
BSA*	spin-labeled bovine serum albumin
BSKG	buffered saline with potassium and glucose, pH 7.4
CAT	catalase
CCl_3CO_2H	trichloroacetic acid
CO	carbon monoxide
CPDA	citrate phosphate dextrose with adenine
CV	coefficient of variability
DEAE	diethylaminoethyl
DHA	dehydroascorbate, dehydroascorbic acid
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
$FeCl_3$	ferric chloride
GPx	glutathione peroxidase

GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
H_3PO_4	phosphoric acid
Hb	hemoglobin
HbA	normal adult human hemoglobin
HbCO	carbon monoxide ligated hemoglobin
HbO_2	oxygen ligated hemoglobin
HbS	sickle hemoglobin
HCOH	formaldehyde
HOCl	hypochlorous acid
ISC	irreversibly sickle cell(s)
K_2HPO_4	potassium phosphate, dibasic
KCl	potassium chloride
KH_2PO_4	potassium phosphate, monobasic
KOH	potassium hydroxide
$L^{\cdot}$, $R^{\cdot}$	alkyl radical
$LO^{\cdot}$, $RO^{\cdot}$	alkoxy radical
$LOO^{\cdot}$, $ROO^{\cdot}$	peroxyl radical
LOOH, ROOH	hydroperoxide
Mb	red cell membrane
Mb*	spin-labeled red cell membrane
$MgCl_2$	magnesium chloride
Na_2HPO_4	sodium phosphate, dibasic
NaCl	sodium chloride

NAD	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NaH_2PO_4	sodium phosphate, monobasic
NaOH	sodium hydroxide
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
NO	nitroxide
NOH	hydroxylamine
$^1O_2$	singlet oxygen
O_2^-	superoxide
·OH	hydroxyl radical
PBS	phosphate buffer saline, pH 8.0
RBC	red blood cell(s)
RBC*	spin-labeled red blood cell(s)
rpm	revolution per minute
RS·	thiyl radical
RSOO·	thiyl peroxyl radical
SCA	sickle cell anemia
SD	standard deviation
SEM	scanning electron microscopy
SOD	superoxide dismutase
TCA	trichloroacetic acid
Tris	tris-(hydroxymethyl)aminomethane
UV	ultraviolet
vs	versus

CHAPTER I

INTRODUCTION

1.1 Free Radical Formation and Antioxidant System in Human Erythrocytes

Free radicals and reactive oxygen species, such as superoxide (O_2^-), hydroxyl radical (·OH), alkyl radical (L· or R·), alkoxyl radical (LO· or RO·), peroxyl radical (LOO· or ROO·), thiyl radical (RS·), thiyl peroxy (RSOO·), hydrogen peroxide (H_2O_2), hydroperoxide (LOOH or ROOH), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$), are oxidants. They are generated endogenously and exogenously throughout our lifetime. Endogenous sources of oxidants are from all the aerobic cellular metabolism such as mitochondrial respiration, phagocytosis, hemoglobin auto-oxidation, and the activities of some oxidases, dehydrogenases, and peroxidases. Examples of external sources of oxidants are natural dietary constituents, UV radiation, hyperoxic air, chemicals, drugs, and environmental pollutants such as automotive exhaust, cigarette smoke, and ozone (Ames, 1983; Cerutti, 1985; Cross *et al.*, 1987; Halliwell & Gutteridge, 1989).

In the past decade, it has become increasingly evident that oxidants are involved in a variety of human diseases as they play roles in cellular destruction: poisoning vital enzymes; bashing nicks, crosslinks, and disruptive additions or adducts into DNA; inciting normally harmless chemicals to toxicants and carcinogens; tearing apart polysaccharides; and touching off peroxidase chain reactions that unravel membranes (Hooper, 1989; Halliwell, 1991). With the recognition that oxidative damage is an unavoidable part of our life, there has been growing interest in elucidating the mechanism of the cellular antioxidant system. Aerobic

organisms are endowed with various antioxidants including enzymes and small biological molecules. In addition, antioxidant vitamins are taken up from nutrient diet. Normally, metabolic activity is able to control or prevent adverse effects of oxidants. The susceptibility of a given organism to oxidative stress is a function of the overall balance between the factors that exert oxidative stress and those that exhibit antioxidant capability. Therefore, oxidative damage can be described as a consequence of excessive oxidative stress or insufficient antioxidant potential (Chow, 1979; Sies, 1991).

Red blood cells (RBC), as oxygen carriers in the body, are especially vulnerable to the potential hazards of an aerobic environment since they are laden with iron, a transition metal conducive to oxygen radical formation, and their membrane with high polyunsaturated lipid are exposed to an endless flux of oxygen. The oxidant challenge almost certainly begins with generation of O_2^-, for which the normal daily oxidation of hemoglobin in circulating erythrocytes serves as a relentless source (Chiu *et al.*, 1982; Caughey & Watkins, 1985). Then, O_2^- is converted into H_2O_2 by enzymatic or non-enzymatic dismutation. The presence of both O_2^- and H_2O_2 creates the possibility for appearance of ·OH, as classically indicated by the Haber-Weiss reaction:

$$O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$$

This equation actually is the sum of following two reactions in which a catalytic iron cycles between two valence states:

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + \cdot OH + OH^-$$

$$Fe^{+3} + O_2^- \longrightarrow Fe^{+2} + O_2$$

Thus, the reaction of H_2O_2 with ferrous iron is the actual ·OH generating step, known as the Fenton reaction. Due to its extreme reactivity, ·OH can damage almost any biological molecule it encounters. Attack of ·OH on biological molecules can proceed by addition, hydrogen atom abstraction, or electron transfer reactions (Halliwell & Gutteridge, 1989). In many cases, carbon-centered radicals (L·) are produced that can react with O_2 to give LOO·.

$$L\text{-}H + \cdot OH \longrightarrow L\cdot + H_2O$$

$$L\cdot + O_2 \longrightarrow LOO\cdot$$

Formation of LOO· is a major chain-propagating step in lipid peroxidation. The process of lipid peroxidation has been shown to be associated with the loss of membrane polyunsaturated fatty acids (Dodge *et al.*, 1966; Jacob & Lux, 1968), the formation of hydroperoxides and other secondary products (Stocks & Dormandy, 1971), the cross-linking of membrane proteins (Haest *et al.*, 1977; Palek & Liu, 1979), and the decreased activity of membrane enzymes (O'Malley *et al.*, 1966; Buckley *et al.*, 1975; Chow, 1978). The changes in lipids, proteins, and enzyme activities of red cell membranes after peroxidant injury inevitably lead to changes in membrane structure and function, and ultimately cause cell hemolysis.

Oxidative damage to hemoglobin also has been shown to cause changes in hemoglobin structure and function (Jacob, 1970; Nagel & Ranney, 1973; Rachmilewitz, 1974). Such changes ultimately result in hemoglobin denaturation and precipitation inside the red cells as Heinz bodies. The physical presence of these inclusion bodies reduces red cell deformability and contributes to the hemolytic event (Rifkind, 1965; Jacob *et al.*, 1968; Chiu *et al.*, 1982).

In addition to being a target for oxidative damage, the mature human erythrocyte is an anucleated cell that lacks protein synthesis machinery and is therefore unable to replace damaged components. However, perhaps as an evolutionary consequence of this deficiency, the erythrocyte devotes much of its metabolic activity toward defense against oxidant challenge.

Erythrocyte antioxidant mechanisms may be divided into two parts: detoxification and damage repair (Hebbel, 1986). The systems that function to detoxify oxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), and vitamin E. The systems considered for damage repair are the hexose monophosphate shunt (also called pentose phosphate shunt) and other enzymes such as methemoglobin reductase, glutathione reductase, and glutathione-S-transferase. It is generally

considered that the primary and major antioxidant systems in RBC are the detoxification systems, since they are involved in the direct elimination of oxidants (Chiu *et al.*, 1982; Sun, 1990). The other systems exist to repair damage by decreasing methemoglobin and peroxide levels, and to the help detoxification system by maintaining a steady supply of reducing equivalents for the primary antioxidant enzymes. A brief review of the known and proposed mechanisms about some of major antioxidants is presented in Appendix II. Details of providing reducing equivalents in reactions that are critical for primary antioxidants are presented in the Discussion section.

1.2 Nitroxide Spin Label

Nitroxide spin label is a synthetic organic compound containing the nitroxide group (>N-O·) with an unpaired electron located close to the oxygen atom. Due to the sterically hindered structure (four alkyl groups around it), the unpaired electron in nitroxide spin labels is relatively stable in both solution and biological environments. Electron paramagnetic resonance (EPR) spectra of nitroxide spin labels are very sensitive to the rate of molecular motion. The rapid isotropically tumbling nitroxide gives a spectrum of three sharp lines. If the rate of molecular motion decreases, differential broadening of the three-line nitroxide spectrum occurs. Both the nitroxide chemical character and EPR techniques render nitroxide spin labels useful as reporters for molecular studies of labeled systems. Nitroxides have been used extensively to study motion in membranes and macromolecules (Likhtenstein, 1976; Berliner, 1979), membrane surface and transmembrane potentials (Mehlhorn & Packer, 1979; Mehlhorn & Probst, 1982), and oxygen concentration in cells (Lai *et al.*, 1982; Morse II & Swartz, 1985). Nitroxides are also under active investigation as metabolically responsive contrast or imaging agents for *in vivo* nuclear magnetic resonance (NMR) or EPR imaging (Brasch, 1983; Keana *et al.*, 1987; Sentjurc *et al.*, 1991). All of these uses have led to an increased need for nitroxides with differing chemical properties, especially in regard to their

distribution and reduction rate in biological systems. Spin label reductions of various nitroxides to diamagnetic hydroxylamines (Chen & Swartz, 1989) have been found to depend upon many factors including the characteristics of the spin label (Swartz *et al.*, 1986; Eriksson *et al.*, 1986; Perussi *et al.*, 1988; Belkin *et al.*, 1987), the system investigated (Stier & Reitz, 1971; Giotta & Wang, 1972; Quintanilha & Packer, 1977; Baldassare *et al.*, 1974; Couet *et al.*, 1985; Chen *et al.*, 1988) and the environmental conditions with particular emphasis on oxygen concentration (Swartz *et al.*, 1986; Keana *et al.*, 1987; Chen *et al.*, 1989).

Fatty acid nitroxide spin labels with the nitroxide moiety on the acyl chain are lipophilic and able to position themselves into the bilayer of membrane. The reduction of fatty acid nitroxide spin labels has been studied in many biological systems such as mitochondria (Quintanilha & Packer, 1977; Mehlhorn & Packer, 1982), *Escherichia coli* membrane vesicles (Baldassare *et al.*, 1974), mouse thymus-bone marrow cells and Chinese hamster ovary cells (Chen *et al.*, 1988). It is generally believed that enzymatic reductions by the electron transfer systems are involved in these tissues and cells.

For human erythrocytes, the reduction of some water-soluble nitroxide spin labels have also been observed (Ross & McConnell, 1975; Craescu *et al.*, 1982; Eriksson *et al.*, 1986; Branca *et al.*, 1988; Mehlhorn, 1991). It has been suggested that ascorbate and glutathione are possible intracellular reductants for the water-soluble nitroxides. However, it has been reported that fatty acid spin labels are stable in normal mice erythrocytes over a time period of 30 min (Deslauriers *et al.*, 1987).

We have monitored the EPR signals of fatty acid spin labels in human normal erythrocyte for a longer period of time under somewhat different conditions, and observed nitroxide reduction with a pseudo first-order initial rate constant of about 4×10^{-3}/min (Fung & Zhang, 1990). This reduction reflects antioxidant activity toward nitroxide radicals in cell membrane and thus can be used to monitor the capacity of intracellular antioxidant system for radicals in human erythrocyte membranes.

1.3 Overview of Sickle Cell Disease Studies

The existence of sickle-shaped erythrocytes in human blood was first discovered by Herrick in the early part of this century (Herrick, 1910). Afterwards, many case reports and small series from which most of the clinical features and pathological changes in the disease have emerged. In general, the clinical manifestations of this disease are chronic hemolytic anemia and occasional painful crisis resulting from tissue infarct caused by vascular occlusion. The alteration of hemoglobin molecule in sickle cells was identified with electrophoretic mobility by Pauling and his colleagues in 1949 (Pauling *et al.*, 1949). Then, Ingram (1956) showed that the molecular abnormality was due to the substitution of a single amino acid, valine for glutamic acid residue at the sixth position of hemoglobin β chain. In the last quarter of a century, understanding of the molecular pathophysiology, diagnosis, and treatment of sickle cell disease has come from striking results in the application of x-ray diffraction, electron microscopy, thermodynamic and kinetic analyses, NMR spectroscopy, restriction enzyme mapping, and differential gene expression studies (Schechter *et al.*, 1987).

The deoxygenated sickle hemoglobin (HbS) has an abnormally low solubility and aggregates to form long fibers, which deform red cells to give their sickle shape (Dobler & Bertles, 1968; Edelstein *et al.*, 1973). Upon oxygenation, the aggregated HbS reverts to the soluble state and the majority of sickled cells regain the normal biconcave shape. However, a fraction of the sickled cells retain the abnormal morphology even under fully oxygenated condition (Bertles and Dobler, 1969). These irreversibly sickled cells (ISC) are dehydrated cells with increased intracellular hemoglobin concentrations (Clark *et al.*, 1978) and increased internal viscosity (Chien *et al.*, 1970; Kaul *et al.*, 1983). They are much less deformable than normal shaped cells (Clark *et al.*, 1980) and may contribute to the initiation of microvascular occlusion in sickle cell disease. It is generally believed that the ISC abnormal morphology is the result of an irreversible alteration of their membrane. Evidence is provided by the findings that ISC maintain their distorted shapes upon re-oxygenation even after the

intracellular hemoglobin is removed (Lux *et al.*, 1976; Smith *et al.*, 1982). Since the 1950s, many abnormalities in sickle cell membranes, such as increased cation leak (Tosteson *et al.*, 1952; Glader & Nathan, 1978; Joiner *et al.*, 1986; Joiner, 1990), increased calcium content (Eaton *et al.*, 1973; Bookchin & Lew, 1980), altered phospholipid distribution and membrane organization (Lubin & Chiu, 1981; Wagner *et al.*, 1985; Zachowski *et al.*, 1985; Mohandas *et al.*, 1985; Choe *et al.*, 1986; Middelkoop *et al.*, 1988), decreased activity of Ca^{+2}-Mg^{+2} ATPase (Dixon & Winslow, 1981; Niggli *et al.*, 1982; Leclerc *et al.*, 1987), abnormal protein phosphorylation (Dzandu & Johnson, 1980; Johnson *et al.*, 1986) and methylation (Ro *et al.*, 1981), have been detected.

Besides its low solubility, HbS is unstable in terms of giving rise to denatured hemoglobin (Asakura *et al.*, 1974). This HbS instability has been directly demonstrated by increased autoxidation of HbS (Hebbel *et al.*, 1988). The denaturation of HbS is believed to occur initially through oxidation to methemoglobin, followed by further oxidation to hemichromes and globin. HbS and hemichromes formed from HbS have a high affinity for binding to cell membrane (Fischer *et al.*, 1975; Fung *et al.*, 1983). The shift of iron from cytoplasm to membrane leads to the exposure of the membrane to oxidative species such as superoxide, peroxide, and hydroxyl radicals, which are the products of heme-iron autoxidation and subsequent reactions. It has been reported that sickle RBC from sickle cell anemia patients spontaneously generate double amounts of oxygen radicals compared with normal RBC (Hebbel *et al.*, 1982). Increased oxidation of lipids (Das & Nair, 1980; Chiu *et al.*, 1982) and skeletal proteins constituents (Rank *et al.*, 1984; 1985) of sickle cell membrane has also been demonstrated. Therefore it is generally believed that sickle cell membranes are particularly susceptible to oxidant damage.

On the other hand, some of the antioxidant systems in sickle cells appear to be abnormal. Generally, individual antioxidant activity were measured in the sickle cell anemia (SCA) studies. For example, it has been reported that SCA patients have increased GPx

activities (Chiu and Lubin, 1979; Beretta *et al.*, 1983; Lachant *et al.*, 1983), but decreased SOD activities (Schacter *et al.*, 1985; 1988), or decreased GPx activities (Das and Nair, 1980) and increased SOD activities (Das and Nair, 1980; Beretta *et al.*, 1983). It is also reported that sickle cells have abnormal pyridine nucleotide contents and impaired methemoglobin reduction (Zerez *et al.*, 1988; 1990). However, it is difficult to detect oxidative species such as superoxide, peroxide and hydroxyl radicals in membranes due to their extreme reactivity or their very short life times. Therefore, it is difficult to assess the effectiveness of the cellular antioxidants on radicals in membranes. In addition, the antioxidants exert concerted efforts toward oxidant stress. The decrease of one antioxidant may be compensated by the increase of another antioxidant.

In this work, the spin label reduction method has been applied to sickle erythrocytes to study the radical reduction efficiency in membranes of sickle cells as compared with normal cells. Besides the measurement of spin label reduction in normal and sickle cells, the concentrations of several major antioxidants, such as SOD, CAT, GPx, vitamin E, and ascorbate, have been determined on the same samples used for the spin label studies in order to determine the effects of these individual antioxidants on the radical reduction rates in erythrocyte membranes.

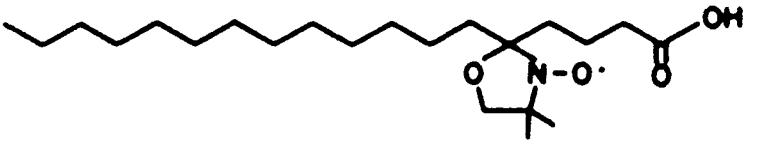
CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

All chemicals used for this dissertation work were of reagent grade.

Fatty acid spin probe, 5-doxylstearic acid (5-DSA), was purchased from either Syva Research Chemicals (Palo Alto, CA) or Aldrich Chemical Co., Inc. (Milwaukee, WI) and used without further purification.



5-DSA

Sodium phosphate (NaH_2PO_4 and Na_2HPO_4), sodium chloride (NaCl), potassium chloride (KCl), potassium hydroxide (KOH), and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Pittsburgh, PA); potassium phosphate (KH_2PO_4 and K_2HPO_4), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), L-ascorbic acid (vitamin C), glutathione (reduced form, GSH), superoxide dismutase (EC 1.15.1.1), glutathione peroxidase (EC 1.11.1.9), ascorbate oxidase (EC 1.10.3.3), xanthine oxidase (EC 1.1.3.22), formaldehyde dehydrogenase (EC 1.2.1.1), formaldehyde (HCOH), xanthine, β-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), β-nicotinamide adenine dinucleotide (reduced form, NADH), β-nicotinamide adenine dinucleotide (NAD), cysteine, N-ethylmaleimide (NEM), and hydrogen peroxide (H_2O_2) from Sigma Chemical Co.

(St. Louis, MO); D-(+)-glucose, ferric chloride ($FeCl_3$), 2,2'-dipyridyl (α,α'-dipyridyl), trichloroacetic acid (CCl_3CO_2H), and phosphoric acid (H_3PO_4) from Aldrich Chemical Co., Inc. (Milwaukee, WI); magnesium chloride ($MgCl_2$) from J. T. Baker Chemical Co. (Phillipsburg, NJ); glutathione reductase (GR) from Calbiochem Corporation (La Jolla, CA); katalase (catalase) from Boehringer Mannheim GmbH (Germany); *d*-α-tocopherol (vitamin E) from Eastman Kodak Co. (Rochester, NY); silicone liquid (200 fluid) from Dow Corning Corporation (Midland, MI); super-heme buffer, lysate reagent, and cellulose acetate plates from Helena Laboratories (Beaumont, TX); absolute ethyl alcohol (200 proof pure ethanol) from Aaper Alcohol & Chemical Co. (Shelbyville, KY).

2.2 Red Blood Cells

Human adult normal RBC were obtained from a local blood bank (Red Cross/LifeSource), with CPDA-1 or ADSOL used for anticoagulant. Sickle RBC samples from adult homozygous patients with sickle cell anemia disease were collected into heparinized, EDTA, or citrate-coated tubes at either Mount Sinai Hospital in Chicago or the George Washington University in Washington, D.C.. Hemoglobin (Hb) electrophoresis was performed on each sickle blood sample in our laboratory and only the blood samples with over 90 % sickle Hb were used for this study.

The RBC were washed with an isotonic buffer solution of either 5 mM sodium phosphate buffer with 150 mM NaCl at pH 8.0 (phosphate buffer saline, PBS), or sodium phosphate buffer (1.4 mM NaH_2PO_4 and 8.6 mM Na_2HPO_4) with 135 mM NaCl, 5 mM KCl, and 11 mM glucose at pH 7.4 and osmolality 290 ~ 300 mmol/kg (buffered saline with potassium and glucose, BSKG). The buffer solutions were gassed with carbon monoxide (CO) at 4 °C for at least 30 min before use. To about 5 ml of packed RBC, 35 ml of PBS were added and mixed gently with a plastic pipette, then centrifuged at 4,000 rpm (1,912 g) for 4 min with a Sorvall RT6000B refrigerated centrifuge. Supernatant containing plasma materials

and buffy coat was removed by aspiration after the centrifugation. The washing of RBC with PBS was repeated two more times, and all steps were performed at 4 °C.

The washed blood cells were checked optically, using optical cells with very narrow light paths (0.01 mm), at wavelengths of 540 nm and 569 nm (Antonini & Brunori, 1971) to ensure that the Hb molecules inside the cells were fully CO-liganded. For cells that needed to be oxygenated, O_2-gassed buffers, instead of CO-gassed buffers, were used to wash the RBC. Again to ensure all Hb molecules inside the cells were O_2-liganded, optical spectra of these cells at 541 nm and 577 nm (Antonini & Brunori, 1971) were obtained.

2.3 Spin-Labeled RBC

5-DSA was stored in ethanol solution at a concentration of about 0.5 mg/ml. Spin labels in ethanol (1.0 ml) were transferred into a small brown container, and ethanol was evaporated to dryness with nitrogen gas at room temperature to give a thin film of spin labels on the container walls. About 10 ml of fatty-acid-free bovine serum albumin (BSA) (2.2 mg/ml) in PBS solution was added to the container. The solution was stirred for at least 4 hr at room temperature to give a spin-labeled BSA (BSA*) solution, with a spin label concentration of about 5×10^{-5} mM, as determined by EPR measurements.

5-DSA were introduced into RBC membranes by mixing 50 μl packed RBC (hematocrit > 95 %) with 1.0 ml of the BSA* solution for about 30 min at 4 °C, then the mixture was washed with cold PBS again to remove excess BSA*. To avoid echinocyte formation (Hubbell & McConnell, 1969; Shiga *et. al.*, 1977), the fatty acid spin labels were not mixed directly with red blood cells.

The spin-labeled cells (about 20 μl) were packed in small hematocrit capillaries, which were used as EPR sample tubes, by a micro-hematocrit centrifuge for 3 min at 4 °C. The heights of the packed cells in the hematocrit tubes were at least 2 cm to ensure proper EPR measurements.

2.4 Lysate

A centrifuge tube containing washed, CO-gassed RBC with hematocrit values greater than 95 % was immersed in liquid nitrogen for about 30 sec to allow complete freezing of the cells, followed by immersion in a water bath at room temperature for about 2 min. Then the thawed sample was centrifuged for 10 min at 16,000 rpm (30,590 g) with a Sorvall RC-5B refrigerated centrifuge, using a SS-34 rotor. The supernatant in the tube was removed, stored on ice and used as membrane-free cell lysate.

2.5 Hemoglobin

Hb was prepared by others in our laboratory according to the method of Dozy *et al.* (1968). In brief, washed normal RBC were lysed in 2 volumes of cold deionized water with gently stirring for 30 min at 4 °C. To every 80 ml Hb solution, 20 ml of cold ammonium sulfate solution (76.7 g/100 ml H_2O, pH = 7.0) were added, and continuously stirred for an additional 30 min at 4 °C. The mixture was then centrifuged at 12,000 rpm (17,210 g) for 10 min. After centrifugation, all the supernatant portions were pooled, and the residues containing the membrane and other precipitates at the bottom of the centrifuge tubes were discarded. The lysate was concentrated to a small volume and desalted by dialysis in 50 mM Tris buffer (pH 8.3). The dialyzed lysate was then loaded on an ion-exchange column containing DEAE Sephadex resin equilibrated with Tris buffer, pH 8.3. Separation of the different Hb was performed by decreasing pH gradient from 8.3 to 7.0 with the Tris buffer and monitoring the absorbance at 280 nm with an ISCO UV monitor. The adult Hb (HbA) fraction was collected and pooled according to the peak under the chromatogram. The purified HbA was CO gassed, frozen dropwise into liquid nitrogen, and stored in a deep freezer (-70 °C).

2.6 Membranes and Spin-Labeled Membranes

White membrane ghosts were prepared from washed RBC according to a published method (Steck & Kaut, 1974). Cells were lysed in 5 mM sodium phosphate buffer at pH 8.0 (5P8) and followed by centrifugation at 16,000 rpm (30,590 g) for 10 min. The supernatant was removed with a tap aspirator. During aspiration, the centrifuge tube was tipped and rotated on its axis so that the loosely packed ghosts slide away from a small hard button, rich in contaminating proteases, which may then be aspirated. Two or three more identical wash cycles were performed to get white membrane ghosts. Subsequently, the white membrane was washed and re-suspended in PBS. Membrane concentrations were calculated according to their absorbance at 280 nm and expressed by protein concentrations, which were determined by modified Lowry methods (Peterson, 1977).

Spin-labeled membranes (Mb*) were obtained by mixing 1.25 ml of white membranes (about 4.3 mg/ml) with 0.75 ml of 5-DSA in PBS for 30 min at 4 °C, and the mixture was washed 3 times with cold CO-gassed PBS to remove excess free spin labels. The 5-DSA in PBS was prepared by evaporating 1.0 ml stock spin label ethanol solution to dryness and adding 1.0 ml PBS to the container with stirring at room temperature for about 1 hr. Spin labels were not transferred to the membrane via BSA* solution, as was done for the RBC samples, since BSA* strongly adsorbs to isolated ghosts, but not to intact red cells (Landsberger *et al.*, 1972).

2.7 EPR Measurements and Data Analysis

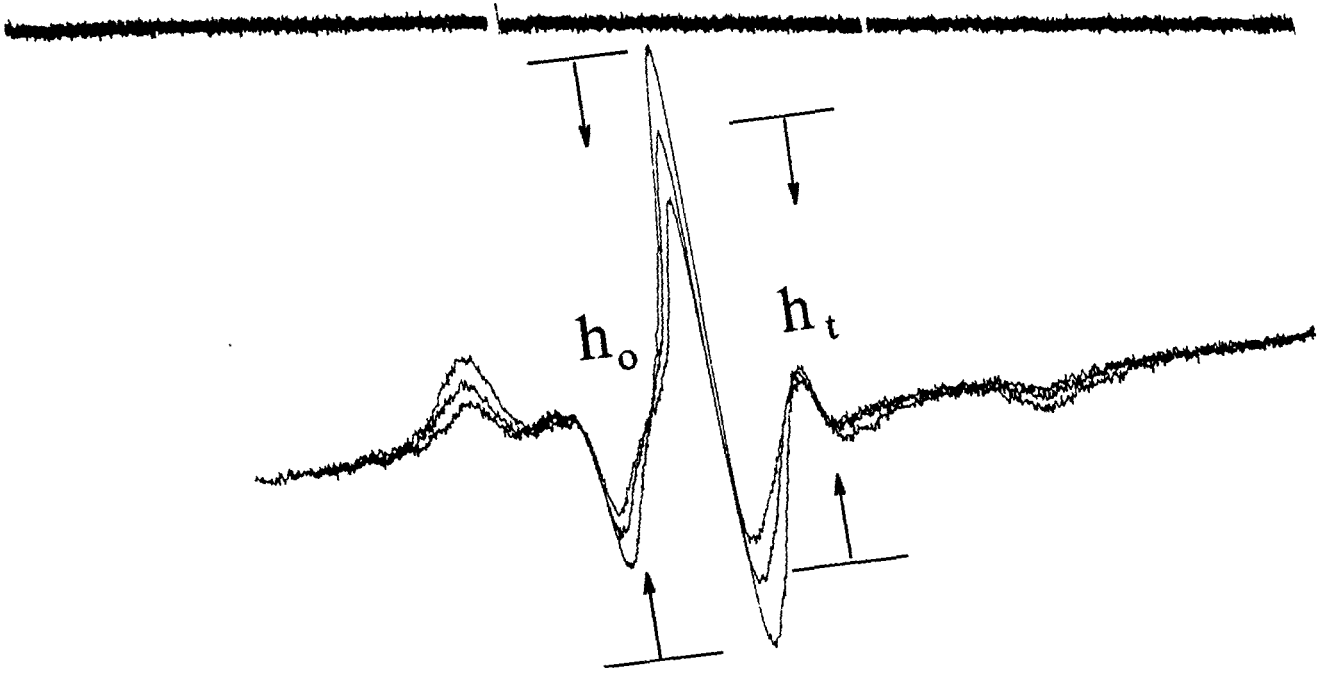
All EPR measurements were performed using a Varian E-Line Century Series EPR spectrometer equipped with Varian E-102 microwave bridge, Varian V-7700 power supply, and IBM variable temperature control unit. A scientific program, ASYST (MacMillan Software), modified for EPR operation, was used for data acquisition and data analysis on a Zenith personal computer. Typical settings for EPR measurements were: field set, 3252.5

gauss; scan range, 100 gauss; field modulation, 100 kHz; modulation amplitude, 1 gauss; time constant, 0.128 sec; receiver gain, 1.25×10^4; scan time, 30 sec (a slower scan time was used initially. A shorter scan time was used to save time. No signal distortion was observed at this scan time.); incident microwave power, 5 mW; and microwave frequency, 8.95 GHz.

The spin-labeled samples were kept at 4 °C, and EPR measurements at 37 °C were carried out as soon as possible after the samples were made. The EPR cavity with a standard quartz EPR tube filled half height of silicon fluid was pre-warmed to 37 ± 0.5 °C. About 2 min after the cold sample was placed in the quartz EPR tube, the sample temperature reached 37 °C, as indicated by a copper-constantan thermocouple inserted in the sample, in about 2 min. The EPR signals of the sample were recorded as a function of time for about 2 hr at 37 °C, with spectra taken about every 20 to 30 min. During the time before the next measurement, the sample was placed in a test tube which contained silicon oil and immersed in a water bath at 37 °C. Some EPR measurements covered a period as long as 22 or 24 hr. For a few samples, the central peak signal was monitored continuously with the following procedures. First, an EPR spectrum was obtained, then the spectrometer was switched to the non-scanning mode to continuously follow the peak height of the central peak. At a later time, the spectrometer was switched to the scanning mode to obtain a full EPR spectrum. Then the spectrometer was returned to the non-scanning mode to monitor the central peak. Since the full EPR spectrum was obtained in about 2 min, no noticeable interruption was detected for the continuous recording of the central peak height (Figure 1). The full EPR spectra obtained in this manner allowed us to check the line-shape and line-width of the EPR signals during the time period of the EPR experiments. This approach provided more accurate measurements of signal reduction, but more laborious and time consuming than the one with spectra taken at different time intervals.

From the EPR spectra, the values of the central peak height at time t (h_t, see Figure 1) were obtained. Since the EPR spectra had equivalent line-shapes and line-widths at different times, the h_t values were proportional to the spin concentrations at time t.

Figure 1 **A typical set of EPR signal reduction data for intact RBC spin labeled with 5-DSA spin label.** At time 0 (left-hand side of the figure), the full spectrum of the spin label was recorded with a central peak height of h_0. Then the central peak height was followed over a period of 2 hr (as shown on the top of the figure). The tracing of peak height was interrupted occasionally during the 2 hr time period to obtain full EPR spectra to check the line-shape and line-width of the signals. EPR spectrometer settings for the full EPR spectra were: field set, 3252.5 gauss; scan range, 100 gauss; field modulation, 100 kHz; modulation amplitude, 1 gauss; time constant, 0.128 sec; receiver gain, 1.25×10^4; scan time, 2 min; incident microwave power, 5 mW; and microwave frequency, 8.95 GHz. The central peak height at time t was designated as h_t. The EPR experiment was done at 37 °C.


h_o
h_t

Double integration of the EPR spectra at different times verified that the spin concentrations were directly proportion to the peak height values.

Pseudo first-order rate constants for spin label reduction reactions were obtained from the slope of a linear regression fit of ln (h_t/h_o) vs time plot, where h_o was the initial peak height of EPR spectrum obtained at t = 0 (see Figure 1). A SYSTAT (SYSTAT, Inc.) statistical program for personal computer was utilized for the linear regression analysis.

2.8 Chemically Treated Samples

2.8.1 NEM-Treated Blood Sample

N-ethylmaleimide (NEM) was quantitatively weighed and dissolved in high phosphate concentration buffers (155 mM NaH_2PO_4 and 103 mM Na_2HPO_4, pH 8.0) to give several stock solutions with concentrations varied from 1.67 to 125 mM. When lower concentrations were desired, the NEM solution at 1.67 mM was used for further dilution. All NEM solutions were made fresh. About 1 ml of washed RBC were mixed with 1.5 ml of NEM solution, at various concentrations, to give final concentrations of NEM ranging from 0.01 to 75 mM. The mixture was allow to stand for 10 min at 4 °C and then washed three more times with PBS. A control sample in high phosphate concentration buffer was prepared at the same time. The supernatant from each washing was optically checked against the characteristic absorbance of NEM at 300 nm. After the third washing, no more NEM was left outside of the cells. NEM-treated and control samples were spin labeled for EPR measurement as described in sections 2.3 and 2.7.

2.8.2 Mb* + Lysate + Antioxidant(s)

The antioxidants selected in this study were enzymes, such as SOD, CAT, GPx, as well as membrane-associated vitamin E, and intracellular biomolecules, such as GSH, NADPH, NADH, and ascorbate. All stock solutions were prepared in relatively high concentrations

based on the dilution factors and the expected final concentrations after adding to Mb* and lysate. For an example, NADPH was dissolved in CO-gassed PBS buffer to give a concentration of 2.88 mM. About 15 μl, 7.5 μl, and 5 μl of NADPH solution were mixed with 30 μl lysate and 15 μl of Mb* with additional 0 μl, 7.5 μl and 10 μl of CO-gassed PBS correspondingly. The final NADPH concentrations in the samples were about 0.74, 0.38, and 0.26 mM, assuming that the lysate contained about 0.02 mM NADPH (Eaton *et al.*, 1972). The control sample consisted of 15 μl of CO-gassed PBS instead of NADPH solution. Another set of control samples which consisted of Mb* and NADPH at concentrations equivalent to those stated above, but excluded lysate was also prepared. Similarly, other antioxidants were used either individually or in various combinations with two more antioxidants.

Since GSH and ascorbate solutions at concentrations higher than 5 mM were highly acidic in PBS, they were freshly prepared with CO-gassed, high phosphate concentration buffers to maintain the pH value at about 8.0. Vitamin E (*d*-α-tocopherol) was dissolved in absolute ethyl alcohol, dried to a thin film with nitrogen gas, and suspended in CO-gassed PBS by swirling with glass beads followed by sonication for about 10 min immediately before use. The droplet of vitamin E was assumed to suspend homogeneously in the sonicated solution.

2.8.3 Addition of Ascorbate Oxidase

Ascorbate oxidase (AO) with 100 units (1 unit oxidizes 1.0 μmole of L-ascorbate to dehydroascorbate per min at pH 5.6 at 25 °C) was dissolved by addition of CO-gassed PBS buffer, at various volumes to give AO solutions of different concentrations. About 5 μl of AO solution were added to 40 μl of lysate, then mixed with 15 μl Mb*. A control sample was made of 5 μl CO-gassed PBS, 40 μl lysate, and 15 μl Mb*.

2.9 Blood Samples for Antioxidant Assays

The same blood samples used for spin labeling were diluted with PBS buffer to give

30 - 50 % hematocrit. A large portion of the blood suspensions was immediately used or stored at -20 °C for a period of no more than two months before subsequent vitamin E and ascorbate determinations. Another portion was hemolyzed by adding a four-fold dilution of deionized water and freeze (with an acetone/dry ice mixture) - thaw (with a water bath at room temperature) 3 times. It was not necessary to remove the cell membrane in the sample. One part of the lysate used for GPx assay was further diluted with an equal volume of GSH diluent containing 2 mM GSH and 50 mM potassium phosphate buffer (pH 7.0) to avoid losing GPx activity (Blanchflower *et al.*, 1986), and the left-over lysate were then stored at 4 °C for the enzyme assays within 36 hr or kept in a freezer (-20 °C) for longer periods.

Before blood suspensions were lysed or the samples were stored in the freezer (-20 °C), the number of cells in the samples was determined by means of CBC5 Coulter Counter.

2.10 Antioxidant Assays

2.10.1 Glutathione

The enzymatic method based on the reaction of formaldehyde (HCHO),

$$HCHO + NAD^+ + H_2O \longrightarrow HCOOH + NADH + H^+,$$

catalyzed by formaldehyde dehydrogenase in the presence of GSH (Koivusalo & Uotila, 1974), was used for GSH assay with slight modifications. A volume of 1.0 ml blood sample (control or NEM-treated) and 6 ml 10 % (w/v) metaphosphoric acid were mixed and filled up to a total volume of 10 ml with deionized water, then centrifuged at 16,000 rpm (30,590 g) for 10 min. The supernatant was neutralized to pH 8.0 by adding 1 N NaOH with vigorous mixing. Then the following solutions were transferred via pipettes into a test tube containing 240 μl of above supernatant: 75 μl of 0.04 M NAD, 50 μl formaldehyde dehydrogenase solution (minimum activity 500 mU/ml), and 2.4867 ml of 33 mM sodium phosphate buffer (pH 8.0). The solutions were mixed well on a rotator and the reaction was initiated by the addition of 48.3 μl of 0.06 M HCHO. The tube was stopped and incubated at 37 °C for 30 min. The

reaction was terminated by adding 100 μl of 0.10 M cysteine in deionized water. Then the absorbance of the reaction mixture at 340 nm was read against a blank. The blank contained all the assay mixture except deionized water was used instead of HCOH. A standard assay (5 - 30 μM) were run in parallel with the sample assay.

2.10.2 Catalase

CAT was assayed by direct measurement of the decrease in absorbance at 240 nm as H_2O_2 was decomposed by CAT (Claiborne, 1985).

The lysate prepared, as described in the section 2.9, was further diluted by adding 50 μl of lysate to 10 ml deionized water. Hb concentration was estimated spectrophotometrically using the millimolar extinction coefficient ϵ_{569} = 13.4/heme for CO condition and ϵ_{577} = 14.6/heme for O_2 condition (Antonini & Brunori, 1971). A volume of 1.0 ml diluted lysate was taken and mixed with 20 μl of pure ethanol for assay. A 10 mM H_2O_2 solution was prepared by adding stock H_2O_2 solution into 50 mM potassium phosphate buffer (pH 7.0). The final concentration was confirmed by measuring the absorbance at 240 nm and calculating the concentration from the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$. The spectrophotometer baseline was set with a blank containing 930 μl of 50 ml potassium phosphate buffer (pH 7.0) and 70 μl of lysate-ethanol mixture in a 1 ml quartz cuvette. The absorbance at 240 nm for the solution containing 30 μl of 50 ml potassium phosphate buffer (pH 7.0), 70 μl of lysate-ethanol mixture, and 900 μl of 10 mM H_2O_2 was measured immediately.

The specific activity of CAT was defined in terms of micromoles of H_2O_2 consumed per min per gram of Hb.

2.10.3 Superoxide Dismutase

The activity of SOD activity was assayed using the cytochrome C and xanthine-xanthine oxidase method (McCord & Fridovich, 1969). In this assay, SOD competes with

cytochrome C for the flux of O_2^- induced by the xanthine and xanthine oxidase reaction. A cocktail containing 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA, 50 μM xanthine, and 10 μM cytochrome C was first prepared. The standard assay was performed in a 3 ml cuvette with 2.7 ml cocktail, 0.3 ml deionized water, and about 1 μl xanthine oxidase to produce a rate of cytochrome C reduction of 0.025 absorbance unit per min at 550 nm.

The SOD was extracted from the lysate, which was prepared with the method described in section 2.9. 0.3 ml of lysate was added to 0.5 ml of ice-cold deionized water, followed by adding 1.0 ml of pure ethanol and 0.6 ml of chloroform. Solutions were mixed after each addition, and finally shaken for at least 1 min. The mixture was centrifuged at 3,000 rpm (1,075 g) for 10 min. SOD was found in the clear top layer, which was collected for assay. 0.3 ml of extract instead of water was used under the same conditions as for the standard assay.

One unit of SOD was defined as the amount of enzyme required to inhibit the initial rate of cytochrome C reduction by 50 %. Results were expressed as units of SOD per gram of Hb.

2.10.4 Glutathione Peroxidase

Indirect spectrophotometric methods of Paglia and Valentine (1967) and modified by Blanchflower *et al.* (1986) were used for GPx determination. The GSH oxidation by H_2O_2 as catalyzed by GPx was coupled with the addition of glutathione reductase and NADPH. The rate of oxidized glutathione (GSSG) formation was then measured by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH was converted to NADP.

The frozen lysate in GSH diluent, as described in section 2.9, was thawed and allowed to stand at room temperature for 10 min before analysis. The following solutions were pre-mixed as reagent mixture: 13.15 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, 0.5 ml of 8.4 mM NADPH, 0.5 ml of 0.15 M GSH, 50 μl of 1.125 M NaN_3, and

50 μl of 120 U/ml glutathione reductase suspension as purchased. About 0.95 ml of reagent mixture and 16.7 μl lysate were transferred via pipettes into a 1 ml quartz cuvette. The enzymatic reaction was initiated by addition of 33.3 μl 2.2 mM H_2O_2, the concentration of which was assured by checking its characteristic absorbance at 240 nm against its molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$. The reaction rate was monitored by continuous recording of the absorbance at 340 nm between 2 and 4 min after the initiation of the reaction. The non-enzymatic oxidation of GSH was determined by simultaneous assay of a system identical to the enzymatic reaction, except that the lysate was replaced by an equal volume of water. The reaction rate of the latter system was subtracted from that of the former to determine the true enzymatic activity.

Enzyme units for GPx activity were defined as the number of micromoles of NADPH oxidized per min per gram of Hb, and were calculated by using a molar extinction coefficient of 6.22×10^3 for NADPH.

2.10.5 Vitamin E

A standard fluorometric α-tocopherol analysis was use to measure the vitamin E concentration, following a modified method (Chiu & Lubin, 1979) of Taylor *et al.* (1976).

A mixture containing 2.0 ml PBS (pH 8.0), 0.5 ml of 25 % ascorbic acid (freshly prepared), 1.0 ml pure ethanol, and 0.5 ml blood sample (30 % - 50 % hematocrit) was pre-incubated at 70 °C for 5 min in a 15 ml test tube with a cover. Following the addition of 1.0 ml of 10 N KOH, the mixture was saponified for 30 min at 70 °C. After the mixture had been cooled down, 4.0 ml of hexane were added, and the saponified mixture was extracted by mixing vigorously for at least 1 min on a vortex. The extracted mixture was centrifuged at 3,000 rpm (1,075 g) for 5 min to separate the phases. A portion of the hexane phase (top layer) was removed for fluorometric measurements at 290 nm excitation and 330 nm emission on a Hitachi F-2000 fluorescence spectrophotometer. For all blanks and standards, water was substituted for the blood sample. Vitamin E standards were prepared by dissolving

α-tocopherol in pure ethanol to give concentrations of 0.95 - 3.8 μg/ml. The concentration of vitamin E in RBC was obtained from direct comparison of the sample fluorescence intensity with that of the standards after correction had been made for the fluorescence intensity of the blank, and was expressed as micrograms of α-tocopherol per gram of Hb.

2.10.6 Ascorbate

The ascorbate concentration was determined by an α,α'-dipyridyl method (Omaye *et al.*, 1979; Okamura, 1980). The assay was based upon the reduction of ferric ion by ascorbate to ferrous ion, which was coupled with α,α'-dipyridyl to form a complex with a characteristic absorption at 525 nm.

A modified method for extracting and measuring ascorbate of RBC was established based on the methods of Omaye *et al.* (1979) and Okamura (1980). To 0.5 ml of CO-gassed blood sample (either frozen-thawed lysate with Hb concentration about 150 mg/ml or RBC suspension with hematocrit about 50 %) in a small centrifuge tube, 2.0 ml of 5 % trichloroacetic acid (TCA) was added with vigorous mixing on a vortex for at least 2 min. The mixture was centrifuged at 11,000 rpm (14,470 g) for 20 min at 4 °C. The following reagents were added in sequence to 1.0 ml of the supernatant: 0.4 ml of 42.5 % H_3PO_4, 0.4 ml of 2 % α,α'-dipyridyl dissolved in 70 % ethanol, and 0.2 ml of 1.5 % $FeCl_3$. The samples were mixed vigorously upon addition of $FeCl_3$ and incubated at 37 °C for 60 min with covers. Then absorbance readings against water at 525 nm were taken. Blank tests were carried out at the same time as samples. Standard solutions of ascorbate, in a range of 0.5 - 10 μg/ml, were prepared in 5 % TCA.

2.11 Statistical Analysis

Data were often analyzed statistically by using a two-sample t test and a paired-sample t test. The two-sample t test is applied for comparison of two means of measurements from two independent samples, assuming that each datum in one sample is in no way associated

with any datum in the other sample. The paired-sample t test does not use the original measurements for the two samples. Instead, the differences between the pairs of measurements are used, assuming that the differences come from a normally distributed population of differences (Zar, 1974). The variability of the data is presented as an average (Avg) with standard deviation (SD), or coefficient of variability (CV), which is defined as the standard deviation divided by the average. Differences at $p < 0.05$ are usually considered significant in biological systems (Zar, 1974).

CHAPTER III

RESULTS

3.1 Spin Label Reduction in Normal RBC

3.1.1 Spin Label Reduction by Intracellular Components

In this study, EPR signals were monitored as a function of time at 37 °C. A typical set of EPR spectra showing signal reduction of 5-DSA in intact RBC over a period of 2 hr, is illustrated in Figure 1. It shows that there is no change in the EPR signal line-shape or line-width among the three full EPR spectra of the same sample obtained at different time intervals during the reduction experiment. Therefore, the amplitudes of the central peak could be used to measure the spin label signal reduction. Figure 2a is a plot of a typical set of data for percent EPR signal reduction $[(h_0 - h_t) / h_0 \times 100\ \%]$. At the end of a period of 22 hr, a majority of the original signal of 5-DSA spin labels intercalated in the membranes of CO-gassed intact RBC diminished, with only about 5 % signal retained. In contrast, EPR signals persisted for the same spin labels intercalated in white membrane ghosts, which were isolated from the same batch of erythrocytes, under the same experimental conditions as used for intact erythrocytes. No signal reduction was observed, within ± 5 %, over a period of 22 hr (Figure 2b). When about 40 μl of lysate, prepared from the same batch of blood sample used for intact cell studies, was added to the Mb* (about 20 μl at about 8 mg/ml concentration), a similar signal reduction profile (Figure 3c) was observed as in intact cells (Figure 3b) over a period of 2 hr. These data suggest that intracellular components, which were not present in the white membrane ghost sample (Figure 3d), were responsible for the spin label signal reductions observed in intact cells.

Figure 2 **Percent EPR signal reduction [(h_0 - h_t) / h_0 x 100 %] of 5-DSA spin label in intact RBC and in white membrane ghosts over a period of 22 hr.** Spin-labeled intact cells (● , line a), spin-labeled white membrane ghosts (■, line b). The same batch of blood was used to prepare the samples.

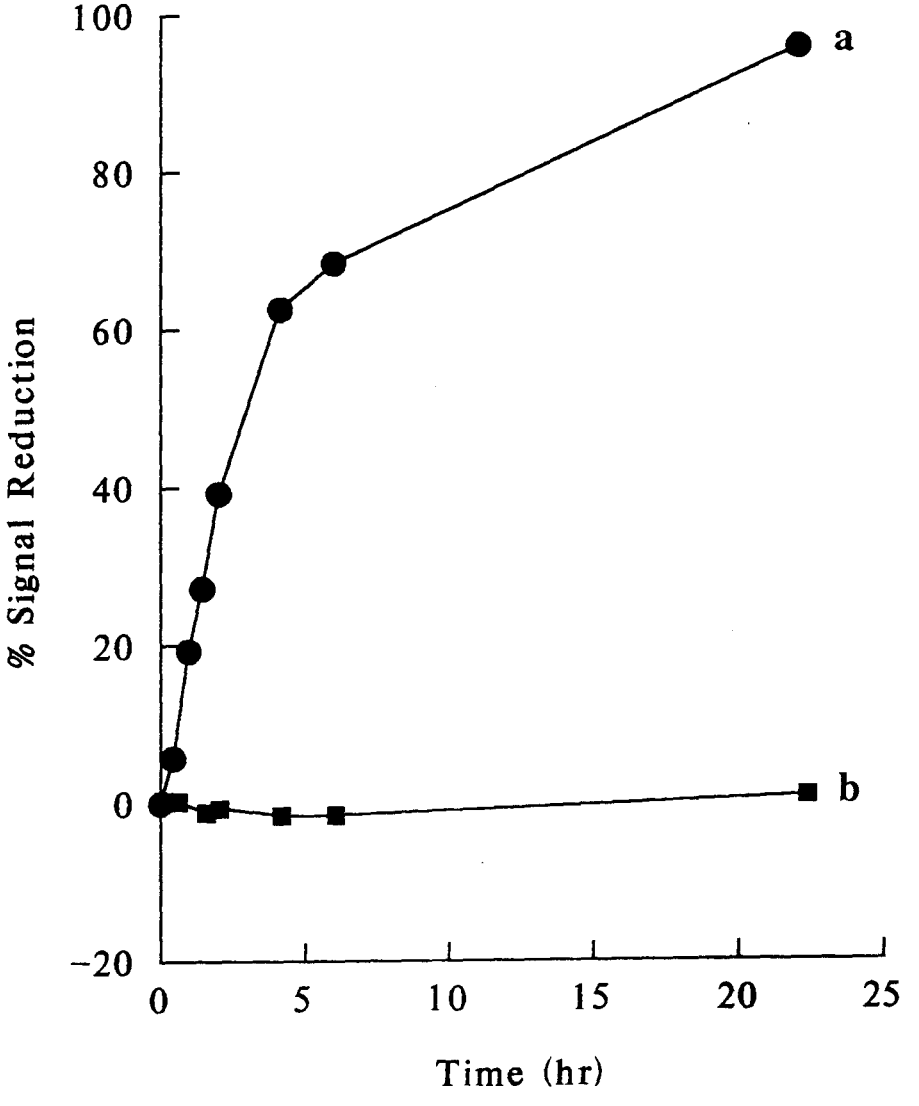
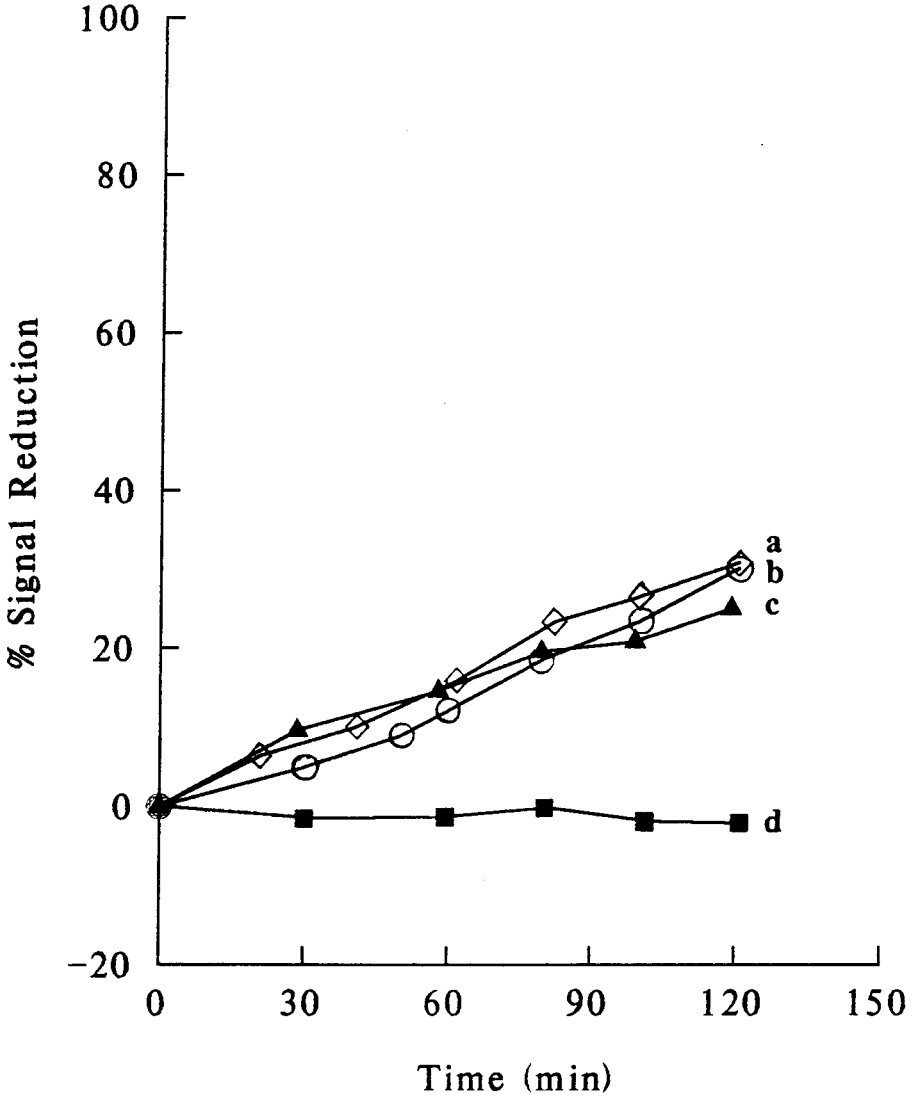

100
80
60
40
20
0
-20
% Signal Reduction
0
5
10
15
20
25
Time (hr)
a
b

Figure 3 **Similarity of signal reduction between spin-labeled membrane ghosts plus lysate or spin-labeled intact RBC plus lysate and spin-labeled intact RBC.** Percent EPR signal reduction over a period of 2 hr for spin-labeled intact cells plus lysate (◇, line a), spin-labeled intact cells (O, line b), spin-labeled membrane plus lysate (▲, line c), and spin-labeled white membrane ghosts (■, line d). Same batch of blood was used to prepare the samples.


100
80
60
40
20
0
-20
% Signal Reduction
0
30
60
90
120
150
Time (min)
a
b
c
d

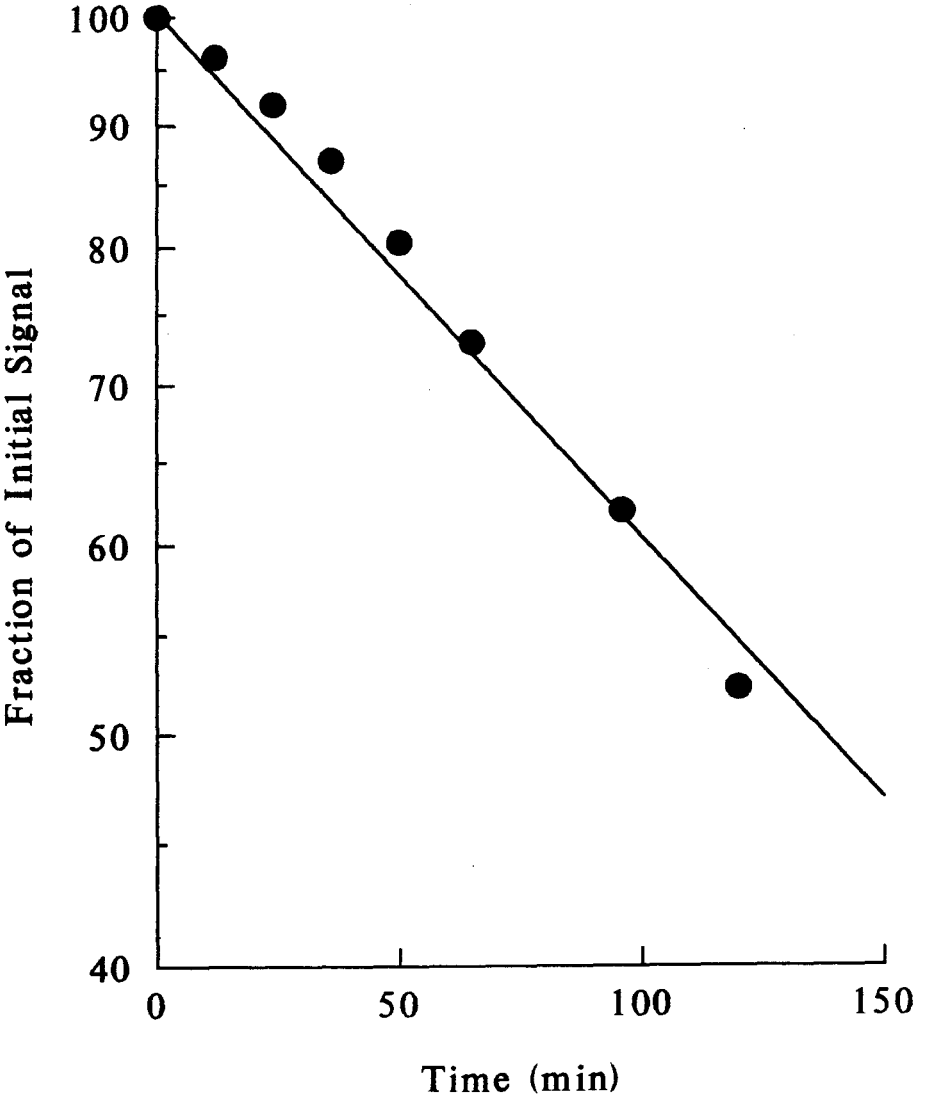
3.1.2 Reduction Rate Constants

In order to obtain rate constants from the signal reduction data presented above, it is necessary to demonstrate that the spin label translocation rate is relatively faster than the spin label reduction rate in the erythrocytes. When fatty acid spin labels were initially introduced into the erythrocyte membranes, they resided in the outer leaflet of the membrane bilayer. In order for them to be reduced by intracellular components, the labels must translocate to the inner leaflet. A system which consisted of spin-labeled intact cells plus lysate was used to examine such a hypothesis. If the translocation movements of the spin labels in membranes were slow relative to EPR measurement time, some labels would locate in the outer leaflet during the EPR experiments. Thus, one would expect that the addition of lysate to the intact cells would result in faster reductions, since with lysate added both sides of the bilayer were in contact with the reducing agents (intracellular components). Figures 3a and 3b show that the spin label reduction obtained from the spin-labeled intact cells plus lysate is similar to that from the intact cells. It is, therefore, concluded that the translocation movements of 5-DSA spin labels in erythrocyte membranes are relatively faster than the spin reduction, and the signal reduction data can be used to provide reduction rate constants.

The 5-DSA spin label reduction in many biological systems follows a first-order reaction mechanism (Baldassare *et al.*, 1974; Chen *et al.*, 1988). In our systems, initial reduction rates (over a period of 2 hr) appeared to follow a first-order reaction mechanism, exhibiting a linear fit in a semi-logarithmic plot with correlation coefficients generally > 0.99. A semi-logarithmic plot of the data for the CO-gassed intact RBC is illustrated in Figure 4. Since the spin label translocation rate is relatively faster than the spin reduction rate, the linear fit for signal reduction data exhibited in Figure 4 represents a pseudo first-order spin label reduction with a rate constant (k) of about 5.1×10^{-3}/min and the correlation coefficient of 0.996 for the linear fit.

As discussed below, the k values varied from batch to batch (each batch was defined

Figure 4 **A semi-logarithmic plot of the fraction of initial signal (h_t/h_0 x 100) versus time for CO-gassed, spin-labeled intact cells.** Raw data were from Figure 1. A linear fit gave a pseudo first-order rate constant for reduction of about 5.1 x 10^{-3}/min, with correlation coefficient of 0.996.


100
90
80
70
60
50
40
Fraction of Initial Signal
0
50
100
150
Time (min)

as the blood sample from one blood bag obtained from a blood bank) of blood samples.

3.1.3 Experimental Uncertainties

To check the reliability and the reproducibility of the EPR measurements, reduction rate constants were compared among the following groups of experiments: (1) multiple EPR samples were prepared from the same spin-labeled samples, and stored at 4 °C until the EPR measurements; (2) multiple spin-labeled samples were prepared from the same batch of blood; (3) samples from the same batch of blood were prepared and measured on different days. Table 1 shows that the k values were about the same within one batch of blood regardless of groups. Data obtained from 18 batches of blood show that the values of CV of reduction rate constants ranged from 1.3 % to 20.0 % (Table 1), with an average CV of about 9.5 ± 5.8 % for individual batches of blood. This indicates that the experimental uncertainty of the rate constants is about 10 %.

3.1.4 Variation of Blood Samples

Different batches of blood samples were used throughout this dissertation study. It was found that the spin label reduction rates vary from batch to batch of blood. The k values of 64 batches of normal blood samples ranged from 1.2 x 10^{-3}/min to 8.4 x 10^{-3}/min, with an average value of 3.8 ± 1.7 x 10^{-3}/min. The CV for batch to batch was 44.7 %.

3.1.5 Sample Characterization

3.1.5.1 Amount of Spin Label

At the end of 24 hr, only about 5 - 20 % of the signal remained. In principle, the higher the initial concentration of spin label in the erythrocyte membranes, the more accurately one can follow the decrease in signal intensities of the spin label at different times. However, spin-spin broadening poses an upper limit to the spin label concentration per cell

Table 1 Reduction Rate Constants from 18 Batches of Blood and the Variation of Rate Constants within the Same Batch of Blood.

Blood[a]	Sample[b]	k[c] (10^{-3}/min)	Average k (10^{-3}/min)	CV (%)
1	a	4.60		
	b	4.69	4.65 ± 0.06[d]	1.3
2	a	6.99		
	b	6.81		
	c	7.11	6.97 ± 0.15	2.2
3	a	8.23		
	b	7.92	8.08 ± 0.22	2.7
4	a	1.64		
	b	1.73		
	c	1.71	1.69 ± 0.05	3.0
5	a	1.28		
	b	1.18	1.23 ± 0.07	5.7
6	a	4.21		
	b	4.61	4.41 ± 0.28	6.3
7	a	3.39		
	b	3.22		
	c	2.98	3.20 ± 0.21	6.6
8	a	3.35		
	b	2.99	3.17 ± 0.25	7.9
9	a	2.85		
	b	3.23	3.04 ± 0.27	8.9
10	a	2.85		
	b	3.23	3.04 ± 0.27	8.9
11	a	6.13		
	b	7.00	6.57 ± 0.61	9.3

[a]Each entry under Blood represents one batch of blood from the blood bank.
[b]Each entry under Sample represents an EPR sample.
[c]k is the pseudo first-order rate constant for spin label reduction.
[d]Standard deviation.

(continued)

Table 1 (continued)

Blood[a]	Sample[b]	k[c] (10^{-3}/min)	Average k (10^{-3}/min)	CV (%)
12	a	7.91		
	b	6.42		
	c	7.05	7.13 ± 0.75[d]	10.5
13	a	2.08		
	b	1.79	1.94 ± 0.21	10.8
14	a	3.46		
	b	2.83	3.15 ± 0.45	14.3
15	a	4.46		
	b	3.44		
	c	3.35	3.75 ± 0.62	16.5
16	a	3.73		
	b	2.98		
	c	3.97		
	d	3.34		
	e	4.21		
	f	4.85	3.85 ± 0.66	17.1
17	a	3.35		
	b	4.21		
	c	4.93	4.16 ± 0.79	19.0
18	a	5.20		
	b	6.91	6.06 ± 1.21	20.0

The average coefficient of variability (CV) of k for multiple samples prepared from the same batch of blood is 9.5 ± 5.8 %.

[a]Each entry under Blood represents one batch of blood from the blood bank.
[b]Each entry under Sample represents an EPR sample.
[c]k is the pseudo first-order rate constant for spin label reduction.
[d]Standard deviation.

that can be used for this study. The spin labels may cluster at higher concentrations to distort the EPR signal. In studies when fatty acid spin labels were introduced directly into red blood cell membrane ghosts, the spin labels clustered in membranes at a spin label-to-lipid ratio of about 1/360, resulting in spin-spin exchange induced broadening in the EPR spectra (Gordon *et al.*, 1985). In our study, the spin label concentrations in cell membrane varied from 5.8 x 10^{-5} M to 2.4 x 10^{-4} M as measured at t = 0. The line-widths of the EPR signals were carefully checked, and no spin broadening was observed in the EPR spectra. In addition, the reduction rate constants were not affected by the different initial spin label concentrations, which is in agreement with the first-order reaction kinetics.

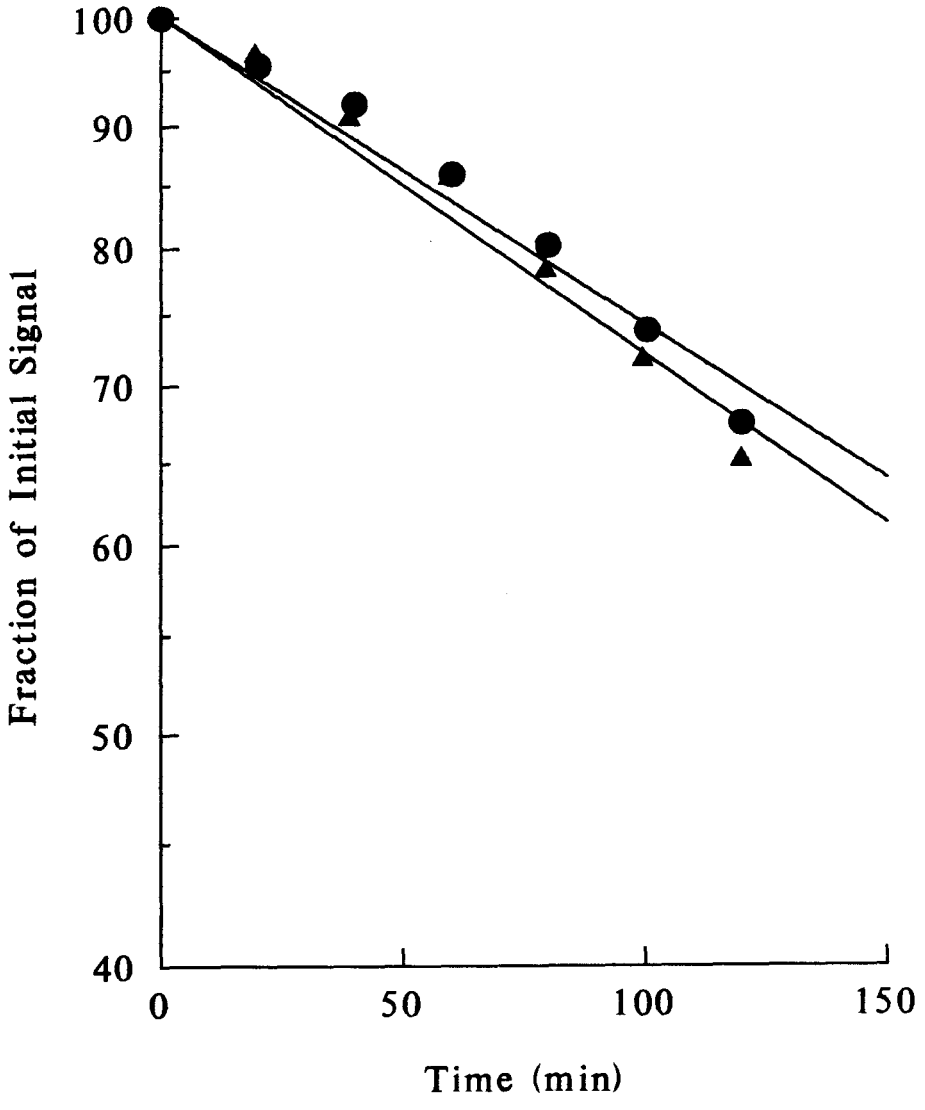
3.1.5.2 Labeling Time

Usually, labeling RBC with BSA* or Mb with free spin label in PBS was allowed for 30 min at 4 °C before washing away the excess spin label solution. However, we found that a labeling time of 10 min was sufficient. Under this condition (10 min labeling), the EPR intensity of spin label signal at t = 0 was 1.4 x 10^{-4} M (7.8 x 10^6 spin labels per cell), similar to 1.7 x 10^{-4} M (9.0 x 10^6 spin labels per cell) when 30 min of labeling time was used. The spin label reductions of CO-gassed intact cells with different labeling times were about the same (Figure 5).

3.1.5.3 Microwave Power

To increase the intensity of EPR signal at one spin label concentration, one can increase the incident microwave power. However, when the microwave power is too high the spin label resonance may be saturated. It has been suggested that a power level of 1 - 10 mW for nitroxide spin label is appropriate to avoid saturation (Marsh, 1981). In this study, 1 mW of microwave power was used for the earlier experiments and 5 mW of microwave power was used for most of the later experiments.

Figure 5 **Spin label reduction of intact RBC with different labeling times.** A semi-logarithmic plot of the fraction of initial signal versus time for spin-labeled intact RBC with labeling time 10 min (▲) and 30 min (●). The reduction rate constant obtained from the linear fit is 3.3 x 10^{-3}/min for 10 min labeling time, with a correlation coefficient of 0.994, and 3.0 x 10^{-3}/min for 30 min labeling time, with a correlation coefficient of 0.994.


100
90
80
70
60
50
40
Fraction of Initial Signal
0
50
100
150
Time (min)

To check whether different levels of microwave power affect the spin label reduction, multiple EPR samples were made from the same spin-labeled sample. EPR spectra were obtained using 1 mW and 5 mW microwave power, with other spectrometer settings held constant. Table 2 lists the reduction rate constants from four parallel runs of CO-gassed RBC using different power levels. A paired-sample t test was applied to show that the differences in rate constants from the samples using 1 mW and 5 mW microwave power were not significant ($p > 0.50$).

3.1.6 Effects of Sample Conditions

3.1.6.1 Anticoagulants

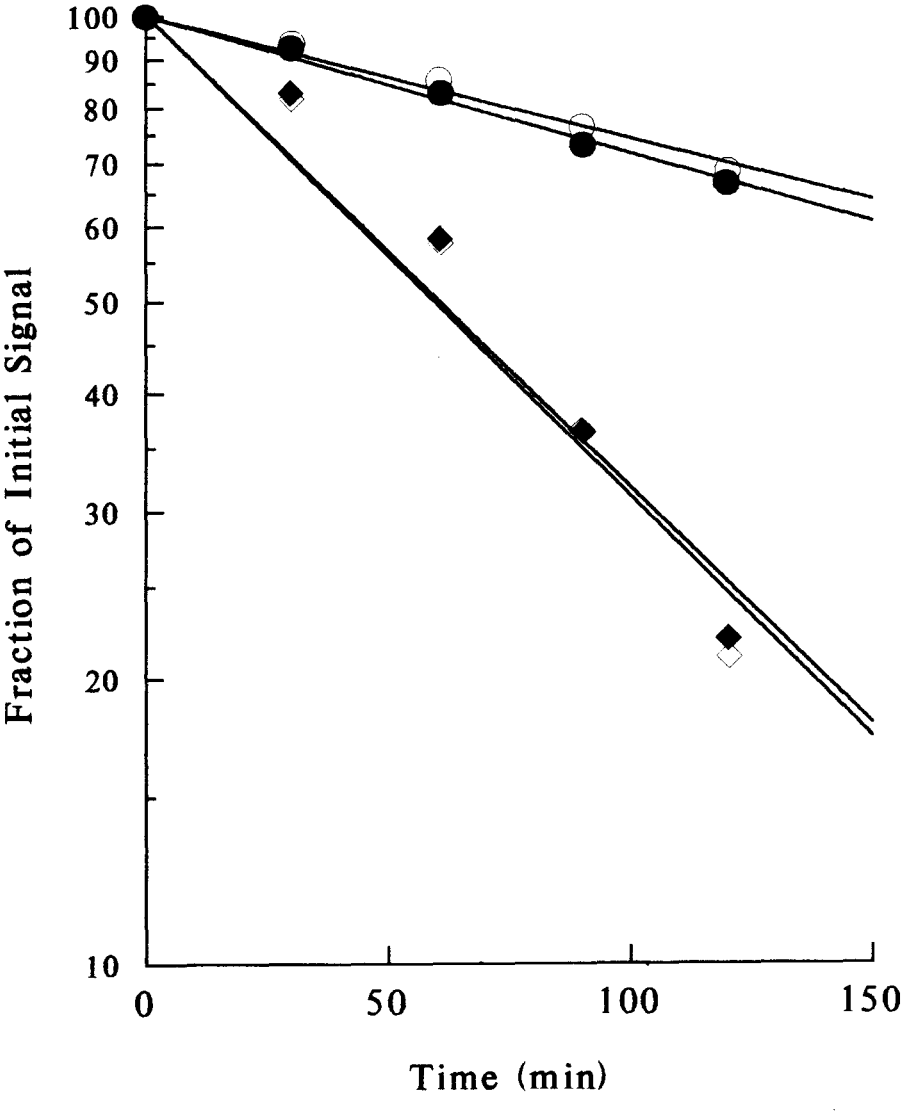
Since different anticoagulants (heparin, EDTA, *etc.*) were used for some of the normal and sickle RBC, the effect of anticoagulants on the spin label reduction rates should be considered. To examine the possible effect, blood samples from the same donors or patients were drawn into heparin coated tubes and EDTA coated tubes at the same time. The blood samples were retained in the tubes for at least 2 hr at 4 °C. Then, the cell washing and spin labeling procedures were followed. Sequentially, spin label reduction was measured, and the reduction rates constants were obtained (Figure 6). For one RBC sample (from a normal donor), the reduction rate constant was 3.4×10^{-3}/min when EDTA was used as an anticoagulant, and was 3.0×10^{-3}/min when heparin was used as an anticoagulant. For another sample (from a sickle patient), the rates were 11.5×10^{-3}/min and 11.7×10^{-3}/min for EDTA and heparin as anticoagulants, respectively. Thus, the results show that anticoagulants have no significant effect on the spin label reduction for both normal and sickle RBC.

Table 2 Spin Label Reduction Rate Constants from Four Parallel Runs of CO-gassed Normal RBC at Two Different Microwave Power Levels

Run	1 mW	5 mW	diff.
1	4.30	3.46	0.84
2	3.73	3.97	-0.24
3	2.94	3.34	-0.40
4	4.86	4.21	0.65

paired-sample t test, $t = 0.682$, $p > 0.50$.

Figure 6 **Spin label reduction of both normal and sickle RBC with different anticoagulants.** A semi-logarithmic plot of the fraction of initial signal versus time for the blood samples from normal donor (●) and sickle patient (◆) with anticoagulant EDTA (closed symbols) or heparin (opened symbols). For the blood sample from normal donor, the reduction rate constant is 3.4 x 10^{-3}/min for EDTA used as anticoagulant, with a correlation coefficient of 0.998, and 3.0 x 10^{-3}/min for heparin used as anticoagulant, with a correlation coefficient of 0.996. For the sample from sickle patient, the rate constant is 11.5 x 10^{-3}/min for EDTA used as anticoagulant, with a correlation coefficient of 0.990, and 11.7 x 10^{-3}/min for heparin used as anticoagulant, with a correlation coefficient of 0.990.


100
90
80
70
60
50
40
30
20
10
Fraction of Initial Signal
0
50
100
150
Time (min)

3.1.6.2 CO-Gassed and O_2-Gassed RBC

It was found that for the same batch of blood cells the spin label reductions for O_2-gassed samples were lower than those for CO-gassed samples. This is in part due to re-oxidation of the spin label from hydroxylamine form (EPR silent) back to nitroxide form (EPR active) (Chen & Swartz, 1989) by O_2 molecules in the system. Figure 7 shows a semi-logarithmic plot of data obtained from typical CO- and O_2-gassed samples. The EPR signal reduction data of the CO-gassed sample followed a linear fit with a correlation coefficient of 1.000. However, the data for the O_2-gassed sample did not give a good linear fit when data points beyond 90 min were included, since the reduction rate began to decrease. If we do not include the data points beyond 90 min, where the reaction could no longer be described by pseudo first-order kinetics, the data followed a relatively good linear fit, with a correlation coefficient of 0.996. From this analysis, a rate constant of 6.4×10^{-3}/min was obtained for the CO-gassed sample, and 3.0×10^{-3}/min for O_2-gassed sample. The average ratio of $k(CO)/k(O_2)$ was 2.3 ± 0.9 for 15 different paired experimental runs.

It is, therefore, important to have erythrocytes fully CO-gassed, as indicated by the conversion of HbO_2 to HbCO in intact spin labeled erythrocytes. Otherwise, the extent of the spin label reduction in cells will be affected by the amount of oxygen molecules in the system.

3.2 Effects of RBC Components that Reduce Membrane Spin Labels

3.2.1 Components with No Effects

3.2.1.1 Vitamin E

Vitamin E is a membrane-associated, lipophilic antioxidant. Its spin label reduction capacity in Mb* was examined with and without lysate. The rate constant for the membrane with lysate was 1.1×10^{-3}/min (Figure 8b). Upon addition of vitamin E, at a final concentration about 11.8 mM, the rate constant was 1.3×10^{-3}/min (Figure 8c), very similar to that without the addition of vitamin E.

Figure 7 **A semi-logarithmic plot of the fraction of initial signal versus time for spin label in intact red blood cells with the red blood cells in CO and O_2 gassed buffer.** The linear fit gives a reduction rate constant of 6.4 x 10^{-3}/min for CO gassed samples (●) with a correlation coefficient of 1.000, and 3.0 x 10^{-3}/min for O_2 gassed samples (▲) during the first 90 min, with a correlation coefficient of 0.996.

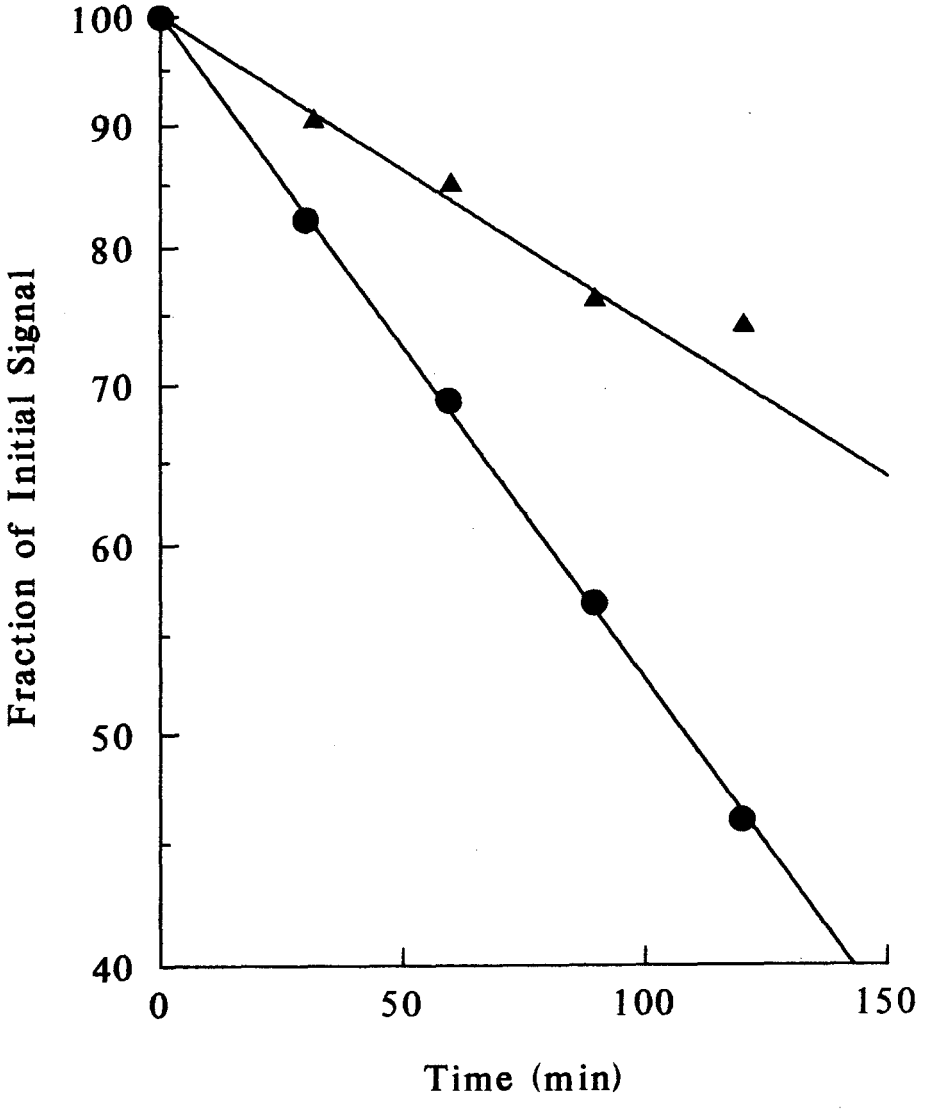
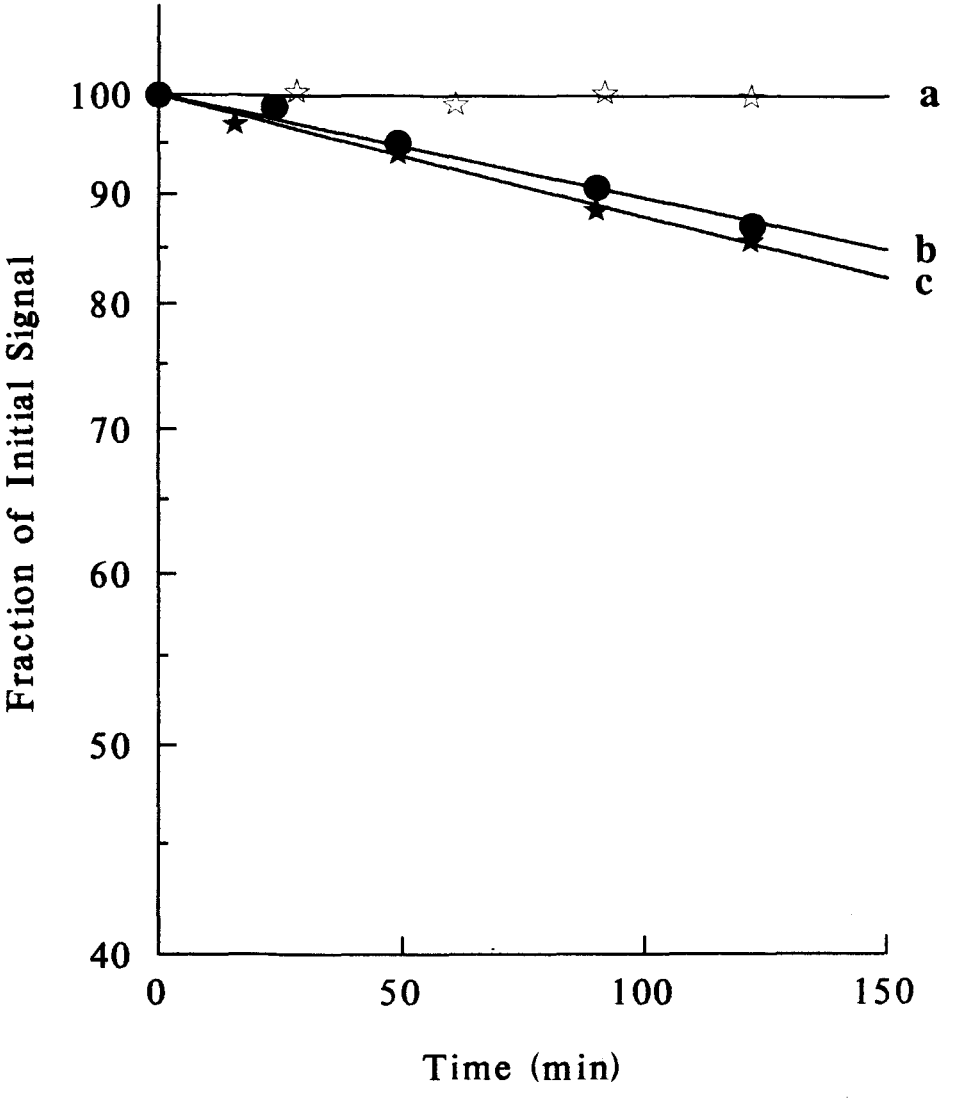

100
90
80
70
60
50
40
Fraction of Initial Signal
0
50
100
150
Time (min)

Figure 8 **Examination of vitamin E effect on spin-labeled membrane ghosts.** A semi-logarithmic plot of the fraction of initial signal versus time for spin-labeled membrane ghosts with 11.8 mM vitamin E (☆, line a), lysate (●, line b), or 11.8 mM vitamin E and lysate (★, line c). The rate constant obtained from the linear fit is 0.02 x 10^{-3}/min for vitamin E in the absence of lysate, with a correlation coefficient of 0.248. The rate constant for lysate sample is 1.1 x 10^{-3}/min, with a correlation coefficient of 0.996, and for vitamin E and lysate is 1.3 x 10^{-3}/mim, with a correlation coefficient of 0.998.


100
90
80
70
60
50
40
0
50
100
150
Fraction of Initial Signal
Time (min)
a
b
c

Samples containing only vitamin E and Mb* in the absence of lysate did not show any detectable reduction (Figure 8a).

3.2.1.2 Glutathione

In order to study the involvement of GSH in the reduction process, two types of experiments were performed by using (1) NEM-treated RBC to block GSH and (2) direct addition of GSH to Mb* in the presence and absence of lysate.

For NEM-treated RBC, the concentrations of GSH in EPR samples were determined. Figure 9 shows a plot of percentage of the remaining GSH concentration and relative spin label reduction rate constant, defined as the rate constant from a treated sample divided by the rate constant of its control, in normal RBC treated with various concentration of NEM. About 1.0 mM of NEM was required to block about 90 % of GSH in intact RBC, but the blockage did not affect the rate constants. Cells treated with more concentrated NEM (10 - 75 mM) gave about same amount of residual GSH, in part due to the detection limit in GSH assay. Nevertheless, the relative reduction rate constants retained the same as the untreated sample (Figure 9).

In another experiment, GSH (4.0 mM) was added to Mb*, and the spin label reduction, monitored over a period of 2 hr, was found to be negligible (Figure 10a). In the presence of lysate, addition of 4.0 mM GSH to lysate which contained about 1.5 mM GSH, based on the physiological value of 3.0 mM (Mehlhorn, 1991) and a dilution factor of the sample, to give a final concentration of 5.5 mM did not significantly enhance the reduction. The rate constant was 1.6×10^{-3}/min (Figure 10c), similar to 1.5×10^{-3}/min obtained from its control (Figure 10b). The results from these experiments showed that GSH did not reduce 5-DSA spin labels in membrane.

Figure 9 **Percentage of remaining GSH concentration and relative spin label reduction rate constant in intact red blood cells treated with various concentrations of NEM.**

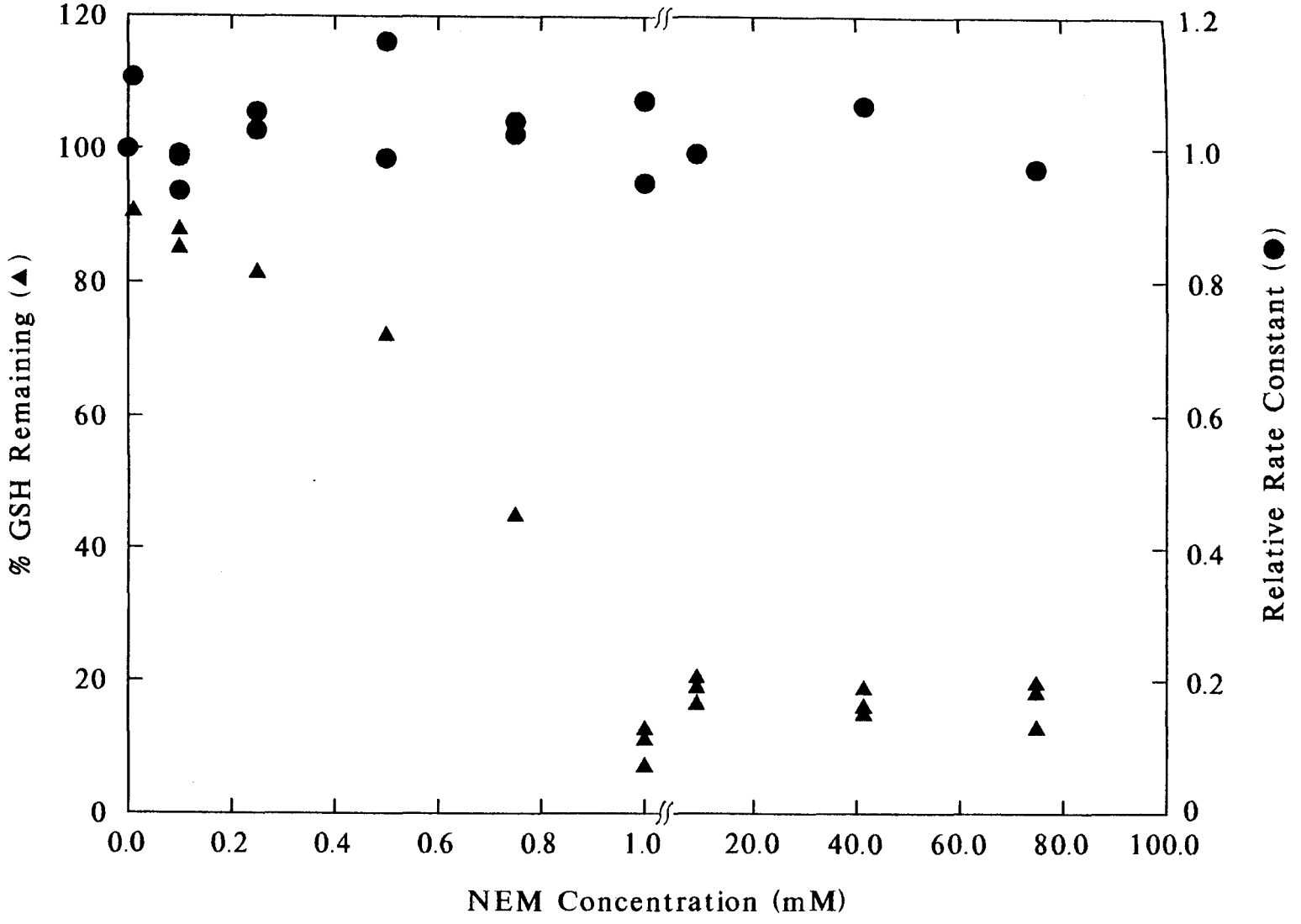
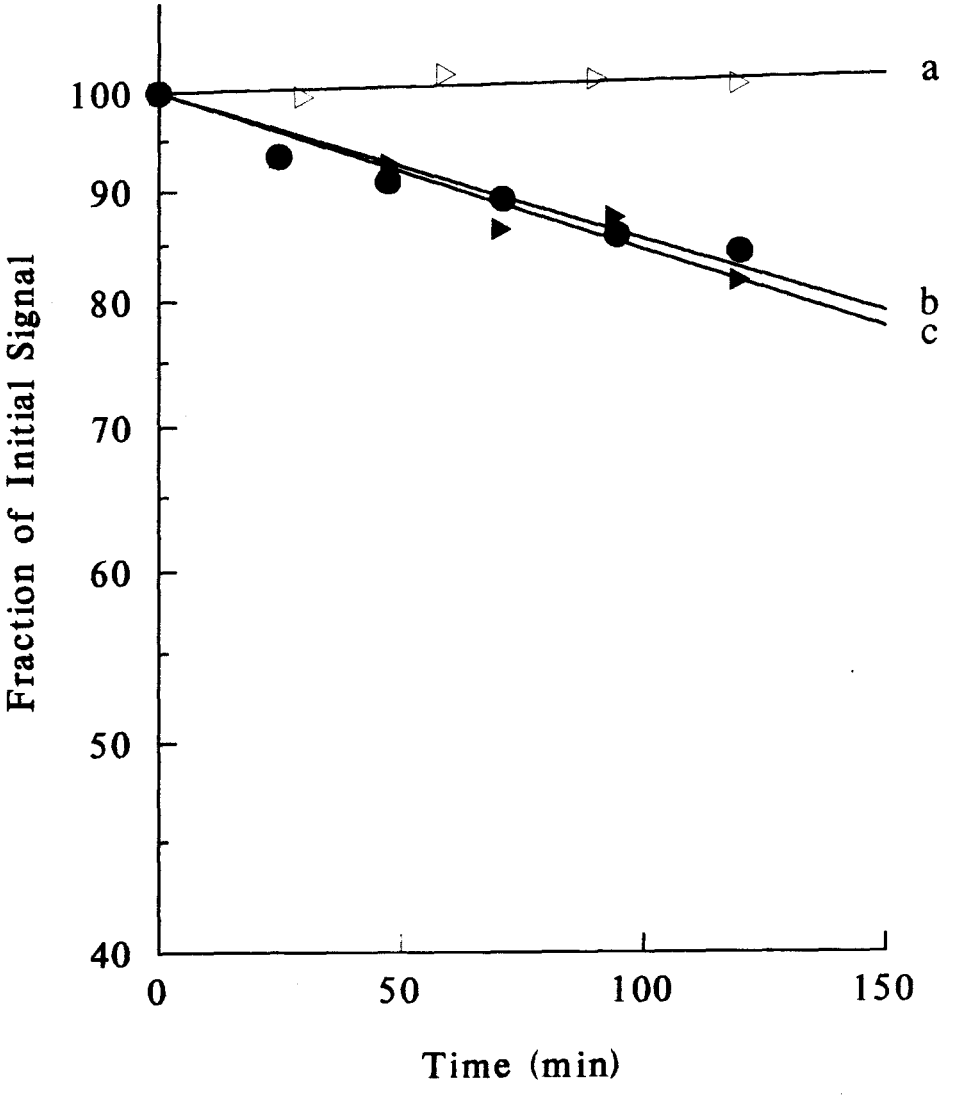

120
100
80
60
40
20
0
1.2
1.0
0.8
0.6
0.4
0.2
0
% GSH Remaining (▲)
Relative Rate Constant (●)
0.0
0.2
0.4
0.6
0.8
1.0
20.0
40.0
60.0
80.0
100.0
NEM Concentration (mM)

Figure 10 **Examination of GSH effect on the spin-labeled membrane ghost in the absence and presence of lysate.** A semi-logarithmic plot of the fraction of initial signal versus time for spin-labeled membrane ghosts with 4.0 mM GSH (▷, line a), lysate (●, line b), or 4.0 mM GSH and lysate (▶, line c). The rate constant obtained from the linear fit is -0.15 x 10^{-3}/min for GSH in the absence of lysate, with a correlation coefficient of 0.854. The rate constant for lysate sample is 1.5 x 10^{-3}/min, with a correlation coefficient of 0.989, and for GSH and lysate is 1.6 x 10^{-3}/mim, with a correlation coefficient of 0.988.


100
90
80
70
60
50
40
Fraction of Initial Signal
0
50
100
150
Time (min)
a
b
c

3.2.1.3 Antioxidant Enzymes

The major enzymes that scavenge reduced products of oxygen, such as SOD, catalase, and GPx, were chosen to study the effects of individual antioxidant enzymes on the spin label reduction by adding them to Mb* in the presence and absence of lysate. The concentrations, Figure 9 legend in terms of activity unit per ml, of each enzyme used in the experiments were at least twice as high as their physiological value. Figure 11 shows that addition of each enzyme, at the highest concentrations used in this study (14,271, 83, and 6,667 units/ml for CAT, GPx, and SOD, respectively), to the Mb* and lysate did not yield additional spin label reduction as compared with control samples. The relative rate constants were 1.0, 1.1, and 0.9 for CAT, GPx, and SOD, respectively. In addition, samples containing Mb* with CAT, GPx, or SOD did not show any observable spin label reduction, indicating these enzymes did not reduce the nitroxide radicals in membrane.

3.2.1.4 Carbonmonoxy Hemoglobin

To determine whether HbCO is involved in the spin label reduction, purified normal Hb in CO ligation was added into spin-labeled membrane in CO-gassed buffer at 4 °C and the EPR signal was monitored at 37 °C. The data from Mb* with 4 different concentrations of HbCO, varied from 70 mg/ml to 200 mg/ml, are displayed in Figure 12. Since the Mb concentration of the samples was about 3 mg/ml, the weight ratio of HbCO/Mb varied from 23 to 67, it was wide enough to cover the physiological ratio of 28, as calculated from known values (335 mg/ml of Hb and 11.8 mg/ml of Mb for packed RBC) (Surgenor, 1974). In all cases, no significant reduction was observed, with fluctuation similar to that by Mb* alone. Therefore, HbCO at concentration of up to 200 mg/ml does not affect spin label reduction over a period of 2 hr.

Figure 11 **Reduction rate constant of spin-labeled membrane and lysate with excess addition of antioxidant enzyme CAT, GPx, or SOD.** Controls contained spin-labeled membrane ghosts and lysate only. For each paired samples, the same batch of blood sample was used. The concentration of enzyme added to the sample was 14,271 unit/ml for CAT, 83 unit/ml for GPx, and 6,667 unit/ml for SOD.

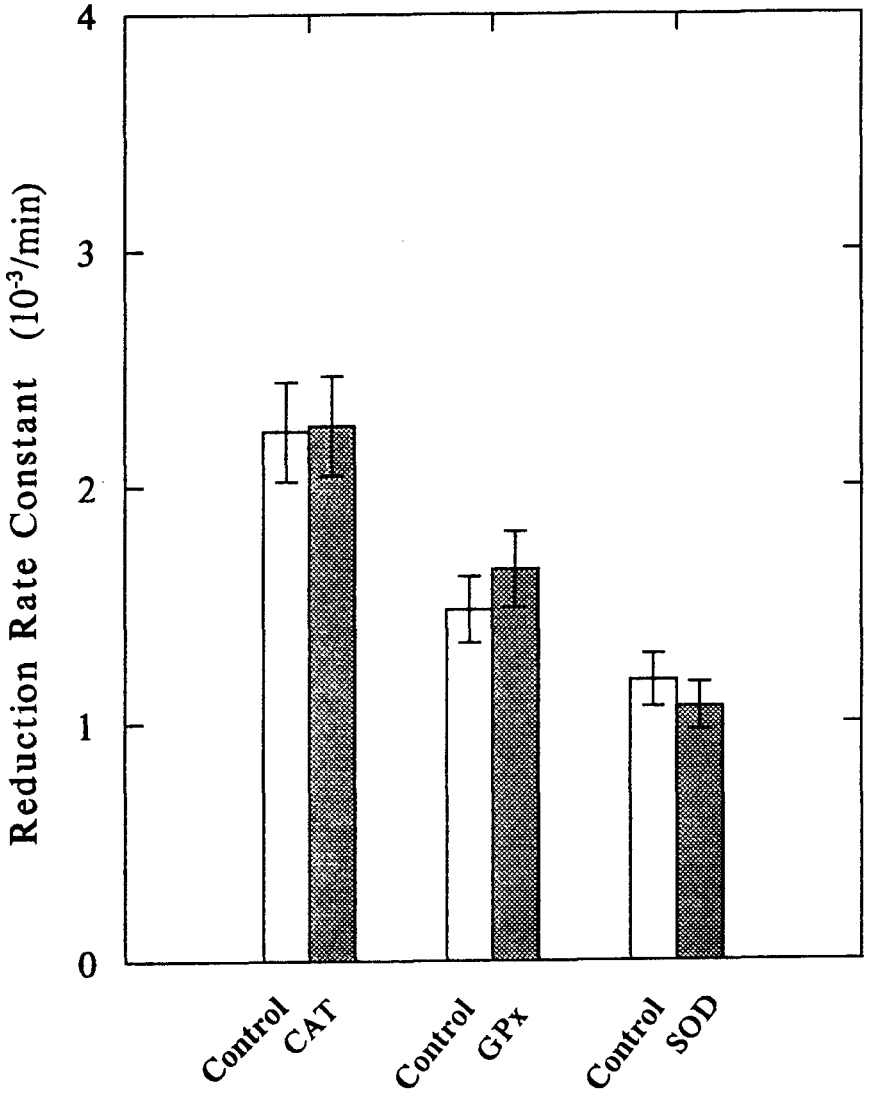
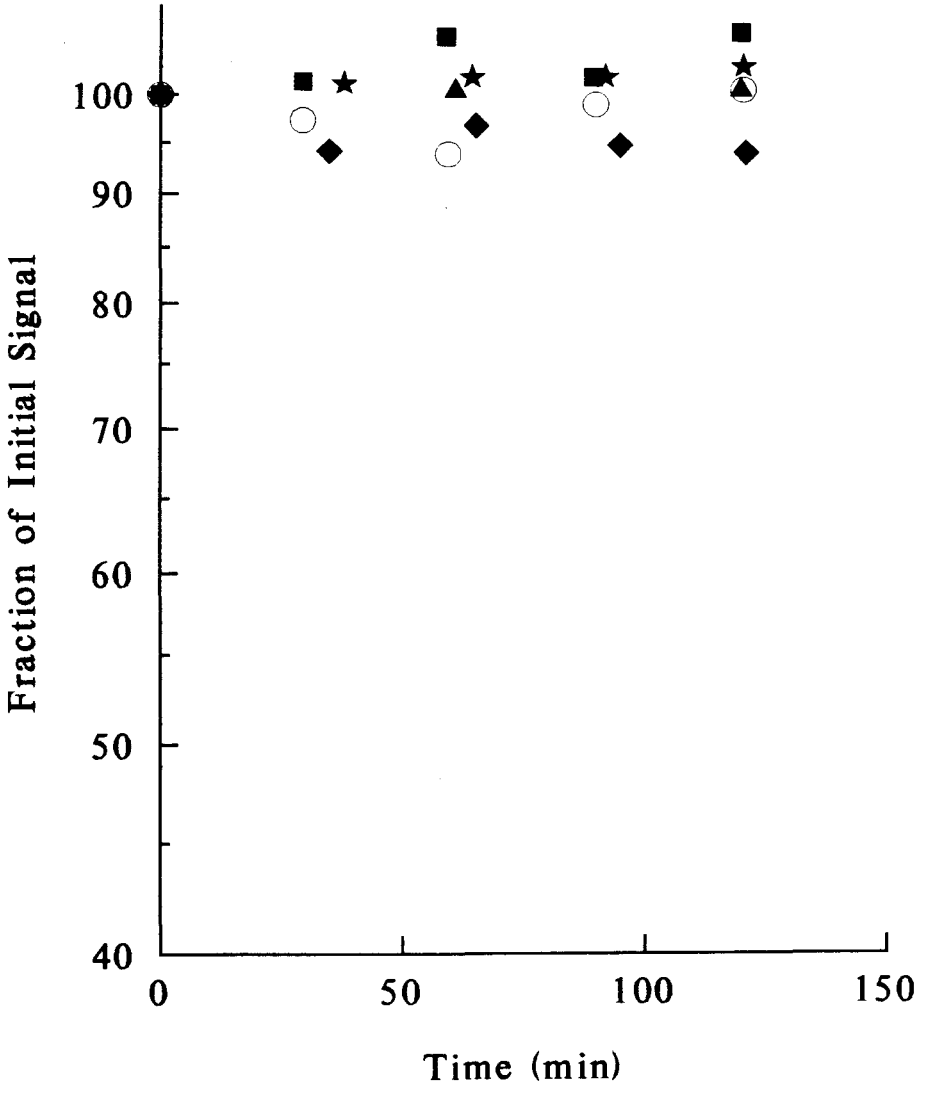

Reduction Rate Constant (10-3/min)
4
3
2
1
0
Control
CAT
Control
GPx
Control
SOD

Figure 12 **Examination of HbCO effect on the spin-labeled membrane ghosts.** A semi-logarithmic plot of the fraction of initial signal versus time for spin-labeled membrane ghosts alone (○) and with HbCO at concentrations of 70 mg/ml (▲), 120 mg/ml (■), 150 mg/ml (★), and 200 mg/ml (◆).


Fraction of Initial Signal
100
90
80
70
60
50
40
0
50
100
150
Time (min)

3.2.2 Components with Effects

3.2.2.1 Reduced Pyridine Nucleotides

Both NADH and NADPH are important reducing equivalents in reactions that are critical in protecting cells against oxidant damage in RBC (Eaton & Brewer, 1974; Zerez *et al.*, 1988). The addition of NADPH or NADH into the lysate significantly increased the reduction rate of Mb*. Figure 13 shows that when 720 μM of NADPH was added to the lysate, the rate constant of Mb* with lysate for one batch of blood was increased from 1.2 x 10^{-3}/min to 2.6 x 10^{-3}/min; and for another batch of blood, the rate constant of sample without NADPH was 1.5 x 10^{-3}/min, and with NADPH was 3.4 x 10^{-3}/min. In both cases, the relative rate constants were about 2.2 upon addition of NADPH. The addition of NADH to lysate, at about the same amount used as for NADPH, also enhanced the reduction rate from 0.7 x 10^{-3}/min to 1.2 x 10^{-3}/min, with a relative rate constant of 1.7. Both NADPH and NADH enhancements were concentration dependent as shown in Figure 14. The apparent second-order rate constant obtained from the slope of the plot for NADPH was 2.2/min·M with a good linear fit (r = 0.992). For NADH, the apparent second-order rate constant was 0.6 /min·M and the correlation coefficients for the linear regression was 0.943. It should be pointed out that there was no EPR signal reduction for Mb* with NADPH or NADH in the absence of lysate. These results indicate that reduced pyridine nucleotides functioned as reducing agents only when they were cofactors for other reactions in the lysate.

The effects of combinations of enzymes and GSH with NADPH in Mb* plus lysate on spin label reduction were then examined. Samples consisting of Mb*, lysate, NADPH, SOD, CAT, GPx, GSH, and other combinations are illustrated in Table 3. The concentrations of each antioxidant added to samples were close to or higher than physiological values. The reduction rate constants from these samples were normalized to the rate constant from Mb* + lysate + NADPH.

Figure 13 **Enhancement of spin label reduction by NADPH in the presence of lysate.** A semi-logarithmic plot of the fraction of initial signal versus time for samples containing spin-labeled membrane ghosts and lysate alone (▲) and with additional 720 μM NADPH (★). The shaded symbols are from blood sample one, and closed symbols are from blood sample two. The rate constant obtained from linear fit for blood sample one is 1.2 x 10^{-3}/min with a correlation coefficient of 0.989, while for the same blood sample with additional NADPH is 2.6 x 10^{-3}/min with a correlation coefficient of 0.994. The rate constant for blood sample two is 1.5 x 10^{-3}/mim, with correlation coefficient of 0.988, and for the same blood sample with additional NADPH is 3.4 x 10^{-3}/min, with correlation coefficient of 0.999.

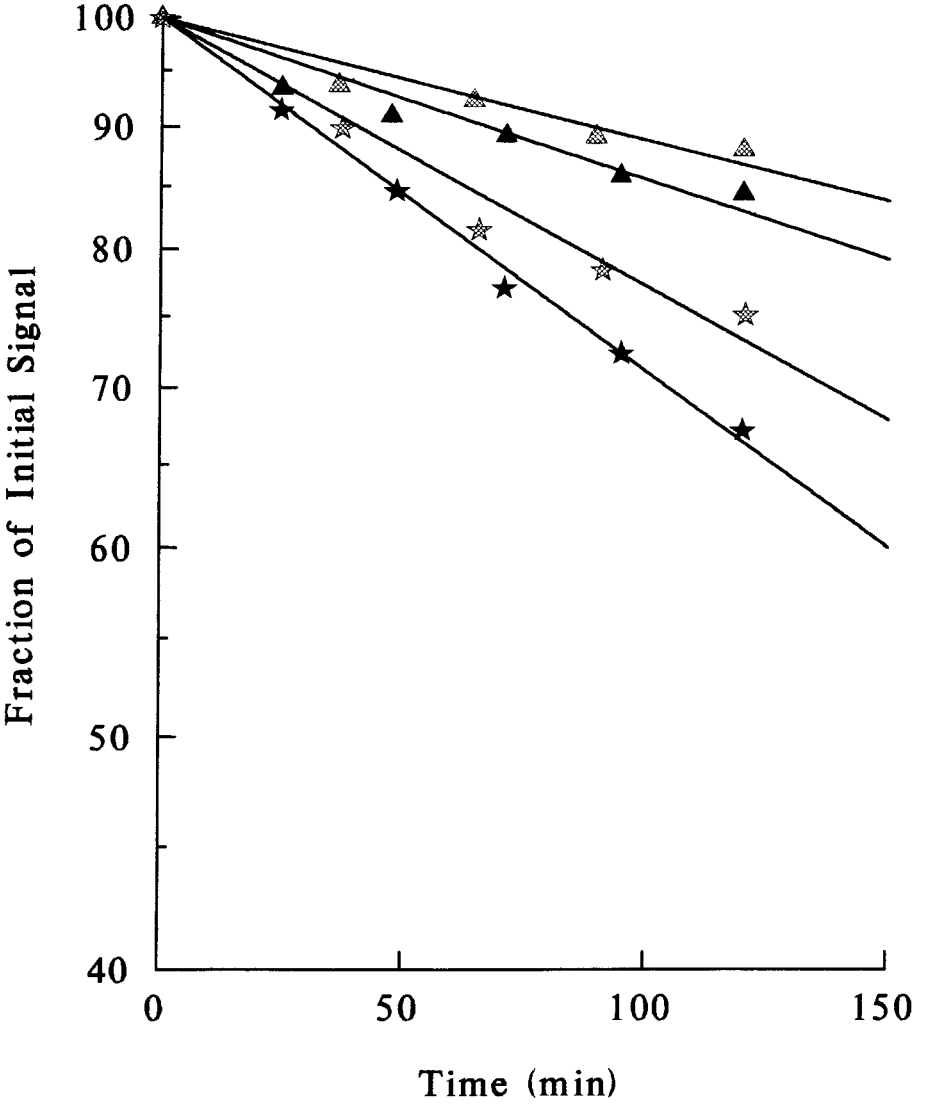

100
90
80
70
60
50
40
Fraction of Initial Signal
0
50
100
150
Time (min)

Figure 14 **NADPH and NADH concentration dependence of spin label reduction in the presence of lysate.** Spin-labeled membrane and lysate with addition of NADPH (★) or NADH (■). Different batches of blood were used for NADPH and NADH experiments.

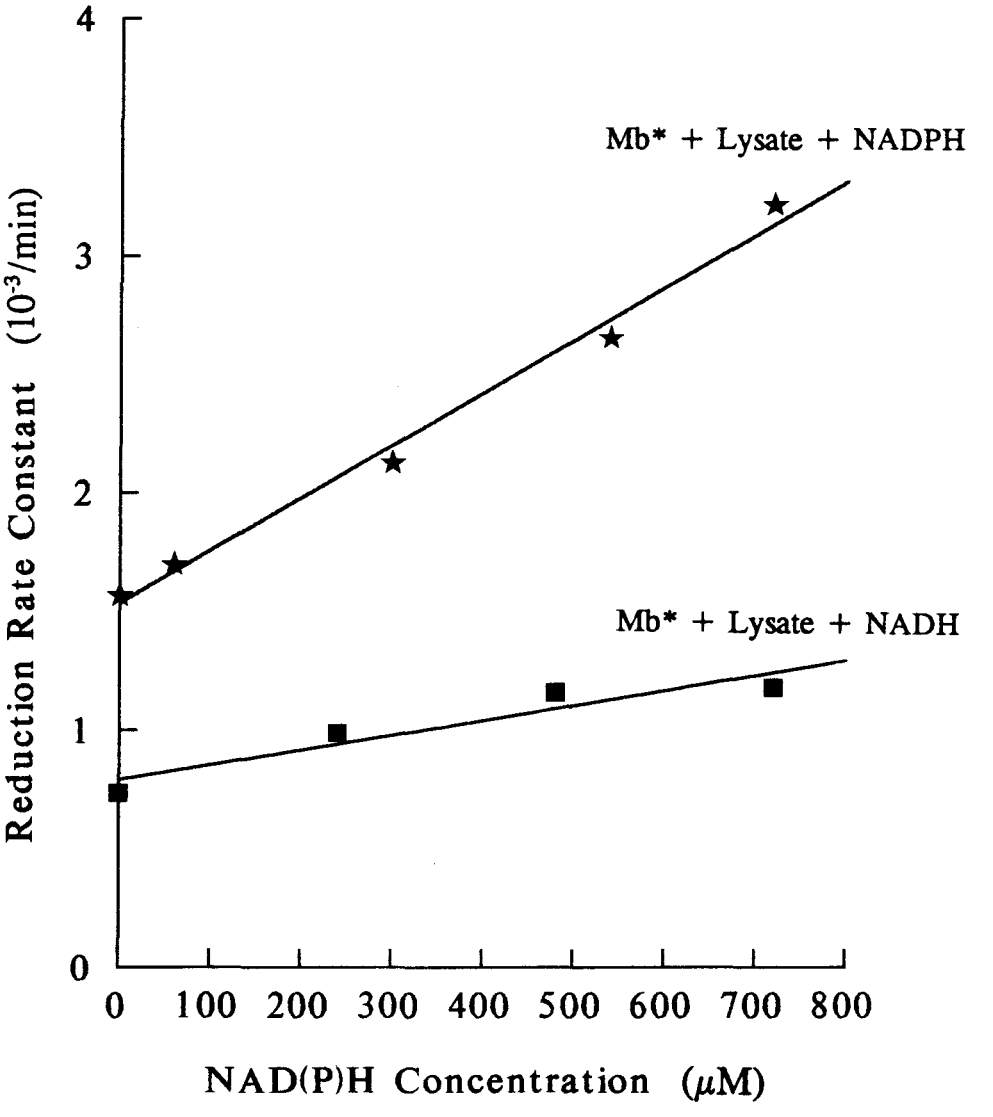

Mb* + Lysate + NADPH
Mb* + Lysate + NADH
Reduction Rate Constant (10^{-3}/min)
NAD(P)H Concentration (μM)
0
1
2
3
4
0
100
200
300
400
500
600
700
800

Table 3 Effects of Enzymes and GSH with NADPH in Spin-Labeled Membrane and Lysate on Spin Label Reduction

System	Relative Rate Constant
Mb* + Lysate + NADPH (720 μM) (control)	1.0
control + CAT (14271 unit/ml)	1.1
control + GPx (83 unit/ml)	0.9
control + SOD (6667 unit/ml)	1.0
control + GSH (4 mM)	1.1
control + CAT (1247 unit/ml) + GPx (18 unit/ml) + SOD (2000 unit/ml)	1.0
control + CAT (1247 unit/ml) + GPx (18 unit/ml) + GSH (2mM)	1.0
control + CAT (1247 unit/ml) + SOD (2000 unit/ml) + GSH (2mM)	1.0
control + GPx (18 unit/ml) + SOD (2000 unit/ml) + GSH (2mM)	1.0
control + CAT (1247 unit/ml) + GPx (18 unit/ml) + SOD (2000 unit/ml) + GSH (2mM)	1.0

The concentration of each antioxidant is the amount of addtion, which does not include the amount in lysate.

The results showed that addition of enzymes and/or GSH gave about the same effects (within ± 10 %) as NADPH alone in the Mb* with lysate. In the absence of lysate, Mb* and NADPH combined with other components showed no detectable spin label reduction over a time period of 2 hr. In Table 4 the relative signals remained of the tested samples with Mb* + NADPH as 100 % are listed.

3.2.2.2 Ascorbate

3.2.2.2.1 In Buffer System

Ascorbate is an aqueous antioxidant, and is known to reduce water-soluble nitroxides in solution. It was found that 5-DSA spin label with 1.0 mM of ascorbate in PBS buffer underwent a pseudo first-order reduction within the first 15 min with a rate constant of 31.0 x 10^{-3}/min and correlation coefficient of 0.996. Over a longer period of time the rate slowed down and did not follow first-order kinetics.

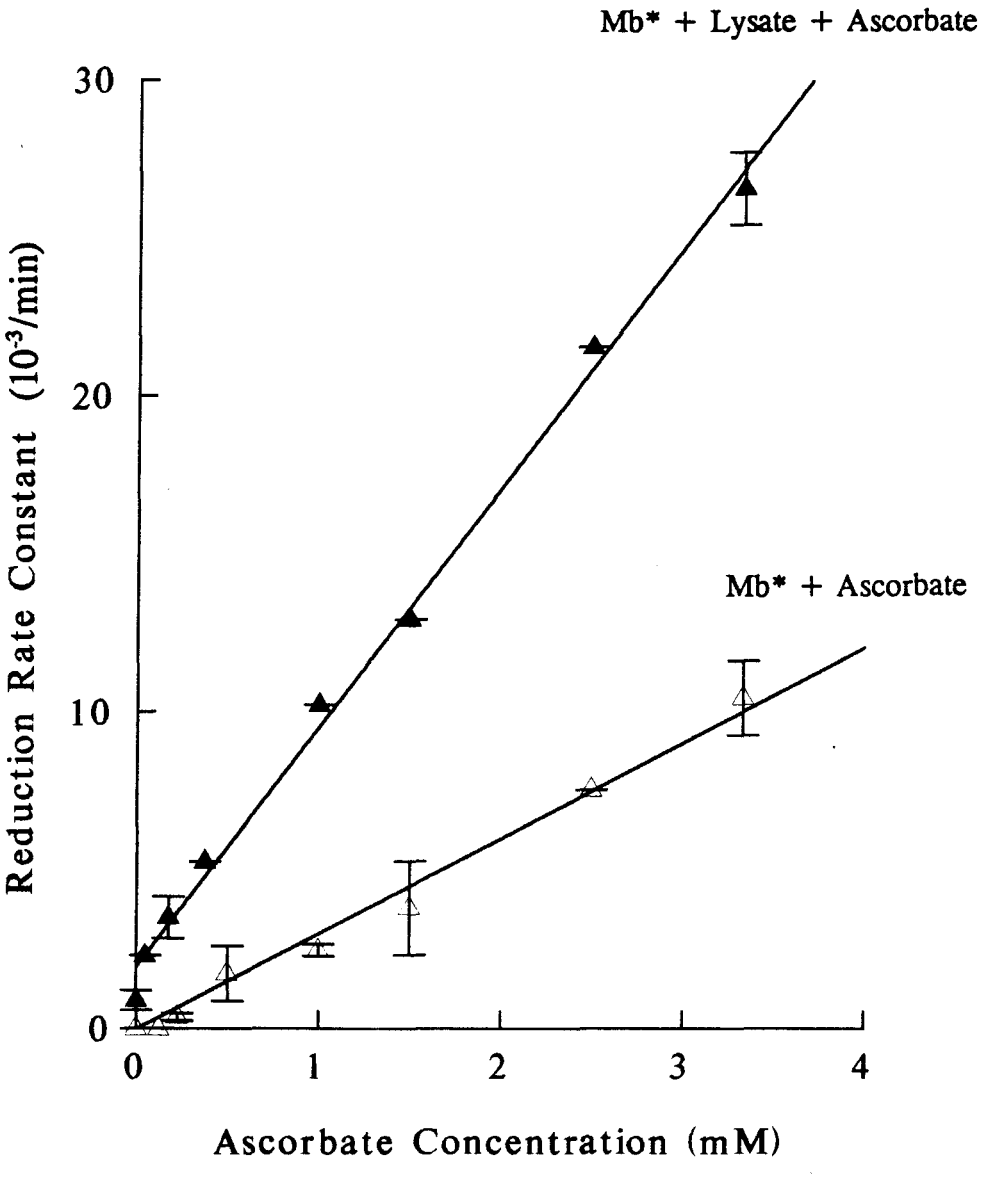
3.2.2.2.2 In Membrane System

Ascorbate was added to Mb* and studied for its reducing ability in membrane systems. It was found that ascorbate, at concentration of 1.0 mM, also reduced the spin label in membrane, but the reduction rate was much slower than that in buffer. The pseudo first-order rate constant was 2.5 ± 0.2 x 10^{-3}/min (n = 3). The spin label reduction of Mb* by ascorbate appeared to be concentration dependent. The reduction rate constant increased to 10.4 ± 1.2 x 10^{-3}/min (n = 3) at ascorbate concentration of 3.3 mM. When ascorbate concentration decreased to the physiological concentration of about 0.1 mM, there was no detectable signal reduction. Plotted in Figure 15 are the pseudo first-order rate constants as a function of ascorbate concentrations. An apparent second-order rate constant obtained from the slope for Mb* + ascorbate was 3.1/min·M, with a correlation coefficient of 0.996.

Table 4 Effects of NADPH with Other Components in Spin-Labeled Membrane on Spin Label Reduction

System	Relative Signal Remaining (%)
Mb* + NADPH (720 μM) (control)	100
control + Hb (125 mg/ml)	96.8
control + GSH (40 mM)	99.5
control + CAT (14271 unit/ml)	99.8
control + GSH (40 mM) + GPx (300 unit/ml)	95.3
control + GSH (40 mM) + SOD (4000 unit/ml)	100
control + Hb (125 mg/ml) + GSH (4 mM) + CAT (1247 unit/ml) + GPx (18 unit/ml) + SOD (2000 unit/ml)	105

Figure 15 **Ascorbate concentration dependence of spin label reduction in the presence or absence of lysate.** Spin-labeled membrane with lysate and addition of ascorbate (▲); spin-labeled membrane with ascorbate (Δ). Error bars represent the standard deviation of 2 or 3 measurements using different batches of blood sample.


Mb* + Lysate + Ascorbate
Mb* + Ascorbate
Reduction Rate Constant (10^{-3}/min)
30
20
10
0
0
1
2
3
4
Ascorbate Concentration (mM)

No detectable spin label reduction was observed when 2 units/ml of AO, which oxidizes ascorbate to DHA, was added to either the spin label in buffer or Mb* system that contained 1.0 mM of ascorbate. Thus, the spin label reduction by ascorbate could be inhibited by AO.

In addition, it was found that in the presence of 4.0 mM GSH the spin label reduction rate constant of Mb* with 1 mM ascorbate increased from 2.5 x 10^{-3}/min to 6.2 ± 0.5 x 10^{-3}/min (n = 2), while the same amount of GSH did not reduce spin label in membrane, as stated in Section 3.2.1.2. At 2.5 mM of ascorbate, the rate constant increased from 7.6 x 10^{-3}/min to 11.8 x 10^{-3}/min in the presence of 4.0 mM GSH. The apparent second-order rate constant obtained from the two data points was 4.7/min·M for Mb* with ascorbate in the presence of 4.0 mM GSH.

Interestingly, the spin label reduction of Mb* caused by the mixture of 1 mM ascorbate and 4.0 mM GSH was not inhibited by AO, even when AO concentration was increased from 2 to 20 unit/ml. The rate constants were 5.5, 5.8, and 5.9 x 10^{-3}/min for 2, 7.6, and 20 units/ml of AO, respectively. As mentioned in the Methods section, the sequence of adding reagents was random, except Mb* was always the last component to be added to the solution. No significant effects on the rate constants were observed when reagents were added in a different order.

To clarify whether GSH could inhibit AO for the oxidation of ascorbate, a parallel experiment was conducted in a UV cuvette while monitoring the characteristic UV peak absorbance range of ascorbate from 225 nm to 325 nm. It was found that the addition of GSH did not significantly affect the UV absorbance spectra of ascorbate, and the peak steadily decreased after AO was added in both cases when GSH was either present or not.

3.2.2.2.3 In Membrane with Lysate System

The ascorbate effect was studied for the spin label reduction of Mb* in the presence

of lysate. Figure 16 presents a plot for relative rate constants of Mb* and lysate treated with AO vs amount of AO added. The results showed that the spin label reduction rates were reduced by about 80 % after treatment of lysate with less than 0.1 unit/ml of AO. At AO concentration of about 1 unit/ml or larger, nearly no spin label reduction was detected with the reduction rate constants fluctuating at a certain low level.

Figure 17a shows the reduction of Mb* with lysate and 3.3 mM ascorbate as a function of time in a typical run. An average rate constant for the membrane with lysate was $1.2 \pm 0.4 \times 10^{-3}$/min (n = 7). Addition of 3.3 mM ascorbate to lysate increased the reduction rate constant to $26.7 \pm 1.2 \times 10^{-3}$/min (n = 3). These rate constants were determined over a period of only 1 hr, because the signal reduction data of samples with ascorbate leveled off beyond that period in a semi-logarithmic plot. It was found that in the presence of lysate ascorbate could reduce spin label in membrane faster than that in the absence of lysate with the same concentration of ascorbate added, as shown in Figure 15. For Mb* + lysate + ascorbate, a linear ascorbate concentration dependence was also observed. An apparent second-order rate constant obtained from the slope was 7.6/min·M with a correlation coefficient of 0.997.

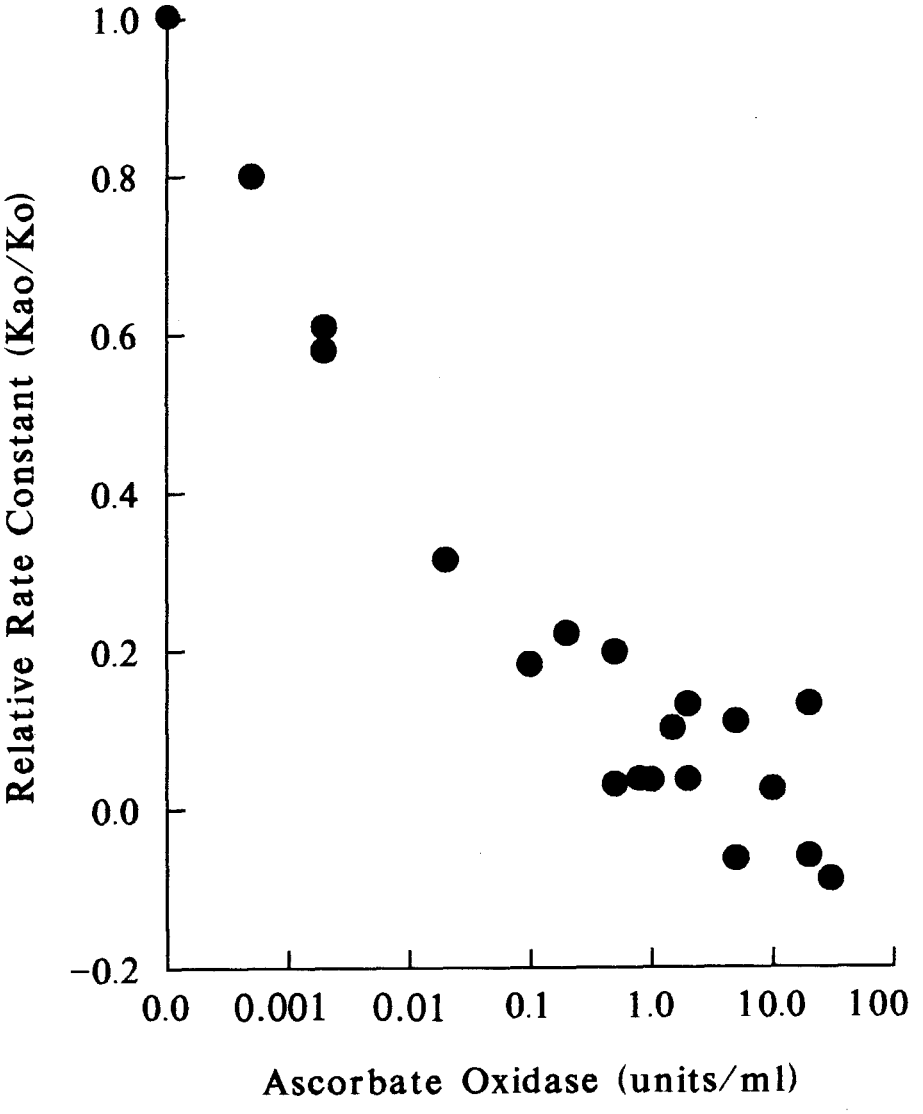
The enhanced reduction with rate constant of 3.0×10^{-3}/min at 0.2 mM ascorbate was inhibited by addition of 2 units/ml AO and showed no detectable reduction.

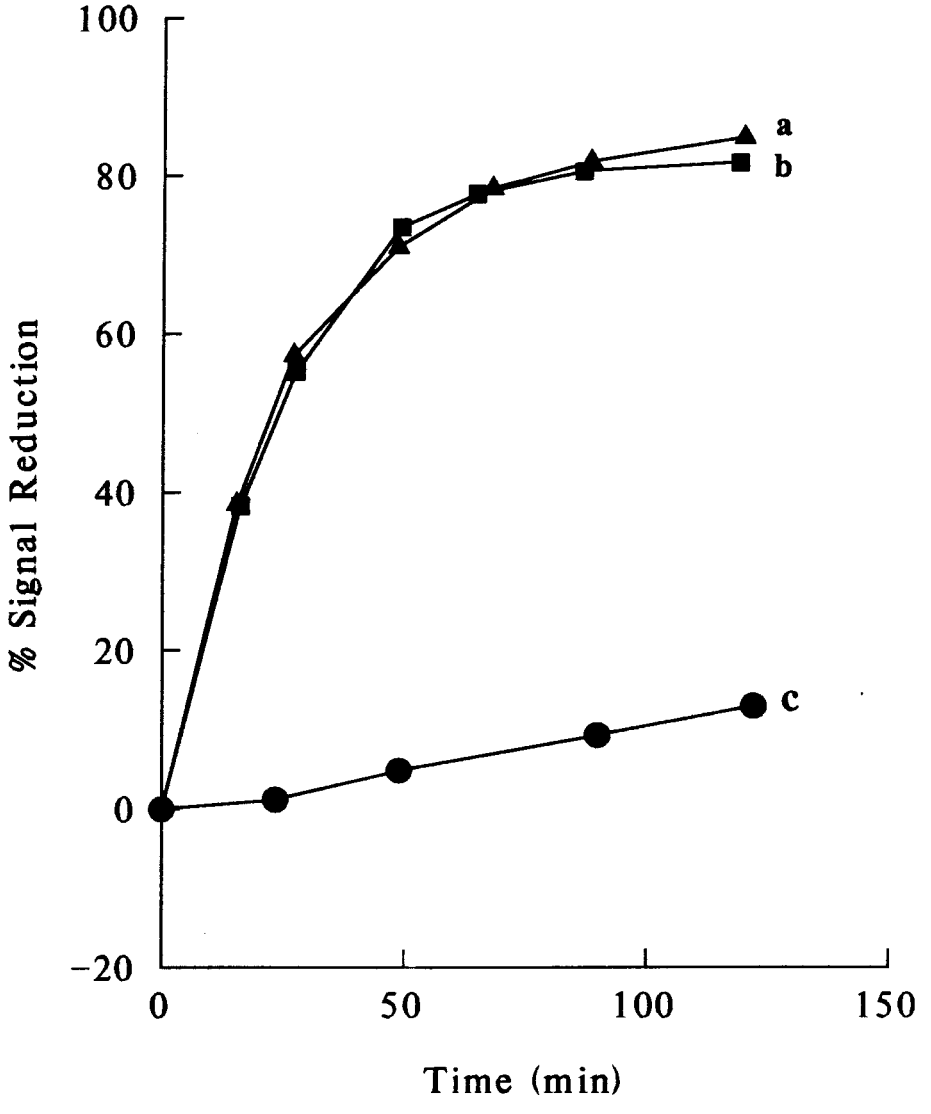
The addition of 11.8 mM vitamin E to samples with 3.3 mM ascorbate did not further increase the reduction (Figure 17b). The rate constant was $26.4 \pm 1.2 \times 10^{-3}$/min (n = 3) as determined over a period of 1 hr.

The combined effects of NADPH and ascorbate were also studied as follows: (1) to study the inhibition effect of AO on the spin label reduction of Mb* with NADPH treated lysate; (2) to measure the ascorbate concentration directly in NADPH treated lysate.

In the presence of 2 units/ml of AO, the reduction rate of Mb* and lysate with 720 μM NADPH was inhibited by only about 50 %, with rate constant reduced from 2.8×10^{-3}/min to 1.4×10^{-3}/min.

Figure 16 **Inhibition of spin label reduction by AO in lysate.** Relative rate constant represents the ratio of rate constant of AO-treated lysate (k_{ao}) to rate constant of the lysate without AO treatment (k_o). Different batches of blood were used to prepare the samples.


1.0
0.8
0.6
0.4
0.2
0.0
−0.2
Relative Rate Constant (Kao/Ko)
0.0
0.001
0.01
0.1
1.0
10.0
100
Ascorbate Oxidase (units/ml)


100
80
60
40
20
0
-20
% Signal Reduction
0
50
100
150
Time (min)
a
b
c

A similar result was observed for lysate treated with 540 μM NADPH. The rate constant reduced from 2.5 x 10^{-3}/min to 1.3 x 10^{-3}/min in the presence of 0.8 units/ml AO.

The results from ascorbate concentration measurement are shown in Figure 18. For one set of samples, lysate samples were mixed with buffer in the absence and presence of 720 μM NADPH and stored at 4 °C overnight. The concentrations of ascorbate were measured the next day and found to be 13.35 and 2.09 μg/gHb for NADPH treated lysate and control, respectively. For another set of samples, ascorbate concentration measurements were done immediately after 720 μM NADPH was added to lysate. The ascorbate concentrations were 19.09 and 11.61 μg/gHb for NADPH treated lysate and control, respectively. Although the sample conditions for these two sets of experiments were not exactly the same and the uncertainty for ascorbate concentration measurement was not determined, the results from both cases showed that the ascorbate levels of NADPH-treated lysate were increased when compared with their controls.

3.2.2.3 Dehydroascorbate

The spin label reduction of Mb* by DHA was studied. It was noticed that a transient delay for DHA to reduce spin label in Mb* existed in the concentration range (1 - 2.5 mM) studied (Figure 19). The reduction rate did not appear to fit the first-order kinetics as well as the other systems studied. In order to compare with previous results, the pseudo first-order rate constants were obtained, despite relatively low correlation coefficient values. The rate constants were 0.5, 4,4, and 10.9 x 10^{-3}/min for 1.0, 1.5, and 2.5 mM of DHA added to Mb* respectively. The apparent second-order rate constant was 4.5/min·M with a correlation coefficient of 0.929. Addition of 4.0 mM GSH to Mb* and DHA increased spin label reduction with a rate constant of 3.6 x 10^{-3}/min at 1.0 mM DHA and 8.0 x 10^{-3}/min at 1.5 mM DHA. The apparent second-order rate constant was 5.1/min·M with a correlation coefficient of 0.968.

Figure 18 **Effect of NADPH treatment on ascorbate concentration in lysate.** For the set one, the lysate samples were mixed with buffer in the absence (□) and presence (▩) of 720 μM NADPH and stored at 4°C overnight. Concentrations of ascorbate in the samples were measured the next day. For the set two, ascorbate concentration measurements were done immediately after 720 μM NADPH was added to the lysate.

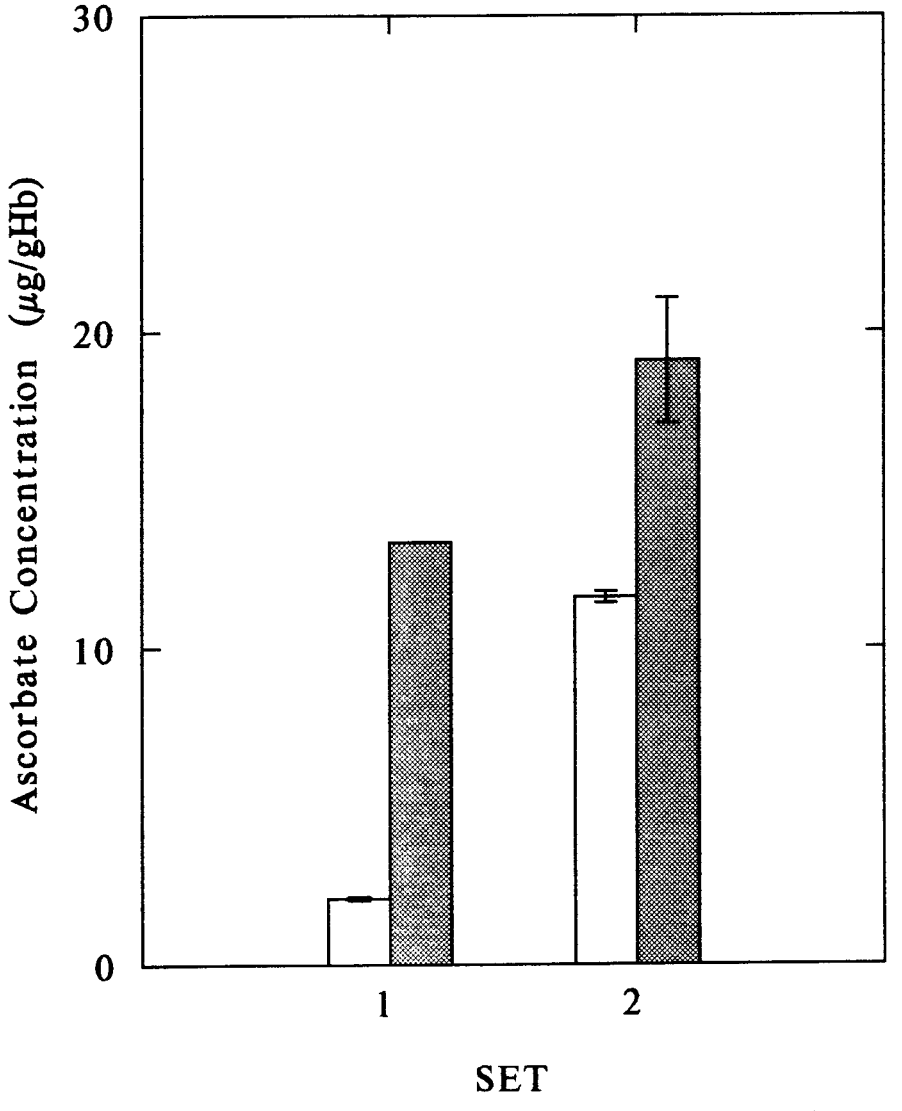
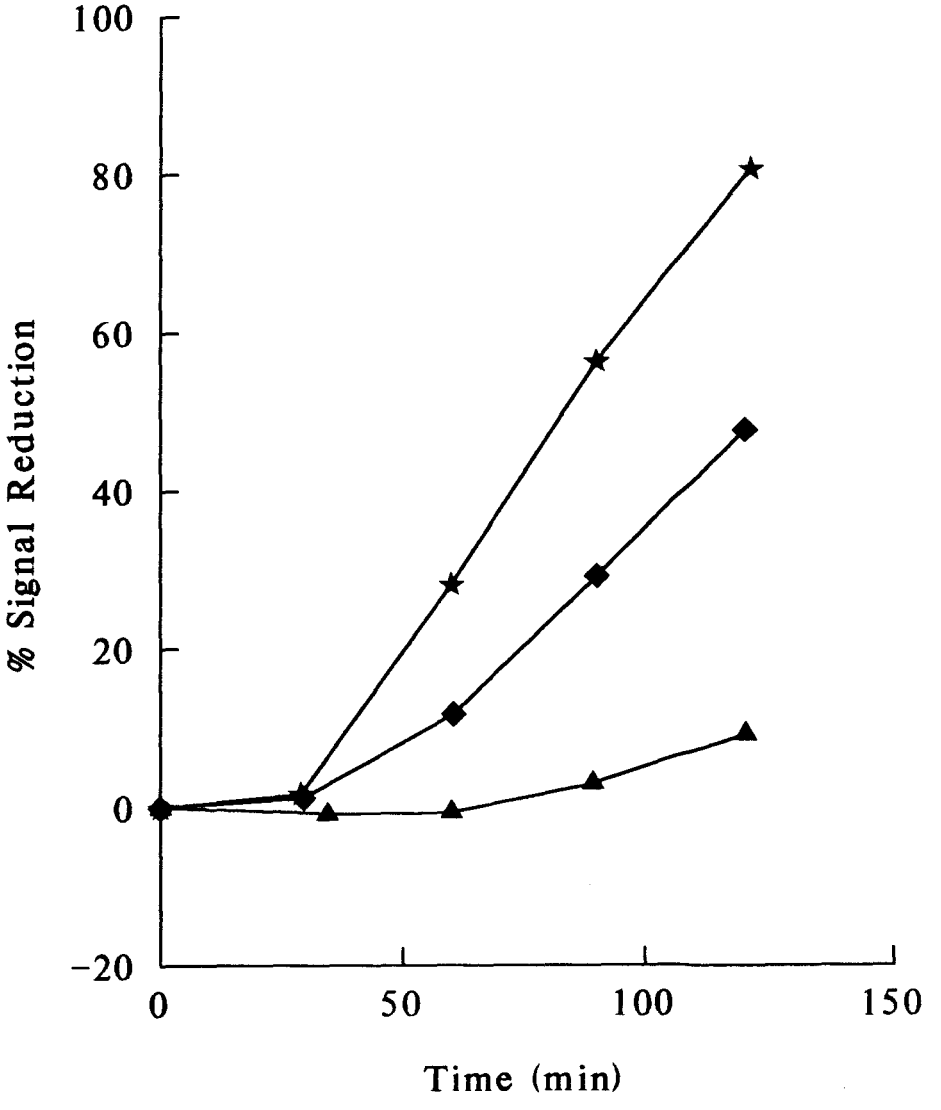

30
20
10
0
Ascorbate Concentration (μg/gHb)
1
2
SET

Figure 19 **Percent EPR signal reduction over a period of 2 hr for spin-labeled membrane with DHA at different concentrations.** DHA concentrations are: 1.0 mM (▲), 1.5 mM (◆), and 2.5 mM (★).


100
80
60
40
20
0
-20
% Signal Reduction
0
50
100
150
Time (min)

The spin label reduction caused by either DHA alone or the mixture of DHA and GSH could not be inhibited by AO. The rate constants for DHA with 10 units/ml of AO were 2.0, 5.5, and 11.7 x 10^{-3}/min for 1.0, 1.5, and 2.5 mM DHA respectively. The rate constants for DHA with 4.0 mM GSH and 10 units/ml AO were 4.0 x 10^{-3}/min and 7.8 x 10^{-3}/min for 1.0 and 1.5 mM DHA.

The addition of 0.5 mM DHA to Mb* with lysate increased the rate constant from 1.1 x 10^{-3}/min to 4.1 x 10^{-3}/min. Addition of 2 units/ml AO to this DHA, lysate and Mb* system reduced the rate constant to 1.3 x 10^{-3}/min.

3.2.2.4 HbCO and Cell-O_2 Condition

As mentioned earlier, the cell condition could affect the reduction rate of spin label. It was noticed that when cells were first washed three times with CO-gassed buffer to fully saturate the Hb, then washed once with un-gassed or O_2-gassed buffer after spin labeling, the HbCO form did not change to HbO_2 as confirmed by optical measurement. However, the reduction rate from this RBC sample was lower than the fully CO-gassed RBC sample and higher than the fully O_2-gassed RBC sample. Figure 20 shows a percentage reduction plot of data obtained from a parallel run of samples under three conditions. Samples were made in duplicates for each condition. The rate constant was 6.9 ± 0.6 x 10^{-3}/min for the fully CO-gassed sample; 3.2 ± 0.3 x 10^{-3}/min for the fully O_2-gassed sample during the first 90 min, and 4.3 ± 0.3 x 10^{-3}/min for the sample under the condition of HbCO and cell-O_2.

3.2.3 Summary of the Effects of RBC Components on Spin Label Reduction

A summary of all the components examined for spin label reduction in membrane and membrane with lysate is presented in Tables 5 and 6. The concentration of each component listed was usually the highest concentration used for the study, especially for the components with no effects on the spin label reduction.

Figure 20 **Percent EPR signal reduction over a period of 2 hr for samples in different conditions.** Fully CO-gassed RBC samples (●), fully O_2-gassed RBC samples (▲), and samples under the condition of HbCO and cell-O_2 (■). Samples were prepared from the same batch of blood and made duplicate for each condition.

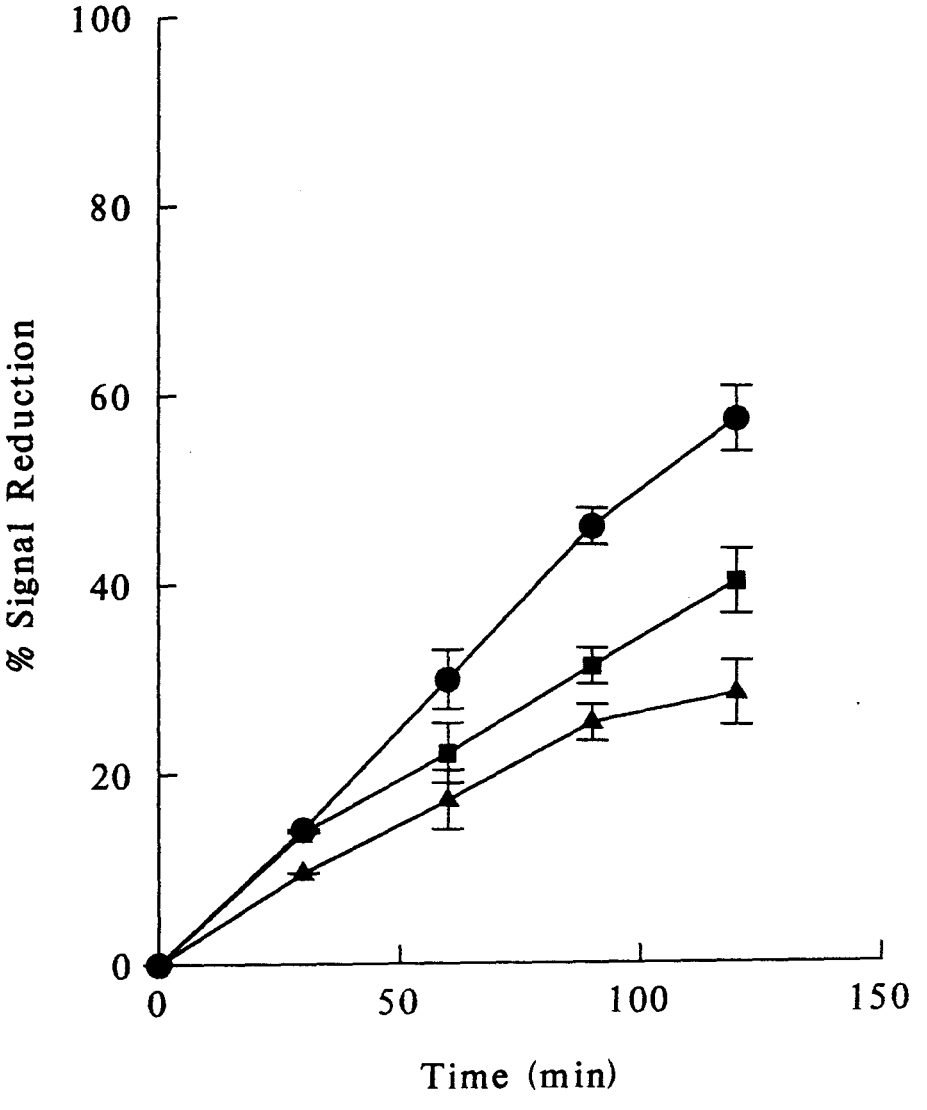

100
80
60
40
20
0
% Signal Reduction
0
50
100
150
Time (min)

Table 5 Summary of the Effects of RBC Components on Spin Label Reduction in Membrane

Component	Conc. of the Material Added	Rate Constant (10^{-3}/min)	Conc. Dependent Rate Constant
Vit E	11.8 mM	ND	
GSH	4.0 mM	ND	
SOD	6667 unit/ml	ND	
CAT	14271 unit/ml	ND	
GPx	83 unit/ml	ND	
HbCO	200 mg/ml	ND	
NADH	720 μM	ND	
NADPH	720 μM	ND	
Ascorbate	0.1 mM	ND	
	1.0 mM	2.5	
	2.5 mM	7.6	
	3.3 mM	10.4	3.1/min·M
Ascorbate + AO	1.0 mM 2 unit/ml	ND	
Ascorbate + GSH	1.0 mM 4.0 mM	6.2	
	2.5 mM 4.0 mM	11.8	4.7/min·M
Ascorbate + GSH + AO	1.0 mM 4.0 mM 20 unit/ml	5.9	
DHA	1.0 mM	0.5	
	1.5 mM	4.4	
	2.5 mM	10.9	4.5/min·M
DHA + AO	2.5 mM 10 unit/ml	11.7	
DHA + GSH	1.0 mM 4.0 mM	3.6	
	1.5 mM 4.0 mM	8.0	5.1/min·M
DHA + GSH + AO	1.5 mM 4.0 mM 10 unit/ml	7.8	

ND = not detectable.

Table 6 Summary of the Effects of RBC Components on Spin Label Reduction in Membrane with Lysate

Conponent	Conc. of the Material Added	Conc. in Lysate[a]	Rate Constant (10^{-3}/min)	Ratio[b]	Conc. Dependent Rate Constant
---	---	---	1.2 ± 0.4	1.0	
Vit E	11.8 mM		1.3	1.1	
GSH	4.0 mM	1.5 mM	1.6	1.1	
SOD	6667 unit/ml	500 unit/ml	1.1	0.9	
CAT	14271 unit/ml	350 unit/ml	2.3	1.0	
GPX	83 unit/ml	4.5 unit/ml	1.7	1.1	
HbCO	NM[c]				
NADH	720 μM	20 μM	1.2	1.7	0.6/min·M
NADPH	720 μM 540 μM	20 μM	2.9 ± 0.4 2.5	2.2 1.7	2.2/min·M

[a]estimated based on physiological values and a dilution factor of 2.
[b]rate constant of sample relative to that of its own control.
[c]not measured.

(continued)

Table 6 (continued)

Conponent	Conc. of the Material Added	Conc. in Lysate[a]	Rate Constant (10^{-3}/min)	Ratio[b]	Conc. Dependent Rate Constant
Ascorbate	0.2 mM	<0.05 mM	3.0	2.8	
	3.3 mM		26.7	22.3	7.6/min·M
Ascorbate + AO	0.2 mM 2 unit/ml		ND[d]		
AO	0.1 unit/ml		0.3	0.2	
	1 unit/ml		ND[d]		
NADPH + AO	720 μM 2 unit/ml	20 μM	1.4	0.5[e]	
	540 μM 0.8 unit/ml		1.3	0.5[e]	
DHA	0.5 mM	< 0.002 mM	4.1	3.7	
DHA + AO	0.5 mM 2 unit/ml		1.3	0.3[e]	

[a]estimated based on physiological values and a dilution factor of 2.
[b]rate constant of sample relative to that of its own control.
[d]not detectable.
[e]rate constant of sample with AO relative to that without AO.

For the components with effects, several concentrations were listed as mentioned in the text to show the concentration dependance. The ratio represented the rate constant of the sample relative to the rate constant of its own control.

3.3 Spin Label Reduction in Sickle RBC

3.3.1 Reduction Rate in Sickle RBC

The reduction rate constants for the blood samples from 7 individual homozygous sickle patients vary significantly from time to time. Some patients were followed up to 3 years during the 5-year period of this study (Table 7). The CV values ranged from 4.7 % (over a period of 20 months) to 47.3 % (over a period of 17 months), with an average of 27.2 ± 15.1 %.

The average rate constants of the blood samples from 31 homozygous sickle patients varied substantially, from 2.7 x 10^{-3}/min to 26.9 x 10^{-3}/min with an average value of 8.8 ± 6.4 x 10^{-3}/min and a CV of 72.5 %.

3.3.2 Spin Label Reduction in Sickle Lysate

Experiments similar to those studied for normal RBC were carried out for sickle RBC. Reduction rates in sickle RBC were determined. Only those with rate constants higher than those from normal were used to prepare membrane ghosts and lysate. Membrane and lysate from normal RBC were used as controls. The systems were (1) Mb*(N) + lysate (N); (2) Mb*(S) + lysate(N); (3) Mb*(N) + lysate(S); (4) Mb*(S) + lysate(S). Three sets of spin label reduction rate constants of these 4 systems are presented in Figure 21. These data show that within a single set of experiment, reduction rate constants from systems (3) and (4) were always higher than those from systems (1) and (2). The rate constants from system (1) to system (4) are 7.0, 8.7, 9.7, and 10.8 x 10^{-3}/min for the first set; 2.0, 2.4, 4.6, and 4.4 x 10^{-3}/min for the second set; and 2.1, 2.6, 4.2, and 4.5 x 10^{-3}/min for the third set.

Table 7 Reduction Rate Constants from 7 Sickle Patients' Blood and the Variation of Rate Constants within the Same Patient from Time to Time.

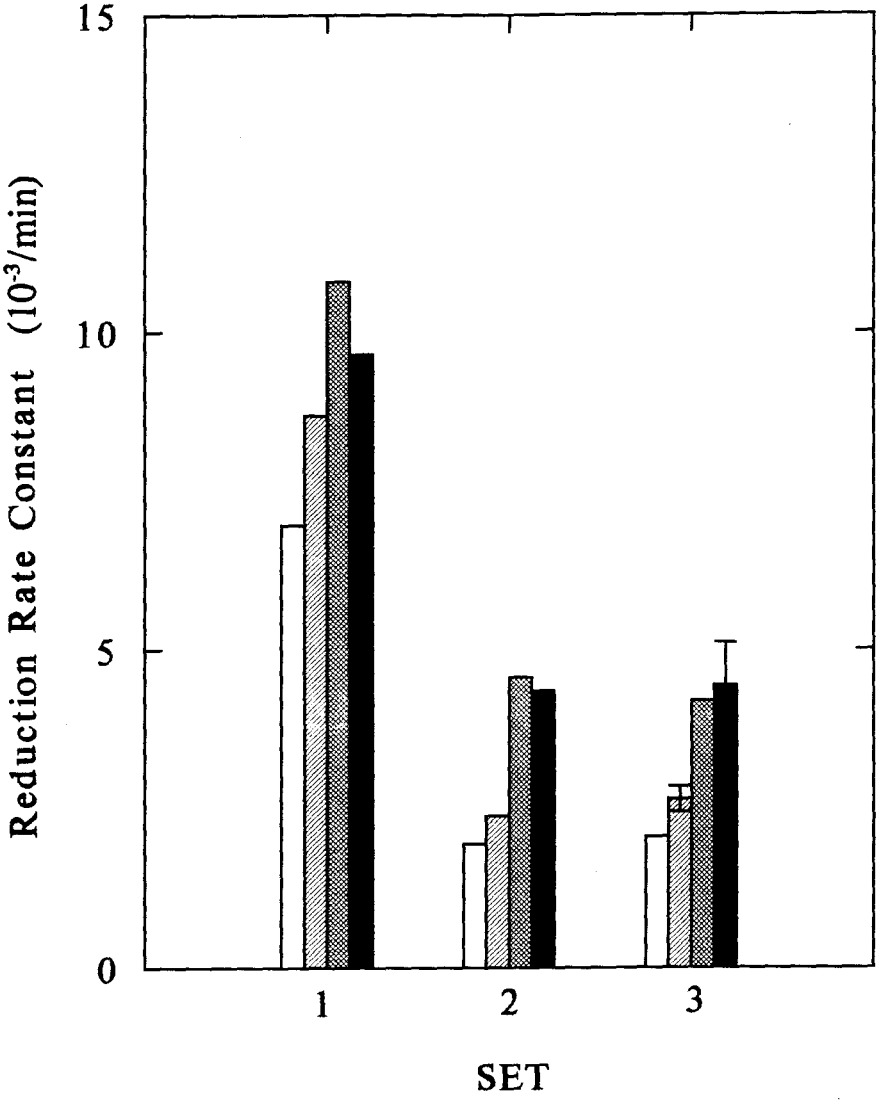
Patient	Date	k[a] (10^{-3}/min)	Avg k[b] (10^{-3}/min)	CV (%)
A	5-26-88	12.54		
	1-23-90	11.47		
	1-23-90	11.69	11.90 ± 0.57	4.7
B	6-08-88	8.58		
	6-21-88	6.76	7.67 ± 1.28	16.7
C	5-03-91	8.61		
	5-09-91	6.66	7.64 ± 1.38	18.1
D	12-15-88	7.97		
	8-29-89	4.48		
	11-20-90	8.19	6.88 ± 2.08	30.2
E	6-04-87	3.39		
	6-16-87	3.81		
	7-14-87	4.92		
	7-28-87	3.50		
	9-01-87	4.04		
	1-30-88	3.78		
	6-07-88	1.37		
	6-16-88	3.52		
	6-28-88	4.42		
	9-06-89	1.79		
	9-10-89	5.28		
	11-15-89	3.95		
	12-06-89	4.87		
	12-06-89	5.51	3.87 ± 1.18	30.6
F	6-04-87	11.76		
	6-16-87	10.57		
	7-14-87	8.75		
	7-28-87	10.11		
	9-01-87	12.10		
	10-06-88	14.75		
	9-06-89	3.32		
	10-26-90	3.30	9.33 ± 4.10	43.0
G	8-29-89	3.42		
	1-29-91	6.85	5.14 ± 2.43	47.3

The average coefficient of variability (CV) of k for the same patient from time to time is 27.2 ± 15.1 %.

[a]k is the pseudo first-order rate constant for spin label reduction.

[b]Average k value of the same patient with standard deviation.

Figure 21 **Spin label reduction in sickle lysate.** The systems were (1) Mb*(N) + lysate (N) (□); (2) Mb*(S) + lysate(N) (▨); (3) Mb*(N) + lysate(S) (▩); (4) Mb*(S) + lysate(S) (■).


15
10
5
0
Reduction Rate Constant (10-3/min)
1
2
3
SET

This suggests that intracellular components play a role in the reducing ability of sickle RBC, and that the sickle membrane had no significant contribution to the high spin label reduction.

3.3.3 Effects of Ascorbate Oxidase in Sickle Lysate

AO was also used to study ascorbate involvement in spin label reduction of sickle RBC. Figure 22 shows the effects of AO (2 units/ml) on spin label reduction in two sickle lysate systems. (1) Sickle lysate was mixed with AO before adding sickle membrane. The reduction rate constant was 1.9 x 10^{-3}/min without AO (Figure 22c), and 0.1 x 10^{-3}/min with AO (Figure 22a). (2) Intact sickle RBC was first spin labeled, then frozen and thawed. The rate constant was 5.9 x 10^{-3}/min without AO (Figure 22d) and 0.5 x 10^{-3}/min with AO (Figure 22b). In both cases, at least 90 % of the spin label reductions were inhibited by AO.

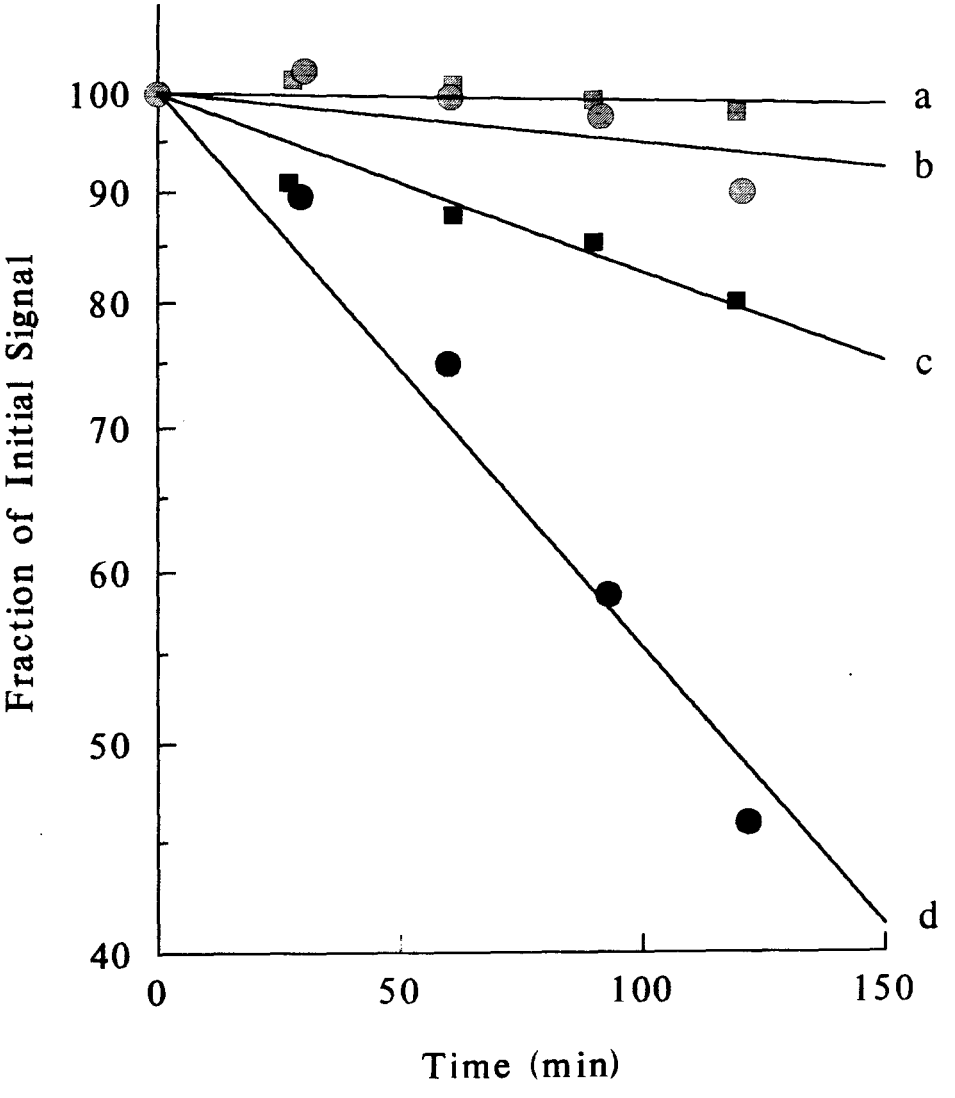
3.4 Spin Label Reduction in Sickle RBC and Correlation with Antioxidant Activities

3.4.1 Antioxidant Levels

The levels of vitamin E and ascorbate and the activities of GPx, SOD, and CAT varied from sample to sample in normal and sickle blood. Thus, the assays of these antioxidants were performed with the same samples as used for spin label experiments. The vitamin E, GPx, SOD and CAT assays were done on the blood samples of one set of sickle patients (n = 19), while the ascorbate measurements were done on another set of sickle patients (n = 3).

The values of vitamin E varied from 5.91 to 37.63 for sickle samples, with an average value of 16.95 ± 8.63 μg/gHb (n = 19), whereas the values from 3.19 to 17.69 were obtained for normal samples with an average value of 10.10 ± 4.56 μg/gHb (n = 18). The experimental uncertainties in vitamin E measurements exhibited an average standard deviation of 2.04 μg/gHb (n = 55). The difference between the average values of normal and sickle samples was statistically significant with p = 0.005.

Figure 22 **The effect of AO on spin label reduction in sickle lysate.** A semi-logarithmic plot of the fraction of initial signal versus time for two sickle lysate systems. (1) Sickle lysate was added to spin-labeled sickle membrane (■); (2) Intact sickle RBC was first spin-labeled then frozen and thawed (●). The closed symbols are from the samples without AO, and shaded symbols are from the samples with AO. For system (1), the reduction rate constant for the sample without AO was 1.9 x 10^{-3}/min, with a correlation coefficient of 0.988 (line c), and with AO was 0.1 x 10^{-3}/min, with a correlation coefficient of 0.476 (line a). For system (2), the rate constant was 5.9 x 10^{-3}/min without AO, with a correlation coefficient of 0.994 (line d), and 0.5 x 10^{-3}/min with AO, with a correlation coefficient of 0.788 (line b).


Fraction of Initial Signal
100
90
80
70
60
50
40
0
50
100
150
Time (min)
a
b
c
d

The values of ascorbate varied from 13.55 to 28.39 for sickle samples, with an average value of 19.24 ± 8.00 μg/gHb (n = 3). The values varied from 2.77 to 11.68 for normal samples, with an average value of 7.65 ± 4.52 μg/gHb (n = 3). The experimental uncertainties in ascorbate measurements exhibited an average standard deviation of 0.88 μg/gHb (n = 6). The difference between the average values of normal and sickle samples was not statistically significant with p = 0.094, probably due to the small sampling size.

The values of GPx level varied from 30.26 to 66.27 for sickle samples, with an average value of 41.52 ± 8.76 μmole NADPH/min·gHb (n = 19). The values varied from 16.23 to 48.23 for normal samples, with an average value of 28.47 ± 7.35 μmole NADPH/min·gHb (n = 19). The experimental uncertainties in GPx measurements exhibited an average standard deviation of 0.94 μmole NADPH/min·gHb (n = 23). The difference between the average values of normal and sickle samples was statistically significant with $p << 0.001$.

The values of SOD level varied from 948 to 2,271 for sickle samples, with an average value of 1,442 ± 404 units/gHb (n = 19). The values varied from 931 to 2,787 for normal samples, with an average value of 1,638 ± 535 units/gHb (n = 19). The experimental uncertainties in SOD measurements exhibited an average standard deviation of 140 unit/gHb (n = 23). The difference between the average values of normal and sickle samples was not significant.

The values of CAT level varied from 93,385 to 167,104 for sickle samples, with an average value of 114,838 ± 17,752 μmole H_2O_2/min·gHb (n = 19). The values varied from 88,808 to 157,808 for normal samples, with an average value of 119,663 ± 19,324 μmole H_2O_2/min·gHb (n = 19). The experimental uncertainties in CAT measurements exhibited an average standard deviation of 2,313 μmole H_2O_2/min·gHb (n = 27). The difference between the average values of normal and sickle samples was not significant.

A summary of average values is shown in Table 8a.

Table 8a Average and Standard Deviation Values of the Spin Label Reduction Rate and Activities of Antioxidants of Normal and Sickle Cells.

		Normal	Sickle	Sickle I	Sickle II
Set I					
Rate Constant (10^{-3}/min)	Avg	3.08	8.64	4.01	12.02
	SD	± 1.11	± 6.32	± 0.60	± 6.49
	n	19	19	8	11
Vit E (μg/gHb)	Avg	10.10	16.95	18.31	15.97
	SD	± 4.56	± 8.63	± 6.82	± 9.96
	n	18	19	8	11
GPx (μmole NADPH/min·gHb)	Avg	28.47	41.52	44.03	39.70
	SD	± 7.35	± 8.76	± 11.49	± 6.08
	n	19	19	8	11
SOD (units/gHb)	Avg	1639	1442	1654	1288
	SD	± 535	± 404	± 512	± 219
	n	19	19	8	11
CAT (μmole H_2O_2/min·gHb)	Avg	119663	114838	121197	110214
	SD	± 19324	± 17752	± 22578	± 12432
	n	19	19	8	11
Set II					
Rate Constant (10^{-3}/min)	Avg	3.08			6.93
	SD	± 0.74			± 1.56
	n	3			3
Ascorbate (μg/gHb)	Avg	7.65			19.24
	SD	± 4.52			± 8.00
	n	3			3

Table 8b Results* of Student t-test of Normal and Sickle Data

	Normal vs Sickle		Normal vs Sickle I		Normal vs Sickle II	
Set I						
	t	(p)	t	(p)	t	(p)
Rate Constant	-3.781	(0.001)	-2.235	(0.035)	-5.935	(<0.001)
Vit E	-2.992	(0.005)	-3.630	(0.001)	-2.172	(0.039)
GPx	-4.977	(<0.001)	-4.239	(<0.001)	-4.282	(<0.001)
SOD	1.273	(0.211)	-0.071	(0.941)	2.062	(0.049)
CAT	0.801	(0.428)	-0.179	(0.859)	1.452	(0.158)
Set II						
Rate Constant					-3.854	(0.018)
Ascorbate					-2.183	(0.094)

*The number presented is the calculated t value and the corresponding probability in parentheses.

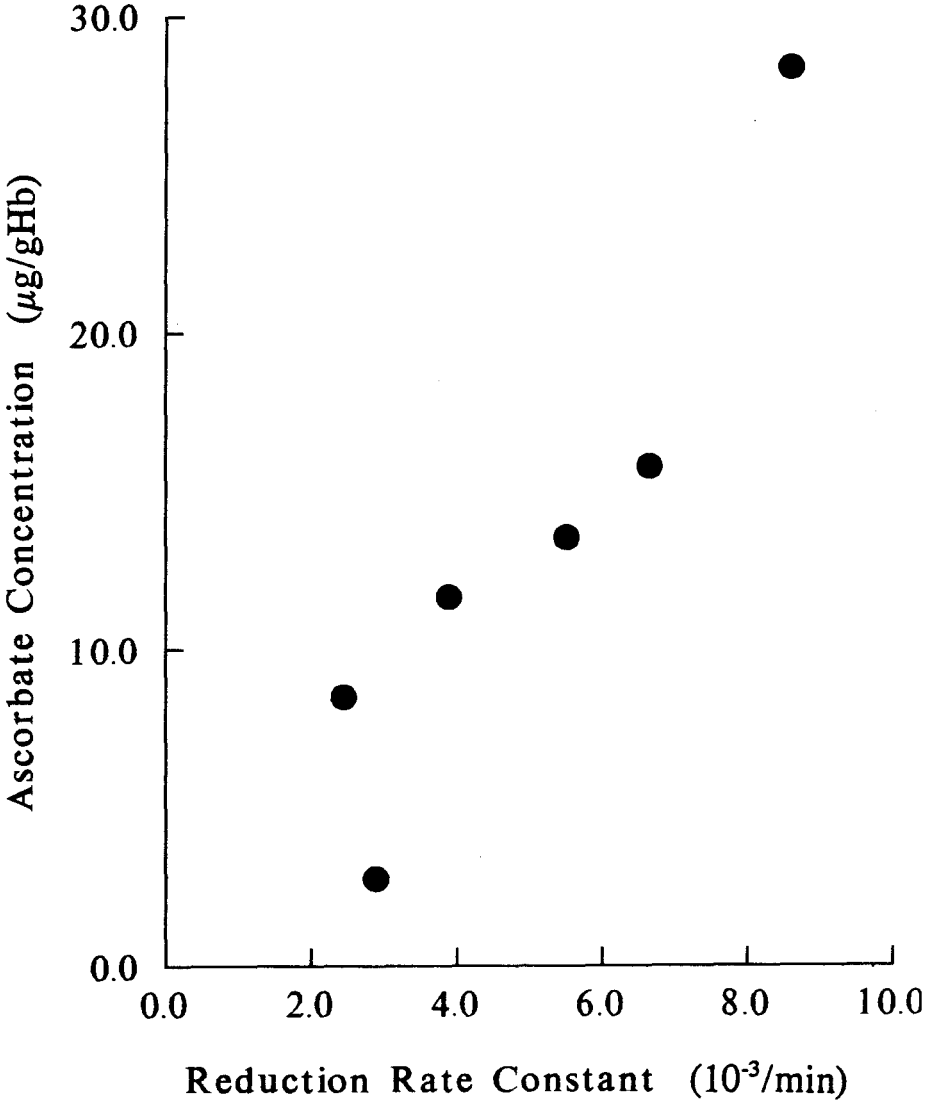
3.4.2 Correlation of Spin Label Reduction with Antioxidant Concentrations or Activities

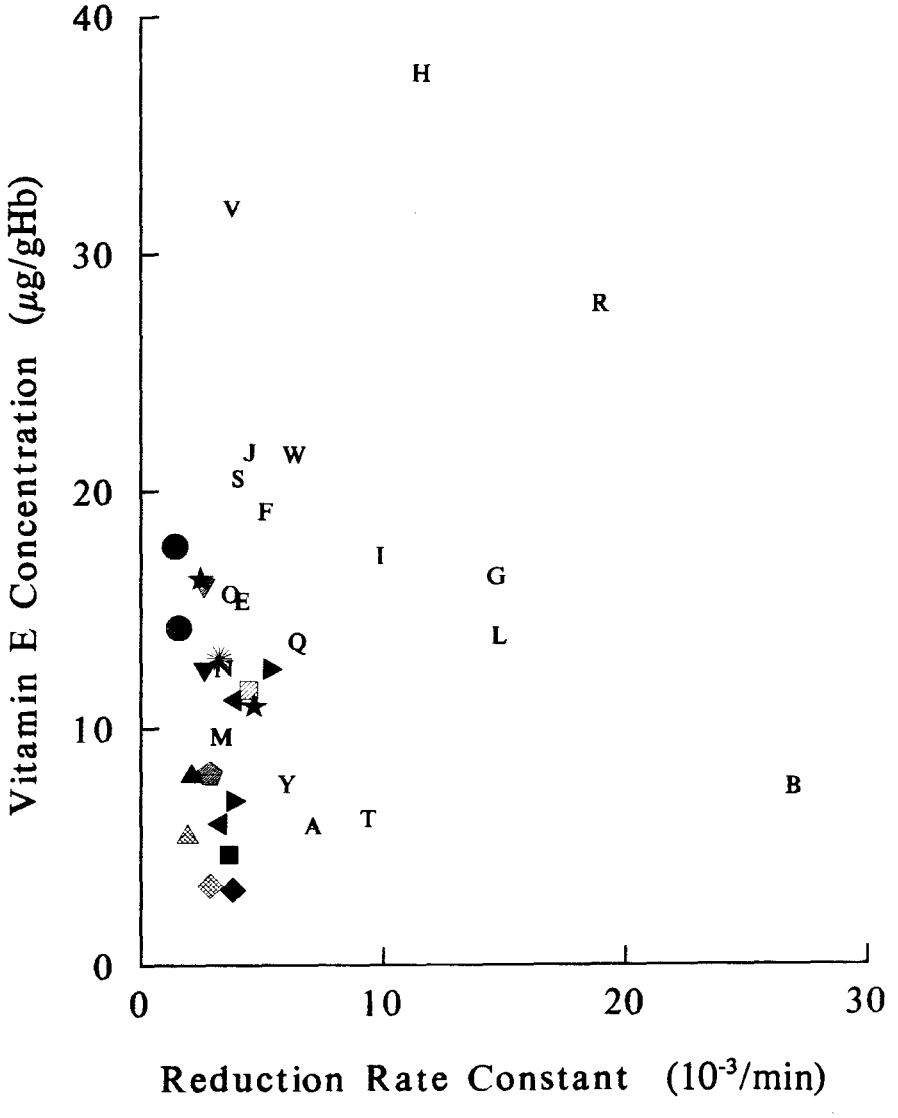
Also shown in Table 8a are the corresponding spin label reduction rate constants of the samples. The average rate constant of the first set (n = 19) of sickle patients is 8.6 ± 6.3 x 10^{-3}/min, whereas the rate constant for normal is 3.1 ± 1.1 x 10^{-3}/min. The average rate constants for the second set (n = 3) are 6.9 ± 1.6 x 10^{-3}/min for sickle and 3.1 ± 0.7 x 10^{-3}/min for normal. The rate constants of these blood samples are in good agreement with the rate constants of a larger population which were presented earlier.

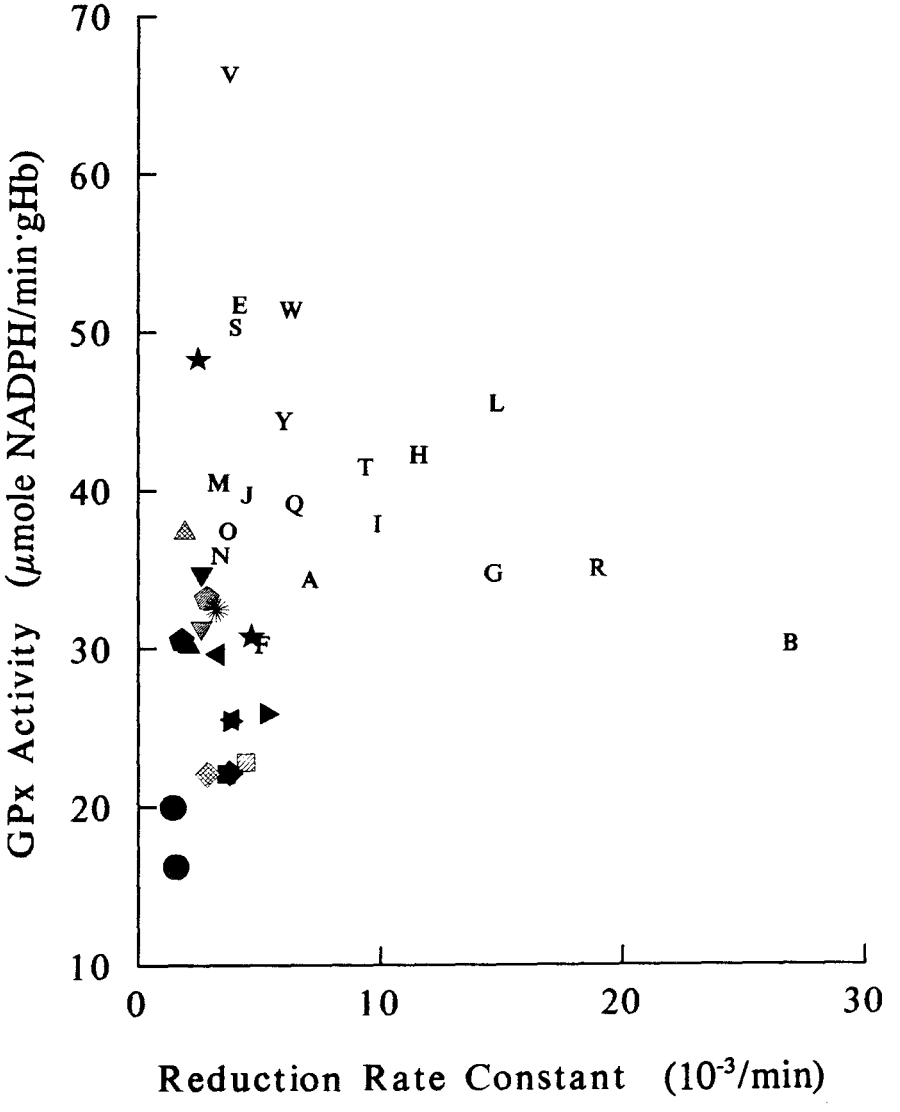
The relationships between spin label reduction rate constant and the level of each antioxidant of an individual blood samples are shown in Figure 23 for both normal and sickle RBC. A strong positive correlation between ascorbate concentrations and spin label reduction rates was obtained with r = 0.929 and p = 0.007 (Figure 23A). For normal cells, the level of vitamin E (Figure 23B), GPx (Figure 23C), SOD (Figure 23D) and CAT (Figure 23E) did not appear to correlate with the spin label reduction rates. A Pearson correlation matrix (Table 9a) showed little correlation between rate constants and the levels of vitamin E, GPx, SOD and CAT in normal cells (p = 0.402, 0.573, 0.206 and 0.369, respectively). However, slight negative correlations were obtained for the sickle cells between spin label reduction rates and the level of GPx, SOD and CAT (Figures 23C, 23D, and 23E; Table 9b), although not very significant (p = 0.080, 0.072, and 0.082, respectively). No correlation was found between vitamin E concentrations and spin label reduction rates in sickle cells (Figure 23B; Table 9b).

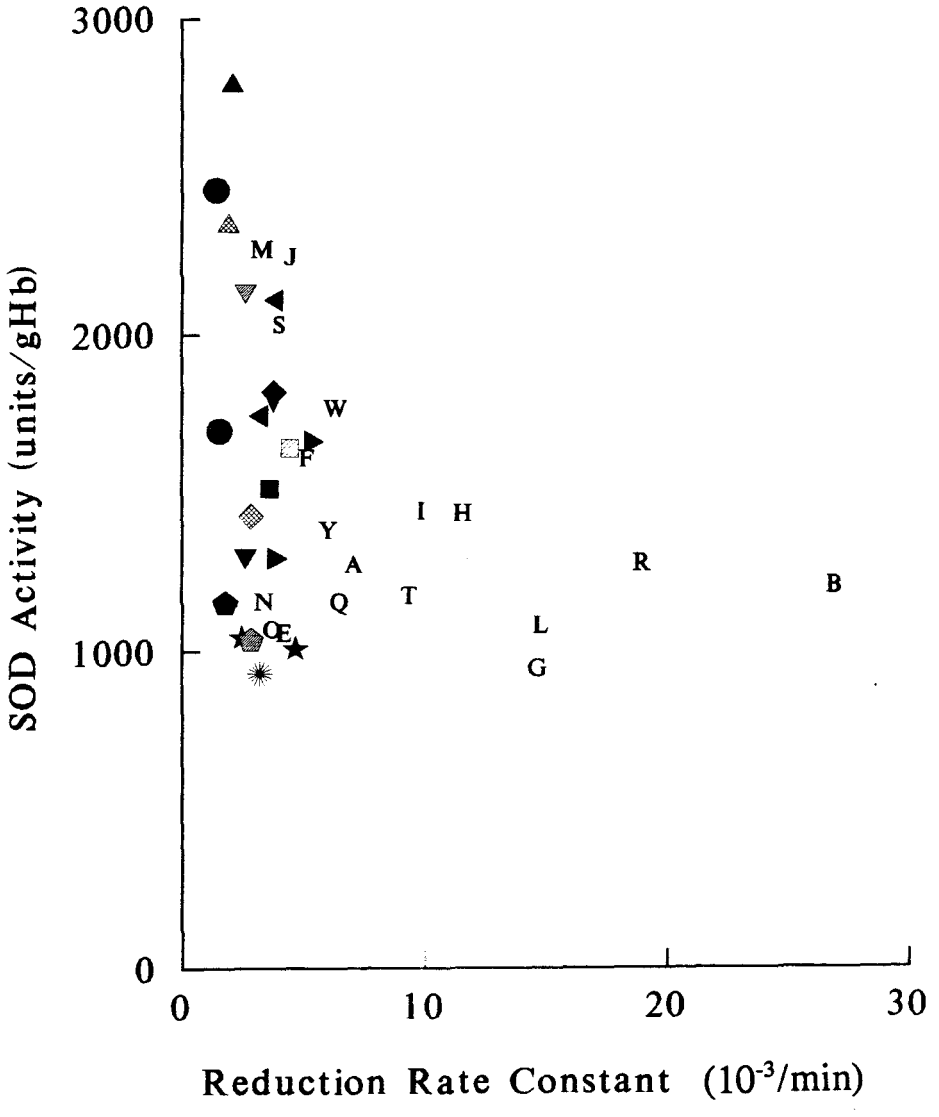
To further determine whether there was a correlation between spin label reduction rate and antioxidant levels in sickle cells, the sickle cells were divided into two groups according to their spin label reduction rate constants. Sickle I had reduction rate constants similar to those of normal cells (less than 6 x 10^{-3}/min) and Sickle II had reduction rate constants significantly higher than those of normal cells (larger than 6 x 10^{-3}/min).

Figure 23 **Relationships between spin label reduction rate constant and the level of each antioxidant of an individual blood sample for both normal and sickle red blood cells.** Ascorbate concentration versus reduction rate constant (A); Vitamin E concentration versus reduction rate constant (B); GPx activity versus reduction rate constant (C); SOD activity versus reduction rate constant (D); CAT activity versus reduction rate constant (E). The different symbols represent the results from different batches of normal blood, and different letter labels represent the results from different sickle patients.


30.0
20.0
10.0
0.0
Ascorbate Concentration (μg/gHb)
0.0
2.0
4.0
6.0
8.0
10.0
Reduction Rate Constant (10-3/min)


40
30
20
10
0
0
10
20
30
Vitamin E Concentration (μg/gHb)
Reduction Rate Constant (10-3/min)
H
V
R
J
W
S
F
I
G
L
Q
E
Q
M
Y
A
T
B


70
60
50
40
30
20
10
0
10
20
30
GPx Activity (μmole NADPH/min·gHb)
Reduction Rate Constant (10-3/min)
V
E
S
W
L
Y
T
H
M
J
Q
I
O
N
A
G
R
B
F


3000
2000
1000
0
SOD Activity (units/gHb)
0
10
20
30
Reduction Rate Constant (10-3/min)
M
J
S
W
F
I
H
Y
A
R
B
N
Q
T
L
G
E

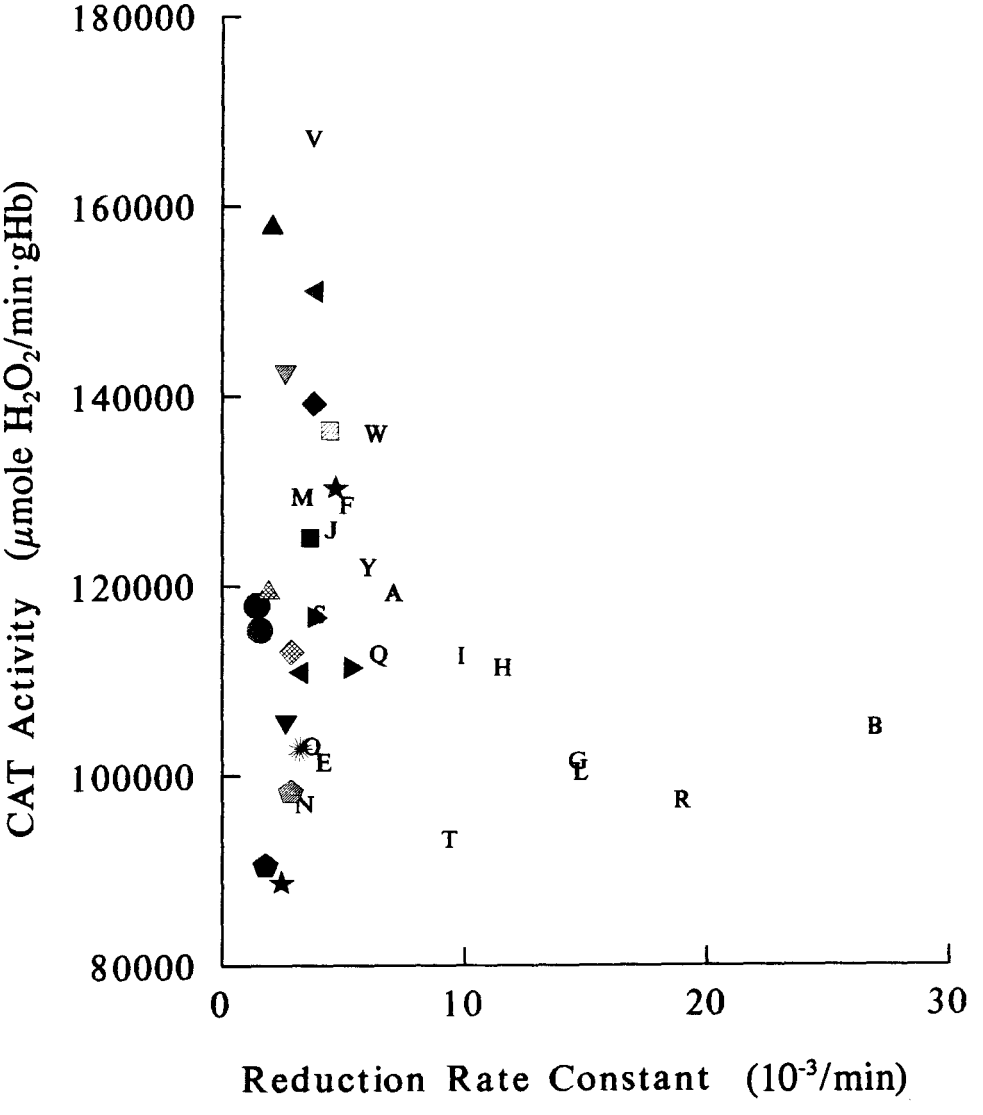

180000
160000
140000
120000
100000
80000
CAT Activity (μmole H_2O_2/min·gHb)
0
10
20
30
Reduction Rate Constant (10^{-3}/min)
V
W
M
F
J
Y
A
Q
I
H
B
G
L
R
E
N
T

Table 9a Pearson Correlation Matrix for Normal RBC

	RATE	CAT	GPx	SOD	VE
RATE	1.000				
CAT	0.218	1.000			
GPx	-0.138	-0.388	1.000		
SOD	-0.304	0.686	-0.256	1.000	
VE	-0.211	-0.160	0.184	-0.001	1.000

Table 9b Pearson Correlation Matrix for Sickle RBC

	RATE	CAT	GPx	SOD	VE
RATE	1.000				
CAT	-0.422	1.000			
GPx	-0.412	0.572	1.000		
SOD	-0.409	0.665	0.301	1.000	
VE	-0.032	0.355	0.394	0.270	1.000

GPx activities in Sickle I and Sickle II (44.03 ± 11.49 and 39.70 ± 6.08 μmole NADPH/min·gHb, respectively) remained significantly higher than that in normal ($p << 0.001$). Similarly, vitamin E concentrations in Sickle I (18.31 ± 6.82 μg/gHb) remained significantly higher than that in normal ($p = 0.001$), and vitamin E in Sickle II (15.97 ± 9.96 μg/gHb) was also higher than normal with $p = 0.039$. However, SOD activities in Sickle II (1,288 ± 219 units/ml) appeared to be significantly lower than those of normal cells (1,639 ± 535 units/ml) with $p = 0.049$.

Better negative correlations were found for the GPx, SOD and CAT levels with the rate constants in Sickle II group ($p = 0.049$, 0.250 and 0.070) than in Sickle I group ($p = 0.552$, 0.867 and 0.805). The vitamin E levels showed no correlation with rate constants in either Sickle I or Sickle II group.

CHAPTER IV

DISCUSSION

4.1 Signal Reduction of Membrane Spin Label in Intact RBC

In general, fatty acid spin labels are considered to be more stable in red blood cells than in any other living cells which contain mitochondria or nucleus. In an earlier study on parasitized erythrocytes using normal mice RBC as control, it was found that 5-DSA was reduced by the electron transport chain in the parasite, but no reduction was observed in the control, non-parasitized cells, over a time period of 30 min (Deslauriers *et al.*, 1987). In this study, the same spin label at a concentration of about 1.4×10^{-4} M was incorporated into the membrane of fully CO-gassed human RBC, and its EPR signal was monitored at 37 °C over a period of at least 2 hr. It was found that the EPR signal was reduced significantly by intracellular components at the end of 2 hr. This observation led us to develop an EPR method to evaluate the efficiency of radical reduction in membranes (Fung & Zhang, 1990). Subsequent studies by others confirmed our finding of fatty acid spin label reduction in RBC (Minetti & Scorza, 1991). These authors also found that when a cluster of spin label occurred, there was a biphasic reduction profile: a faster reaction phase corresponding to the clustered spin label reduction with a half-life of 60 min, and a slow reaction phase corresponding to the reduction of the spin labels dispersed in membrane with a half-life of 170 min. The slow half-life time agreed well with our average rate constant of 3.8×10^{-3}/min, corresponding to a half-life time of about 180 min ($t_{1/2} = \ln 2/k$).

As stated in our published paper (Appendix I), since the measurements reflected the extent of radical reductions occurring in cell membranes, the method could be used to

measure the ability of intracellular antioxidant system to defend against oxidants in membranes of RBC. A pseudo first-order radical reduction rate constant can be used as an index for the effectiveness of intracellular antioxidant system toward the radicals in RBC membranes. The experimental uncertainty for measuring the reduction rate constants is about 10 % (Table 1). However, the spin label reduction rate constants obtained from normal RBC under similar conditions varied from batch to batch, with a CV of about 45 %. Our published work also indicated that the radical reduction depended upon the position of nitroxide radicals on the hydrocarbon chain and the amount of oxygen in the system (Fung & Zhang, 1990).

The RBC-CO used for this study had practical advantages, although CO condition is not a physiological condition. RBC-CO are stable under common experimental conditions for at least several days since Hb has a very high affinity for CO; thereby, the complication of having various species of Hb (e.g. oxyHb, deoxyHb, metHb, *etc.*) was eliminated. Furthermore, the effect of re-oxidation of reduced spin labels by oxygen is also avoided.

It was noticed that the reduction rate constant of RBC sample with HbCO and cell-O_2, which was obtained from CO-gassed RBC washed with un-gassed or O_2-gassed buffer once after spin labeling (sample 1), was higher than that of the fully O_2-gassed RBC sample (sample 2) (Figure 20). A possible reason is that the CO in the cells of sample 1 is not completely replaced by O_2 after a single washing. Thus, the amount of O_2 in sample 1 to re-oxidize spin labels was less than that in sample 2. The results may also imply that the HbCO and HbO_2 have different contributions toward spin label reduction.

4.2 Position of Spin Label in Membrane

Fatty acid nitroxides, especially doxyl stearic acids, are highly lipophilic and readily partition into the bilayer of cell membranes. It is generally accepted that spin labels are randomly distributed in the two leaflets of the membrane bilayer, although the precise distribution of fatty acid spin labels in the two leaflets is not clear. Our results showed that

spin label translocation movement is relatively faster than the spin reduction, providing additional evidence that the fatty acid spin labels distribute between two leaflets within the experimental time period.

The study of spin label location in egg phosphatidylcholine vesicles using the nuclear spin-lattice relaxation method revealed that the nitroxide groups on the stearic acids are located nearer to the membrane exterior than the analogous positions of the phospholipid acyl chains, and the nitroxide group of 5-DSA positions itself close to the head group of the membrane bilayer (Ellena *et al.*, 1988). The electron-electron double resonance study of interaction between [^{14}N]-16-DSA and [^{15}N]-5-DSA pairs provided information on vertical fluctuation of the 16-DSA nitroxide group toward the membrane surface. The cause of vertical fluctuation is attributed to excursions of the terminal methyl region of the 16-DSA toward the membrane surface. 5-DSA appears rotationally immobilized with less influences by the length and unsaturation of lipid alkyl chains (Feix *et al.*, 1984; 1987).

4.3 Kinetics Expression of Spin Label Reduction

It is known that the nitroxide (>N-O·) is generally reduced to the corresponding hydroxylamine (>N-OH). However, the reaction is reversible, and the product can be oxidized back to the nitroxides under aerobic condition (Gaffney, 1976; Marsh, 1981; Chen & Swartz, 1989). The global reaction may be represented by Eq. 4.3.1.

$$>N\text{-}O^{\cdot} + R \rightleftharpoons >N\text{-}OH + O \qquad (4.3.1)$$

where R is a reducing agent which can reduce nitroxide to hydroxylamine, and O is the oxidized form of R, or other oxidant which can re-oxidize hydroxylamine. Since the CO treatment of systems was used in this study to limit the oxidation of hydroxylamine by O_2, the reverse reaction of nitroxide reduction by O_2 could be ignored. Therefore, the reaction rate for (4.3.1) can be expressed as

$$d[NO]/dt = k_r[NO][R] \qquad (4.3.2)$$

where k_r is nitroxide reduction (forward reaction of Eq. 4.3.1) rate constant. When [R] is not changing significantly during the course of measurement, a pseudo first-order condition is satisfied. Eq. 4.3.2 can then be written as

$$d[NO]/[NO] = kdt$$

or,

$$\ln \{[NO]_t/[NO]_0\} = -kt \qquad (4.3.3)$$

Since the nitroxide spin label concentrations at different times were directly proportional to the central peak heights (h) of EPR signal, Eq. 4.3.3 is equivalent to

$$\ln (h_t/h_0) = -kt$$

where h_t is h at time t and h_0 is the initial h. Therefore, a pseudo first-order rate constant for spin label reduction can be obtained from the slope of a linear fit of $\ln (h_t/h_0)$ vs time plot. Since the pseudo first-order rate constant contains the term of concentration of reducing agents, the rate constants obtained by this method reflect the reducing potency of the corresponding components in RBC. This deduction provides the mechanistic background for the apparent second-order rate constant when a specific component is isolated out to examine its concentration dependence of a pseudo first-order rate constant.

4.4 Identification of RBC Intracellular Components that Reduce Membrane Spin Labels

4.4.1 Components with no Effect on Spin Labels in Membrane

In order to study RBC component(s) responsible for the spin label reduction in cell membrane under CO condition, individual components or combinations of various components were added to spin labeled membrane in CO-gassed buffer. As summarized in Table 5, addition of excess (higher than the physiological levels) vitamin E, GSH, CAT, GPx, SOD, NADH, NADPH, or HbCO individually to Mb* did not show any detectable reduction over a period of 2 hr. The combination of some of these components with the Mb* also did not show spin label signal reduction (Table 4).

The concentrations of each component used for this study were considered from two aspects: (1) experimental convenience and (2) appropriate concentration range to cover their corresponding physiological values. The physiological values were chosen from the literature reports for normal RBC. Usually, the concentrations of components added to the samples were at least twice as high as their physiological values, some of them were even up to several orders of magnitude higher.

Vitamin E is a membrane-bound molecule and actually exists in the isolated white membrane ghost, at a concentration of 0.61 μg/mg protein, as determined by vitamin E assay. The Mb* was stable for at least 24 hr. Thus it is not surprising that the added vitamin E did not reduce spin label in membrane.

The antioxidant enzymes, SOD, CAT, and GPx, do not show reducing ability for nitroxide spin label in membrane.

GSH and sulfhydryl groups are considered to be involved in some water-soluble nitroxides reductions in rat tissue homogenates (Couet *et al.*, 1985), in human erythrocytes (Branca *et al.*, 1988), and in human Hb solution (Perussi *et al.*, 1988). However, the results from both GSH depleted RBC and direct addition of GSH to Mb* showed that GSH is not a major reducing agent responsible for 5-DSA spin label reduction (Figures 9 and 10). It should be noted that at high concentration of GSH the pH value changed from neutral to acidic in buffer with low phosphate concentration. To avoid acidic condition, either buffer with high phosphate concentration or GSH solution neutralized by NaOH was used. However, both cases had limitations. High salt concentration and osmolality of the system may significantly alter the experimental condition. Therefore, it is unrealistic to raise the GSH concentration above 40 mM, beyond the buffering capacity of high phosphate concentration buffer (0.155 M NaH_2PO_4 and 0.103 M Na_2HPO_4).

NADH and NADPH are common hydrogen donors in many biological reactions. However, they did not provide hydrogens to nitroxides directly to reduce spin label in

membrane.

Recently, it was reported that Hb in the ferrous form may be responsible for the nitroxide reduction in human RBC based on the experiments of fatty acid spin labels directly adding to Hb under hypoxia condition (Minetti & Scorza, 1991). In this study, the spin label in a mimic system is similar to that in intact RBC. Our results show that HbCO does not appear to be involved in the reduction of spin label in membrane. However, it is not clear whether HbO_2 is involved in the reduction.

The combinations of GSH, SOD, CAT, GPx, NADPH, and HbCO with Mb* did not appear to be responsible for the spin label reduction in membrane (Table 4). It does not rule out the involvements of these antioxidants in spin label reduction when combined with other intracellular components.

4.4.2 Components with no Effect on Spin Labels in Membrane with Lysate

Since antioxidants in RBC coexist in the cellular compartment, any single component which did not reduce spin label in isolated condition may be capable of reducing spin label when mixed with other cellular components. Therefore experiments were designed to test the effects on the reduction rate by systematically adding a single component to lysate. We found that the addition of vitamin E, GSH, SOD, CAT, or GPx to Mb* and lysate to spin labeled membranes did not enhance the reduction rate constant of membrane with lysate alone (Table 6). The fact that SOD, CAT, or GPx does not enhance the reducing ability even under the condition when all the intracellular components are available may suggest that those enzymes are not responsible for the spin label reduction.

4.4.3 Components with Effects on Spin Labels in Membrane

Ascorbate at concentrations higher than 0.1 mM, the upper limit of physiological ascorbate concentration for normal RBC (Mehlhorn, 1991), produces detectable reduction of

spin labels in membrane (Table 5). The pseudo first-order rate constant of spin label reduction is ascorbate concentration dependent (Figure 15), with an apparent second-order rate constant of 3.1/min·M. When 1.0 mM concentration of ascorbate was added to the Mb*, the first-order rate constant was about 3 x 10^{-3}/min. However, the reduction became undetectable when 2.0 units/ml of AO was added to the system containing 1 mM ascorbate. This suggests that (1) the spin label reduction caused by ascorbate can be inhibited by AO; (2) when ascorbate is oxidized under this condition, it can no longer reduce the spin label in membrane, which, however, seems to be inconsistent with the DHA finding, discussed below.

Interestingly, in the presence of 4.0 mM GSH the spin label reduction by ascorbate was enhanced, although GSH itself had been demonstrated that it was not capable of reducing the spin label directly. The enhancement was also ascorbate concentration dependent as the apparent second-order rate constant increased from 3.1/min·M to 4.7/min·M in the presence of 4.0 mM GSH. Furthermore, in the presence of excess GSH, the spin label reduction by ascorbate could no longer be suppressed by addition of AO. The results from a UV experiment revealed that ascorbate still underwent the oxidation catalyzed by AO in the presence of GSH. Therefore, this suggests that the complex of the oxidized form of ascorbate, probably DHA, with GSH has a high reducing ability and can reduce nitroxide spin labels in membrane by a process which cannot be inhibited by AO. A similar postulate was proposed based on a different approach (Mehlhorn, 1991).

DHA is considered as the oxidized form of ascorbate. In general, the amount of DHA which exists in the biological systems is only about 2 - 5 % of total ascorbate (Okamura, 1980; Tangney *et al.*, 1989). DHA is susceptible to rapid decomposition, and can be reduced back to ascorbate through DHA reductase (Rose, 1990), or by thiols (Mehlhorn, 1991). In addition, DHA can spontaneously degrade by undergoing a biologically irreversible opening of the lactone ring to form diketogulonic acid, which lacks antiascorbic or other metabolic activities (Rose, 1990).

It was observed that the kinetics of the spin label reduction by DHA differs from that by ascorbate (Figure 19). The reduction profile has an initial lag followed by a fast reaction phase. When first-order kinetics analysis was applied, the rate constants obtained were in the same order as those corresponding to ascorbate. However, the concentration dependent rate constant obtained for DHA (4.7/min·M) was higher than that for ascorbate (3.1/min·M), but almost the same as that for ascorbate in the presence of GSH (4.5/min·M). Thus, the aforementioned involvement of GSH in spin label reduction by ascorbate may be due to GSH-DHA complex, which cannot be inhibited by AO.

It is likely that DHA is even more efficient in reducing spin label in membrane than the reduction rate constants indicated, since the initial lag of the reduction profile of DHA makes the obtained rate constant lower than it should be. In addition, the reduction rate caused by DHA can be enhanced by addition of GSH. In the presence of 4.0 mM GSH, the DHA concentration dependent rate constant obtained was 5.1/min·M, a moderate increase from 4.5/min·M in the absence of GSH. Therefore, some of the roles of GSH in spin label reduction are established by these experiments, although the precise mechanisms are still unknown.

4.4.4 Components with Effects on Spin Labels in Membrane with Lysate

4.4.4.1 Involvement of NADH and NADPH

Both NADH and NADPH did not produce any detectable reduction of spin label in the absence of lysate. However, the addition of 720 μM of NADH or NADPH to the system with membrane and lysate increased the rate constant significantly (Table 6). The rate constants obtained from the systems with various concentrations of NADH or NADPH showed linear concentration dependence, with an apparent second-order rate constant of 0.6/min·M or 2.2/min·M, respectively. However, at the physiological concentration of NADH and NADPH in lysate (about 20 μM), the NADH and NADPH contributions to the rate constant

are small, of only 1.2 x 10^{-5}/min and 4.4 x 10^{-5}/min respectively. The average rate constant for spin label reduction, however, is in the order of 10^{-3}/min. Thus, NADH and NADPH are probably cofactors for regenerating ascorbate in the lysate, as will be discussed later.

4.4.4.2 Ascorbic Acid is the Major Reductant in RBC Lysate

The role of ascorbate in biological processes has often been evaluated with AO. It should be pointed out that AO is not capable of penetrating the membrane and accessing the sealed membrane compartment. Thus, the enzyme inhibitory effects cannot be used to elucidate the role of ascorbate as a free radical scavenger in tissue homogenates or cell lysate that contain resealed membrane (Mehlhorn, 1991). In this study, AO was added to lysate or other systems with ascorbate prior to the addition of Mb*, to avoid the potential complication of ascorbate that might be entrapped in AO-inaccessible resealed membrane. It was found that at least 90 % inhibition of spin label reduction could be achieved by introducing AO into cell lysate (Figure 16). Therefore, ascorbate is a major reductant for 5-DSA reduction in normal RBC.

In the system with lysate, ascorbate yielded an apparent second-order rate constant of 7.6/min·M, significantly higher than that of 3.1/min·M in the absence of lysate and GSH. It is also higher than that of 4.7/min·M in the presence of 4.0 mM GSH but absence of lysate. It shows that components other than GSH in the lysate are enhance the ascorbate reducing ability.

4.4.4.3 Regeneration of Ascorbate in RBC Lysate

In RBC, ascorbate accumulated when the cells were incubated with DHA, and the DHA reduction to ascorbate in RBC, which was shown to be mediated by enzymatic reactions, was greater than that by equivalent concentrations of GSH in buffer (Christine *et al.*, 1956). It has been suggested that exogenous ascorbate may be internalized by first oxidizing

ascorbate to DHA on or near the surface of RBC, then migrating DHA through the lipid portion of the cell membrane, and finally reducing DHA back to ascorbate within the cell (Wagner *et al.* 1987). Furthermore, the entrapment of ascorbate seemed to be enzymatically mediated, but it is not clear whether the rate of entrapment is controlled by enzymes (Wagner *et al.* 1987).

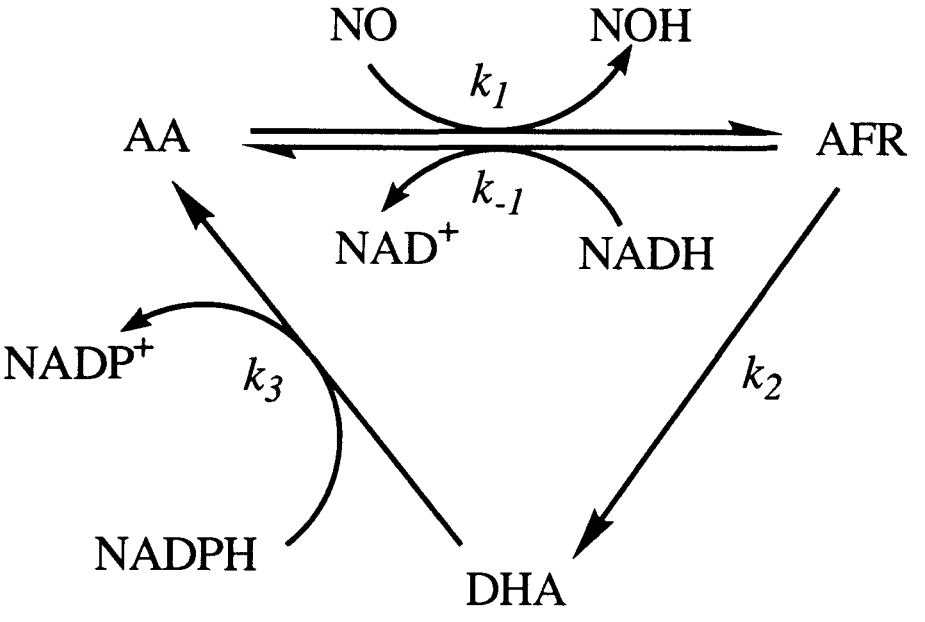
Ascorbate reacts with free radicals resulting in a relatively stable intermediate, ascorbate free radical (AFR), which is a biologically non-hazardous free radical, and can serve both as a one-electron oxidant and as a one-electron reductant (Alcain *et al.*, 1991). AFR can be further oxidized in a slow autoxidation to DHA which is susceptible to rapid decomposition. AFR can also be reduced, enzymatically by AFR reductase, to regenerate ascorbate. DHA can be reduced back to ascorbate through DHA reductase (Rose, 1990), or by thiols (Mehlhorn, 1991).

Choi and Rose (1989) found that in the homogenates of rat colon, NADPH was the most effective cofactor in reducing DHA to form ascorbate as compared with other reducing equivalents such as GSH and cysteine. Nevertheless, the regeneration of ascorbate from AFR is carried out by the NADH-dependent AFR reductase as a part of the transmembrane redox system, which has been found in some biological cells and tissues (Alcain *et al.*, 1991). To date, the efficiency of ascorbate regeneration in human RBC has not yet been resolved (Mehlhorn, 1991). A summary of ascorbate regeneration scheme is shown in Figure 24.

In this study, the finding that the pseudo first-order rate constant of spin label reduction is NADH and NADPH dependent may suggest a regeneration of ascorbate in the RBC lysate. Assuming that the mechanism elucidated in Figure 24 is suitable for ascorbate regeneration in RBC, the effects of adding NADH or NADPH to lysate on the spin label reduction rate constant may be related to the regeneration of oxidized ascorbate. As shown in Figure 24, the reaction rate of the disappearance of ascorbate (AA) can be expressed as

$$-d[AA]/dt = k_1[AA][NO] - k_{-1}[AFR][NADH] - k_3[DHA][NADPH] \qquad (4.4.4.3.1)$$

Figure 24 **Scheme of ascorbate regeneration.** AA, ascorbate; AFR, ascorbate free radical; DHA, dehydroascorbate; NO, nitroxide radical; NOH, hydroxylamine; NAD(H), oxidized (reduced) forms of nicotinamide adenine dinucleotide; NADP(H), oxidized (reduced) forms of nicotinamide adenine dinucleotide phosphate. The reduction of NO by AA yields AFR. Then, AFR is either enzymatically reduced back to AA or auto-oxidized to DHA, while DHA can be also enzymatically reduced to regenerate AA.


NO
NOH
k_1
AA
AFR
k_{-1}
NAD^+
NADH
$NADP^+$
k_3
k_2
NADPH
DHA

Then, when a steady state is achieved, we have

$$-d[AA]/dt = 0, \text{ and}$$

$$k_1[AA][NO] = k_{-1}[AFR][NADH] + k_3[DHA][NADPH] \quad (4.4.4.3.2)$$

For a pseudo first-order reaction rate constant, we have

$$k = k_1[AA] \quad (4.4.4.3.3)$$

Combining (4.4.4.3.2) and (4.4.4.3.3),

$$k = k_{-1}[AFR][NADH]/[NO] + k_3[DHA][NADPH]/[NO] \quad (4.4.4.3.4)$$

A plot of k vs added [NADH] would be linear and yield a slope as (k_{-1}[AFR]/[NO]). Similarly, a plot of k vs added [NADPH] would also be linear and yield a slope as (k_3[DHA]/[NO]). The results from this study showed that the spin label rate constants increased linearly as the concentrations of NADH or NADPH was increased. These are in a good agreement with the scheme in Figure 24. Moreover, the slope from the plot of rate constant vs NADPH concentration is relatively steeper than that from the plot with NADH (Figure 14), probably indicating that more ascorbate is regenerated through the DHA reduction pathway than the AFR reduction pathway.

Experimentally, it has been shown that the NADPH treated lysate had a higher concentration of ascorbate than its control sample (Figure 18). In addition, the fact that in the presence of 720 μM NADPH the spin label reduction of membrane and lysate was only inhibited partially (~50 %) by AO (Table 6) may also support the ascorbate regeneration mechanism. Since AO oxidizes ascorbate to DHA, which can be reduced back to ascorbate by NADPH, the introduction of a large amount of NADPH will establish an equilibrium in which a considerable level of ascorbate could be retained in samples treated with AO.

4.5 Speculations on 5-DSA Spin Label Reduction by Ascorbate in RBC

As discussed in the previous sections, it is conceivable that the spin label molecules are dispersed in the bilayer of membrane. It was also concluded that ascorbate is a major

reducing agent. However, to accept such a conclusion, a critical question must be resolved: How can the lipophilic spin label, with its nitroxide group buried in the membrane, be reduced by water-soluble ascorbate in the cytoplasm? One way or another, the spin label and the reducing agent (ascorbate) should encounter each other. Some possibilities are presented to account for this:

1) There is a fast equilibrium of spin label distribution between membrane and cytoplasm, and only those in cytoplasm are reduced by ascorbate.

2) All spin label molecules are dispersed in the bilayer of membrane, and no reduction occurs when they are buried in the lipid bilayer. However, a vertical motion of the 5-DSA may bring the nitroxide group of the spin label close to the membrane bilayer surface, and the reduction occurs when the nitroxide is close to the membrane surface.

3) Spin label is located in membrane, and ascorbate can penetrate into the bilayer to a certain depth depending upon the hydrophilicity of the environment. Therefore, the reduction rate is controlled by the effective concentration of ascorbate in the bilayer of the membrane.

For the first hypothesis, there should be a considerable amount of free spin labels in the cytoplasm in order to achieve a substantial reduction rate. Therefore it would have noticeable sharp signals, characteristic rapid isotropic motions, in the EPR spectra. However, no such signals were observed in this study (Fung & Zhang, 1990). Although it was reported that such peaks were observed (Minetti & Scorza, 1991), the peak size seems too small to account for the substantial reduction rate.

For the second hypothesis, it is possible to have a considerable concentration of ascorbate near the membrane surface. The lipid head groups in the bilayer consist of about 10 atoms, including a variety of polar groups that can function as either hydrogen-bond acceptors or donors to accommodate molecules with hydroxyl groups, such as ascorbate

molecules. The spin label reduction rate is limited by the vertical motion of spin labels in the membrane bilayer, since only the spin labels with their nitroxide groups close to the membrane surface can contact with ascorbate and be reduced. It has been reported that both 5-DSA and 16-DSA in model membrane can quench a fluorescent probe in which the fluorophore is at second position of the fatty acid acyl chain near the head group. In this case, the quenching efficiency for 5-DSA is higher than that for 16-DSA (Wardlaw *et al.*, 1987). This suggests that the vertical motions of fatty acid acyl chains occur in the bilayer, which in extreme cases may result in the terminal methyl group approaching to the bilayer surface by chain bending. One would expect that the deeper the nitroxide group on the fatty acid chain, the less chance to get close to the membrane surface. Consistent with this, our results showed that the reduction rate constant for 16-DSA in CO-gassed intact RBC was only about half of the value for 5-DSA (Fung & Zhang, 1990).

For the third hypothesis, it is proposed that the distribution of ascorbate in the membrane is a function of hydrophobicity, i.e. the higher hydrophobicity the environment, the lower concentration the ascorbate. This mechanism can also explain the observation that when the nitroxide group is located deeper in the bilayer, the reduction rate is slower. It has been suggested that the principal rate-limiting step for the reduction of nitroxide moiety deep in the membrane of tissue cells appeared to be the diffusion of reducing equivalents from the surface into the hydrocarbon region of the membrane (Chen *et al.*, 1988). However, the reducing equivalents responsible for the spin label reduction in these cells were from the mitochondrial respiratory chain. It will be interesting to find out whether ascorbate is actually able to diffuse into hydrophobic environment.

4.6 Efficiency of Radical Reduction in Normal and Sickle Cell Membranes

4.6.1 Significant Difference of Spin Label Reduction between Normal and Sickle RBC

The results showed that the average spin label reduction rate constant of sickle RBC from 31 patients ($8.8 \pm 6.4 \times 10^{-3}$/min) was significantly higher than that obtained from 64 batches of normal RBC ($3.8 \pm 1.7 \times 10^{-3}$/min). It appears that sickle RBC could reduce nitroxide radicals in cell membrane more efficiently than normal RBC. This has not been reported by others to date and is a surprising finding.

Although the reduction rate constants of both normal and sickle RBC varied from batch to batch and patient to patient, the sickle RBC from 31 patients had a much wider range of reduction rate constants than normal RBC. The spin label reduction rate constants of some sickle patients' RBC were similar to those of normal RBC, while others were significantly higher than those of normal RBC. Rate constants of blood samples from the same patient could also vary significantly from time to time (Table 7). It will be interesting to correlate the radical reduction rate with the severity of symptoms experienced by homozygous sickle cell disease patients. The sickle bloods used in this study were obtained from patients under different conditions such as during clinical visits, hospitalization, or special arrangement. The clinical symptoms of each patient at the time when blood samples were collected were not well documented. Some sickle patients were in pain crisis, and some were asymptomatic. The Hb electrophoresis allowed us to confirm that all of the blood samples subjected for this study were homozygous SS, and the patients had not undergone transfusion for a period of time. Based on the limited information on the clinical symptoms for a few patients, it appears that the higher reduction rate constants may be attributed to more severe sickle syndrome.

Smoking habits, medication, or the gender of the blood donor may also affect the radical reduction rate.

4.6.2 Spin Label Reduction in Sickle RBC Lysate

The results showed that the lysate prepared from sickle RBC which have high spin label reduction rate constants also gave higher reduction rates than lysate prepared from normal RBC, in spite of spin labels in normal or sickle membrane (Figure 21). It clearly demonstrated that the antioxidant components were located in the cytoplasm instead of membrane, and it is the same for both normal and sickle cells. It also suggests that sickle membranes do not play an important role in the spin label reduction.

Furthermore, the fact that AO can suppress at least 90 % of the reduction rate constant in sickle lysate (Figure 22) indicates that ascorbate is also a major reducing agent for sickle RBC.

4.7 Effectiveness of Antioxidant Activities on Spin Label Reduction

4.7.1 Levels of Antioxidants in Normal and Sickle RBC

In general, SOD, CAT, GPx and vitamin E are considered as principal antioxidants in human erythrocytes (Chiu *et al.*, 1982; Hebbel, 1986). Among them SOD and CAT are located within the cytoplasm, while Vitamin E is membrane located, and GPx is weakly membrane bound (Duchon & Collier, 1971; Ursini & Bindoli, 1987). The activities for each of the antioxidant enzymes (SOD, CAT, GPx) in normal RBC have been reported in a wide range by different investigators, in part due to different methods used for enzyme assays (Faraji *et al.*, 1987; Schacter *et al.*, 1988; Goldstein & Czapski, 1991). Enzyme activities of normal RBC measured in this study were well within the range of the values reported in the literature. The average concentration of vitamin E in normal RBC was also similar to the reported value which was obtained using the same method (Chiu & Lubin, 1979).

The activities of SOD, CAT and GPx and the level of vitamin E in sickle RBC have been reported as different from normal RBC. However, the reported results are not consistent in the literature. For example, it has been reported by Das and Nair (1980) and Beretta *et al.*

(1983) that sickle red blood cells had higher SOD activities and lower CAT activities than normal RBC. However, Schacter *et al.* (1985; 1988) reported that sickle cell anemia patients with moderate and severe symptoms had lower SOD activity than those with mild symptoms and normal controls. Reported GPx activities in sickle cells are also controversial, as Das and Nair (1980) reported lower activities while others reported higher activities (Chiu & Lubin, 1979; Beretta *et al.*, 1983; Lachant *et al.*, 1983). The level of vitamin E in sickle RBC as compared with normal RBC has also not been consistent, since both lower (Chiu & Lubin, 1979) and higher (Tangney *et al.*, 1989) levels were reported. The inconsistency about the differences of the antioxidant activities between normal and sickle RBC probably stem from different population groups studied.

To avoid such a problem, in this study, the levels of these four antioxidants were measured using the same blood samples, which consisted of a relatively large population and their spin label reduction rate constants also represented a wide range of rate constants from a large population.

The results obtained from this study show that the average activities of GPx and the level of vitamin E in sickle RBC were significantly higher than those in normal RBC ($p = 0.001, 0.005$). The average activities of SOD and CAT in sickle RBC were lower than normal RBC, but the differences were not statistically significant ($p = 0.21, 0.43$). Our results of high GPx activities in sickle RBC are in accordance with the findings by most authors (Chiu & Lubin, 1979; Beretta *et al.*, 1983; Lachant *et al.*, 1983). Increased vitamin E levels found in sickle RBC is in agreement with the finding by Tangney *et al.* (1989).

In RBC, besides the four antioxidants discussed above, ascorbate is considered as a reducing agent. Its abundance (about 0.1 mM) also enhances its importance in the system. Therefore, its function in the system is not negligible.

Although the amount of ascorbate in normal blood distributed to erythrocytes is about the same as to plasma (Barkhan & Howard, 1958), the role of ascorbate as antioxidant is

generally studied in blood plasma (Jain & Williams, 1985; Frei *et al.*, 1989). In erythrocytes, ascorbate is considered as a synergist in sparing and maintaining the concentration of vitamin E, thereby preventing lipid peroxidation (Niki, 1991). On the other hand, ascorbate is suspected to function as a prooxidant in the presence of ferric ion by reducing ferric ion to ferrous ion which decomposes peroxides to generate hydroxyl radicals (Hebbel, 1986), though the prooxidant action of ascorbate was only observed in some model experiments with free iron (Block *et al.*, 1991).

In sickle cell studies to date, the concentrations of ascorbate in sickle patients have all been measured in their blood plasma (Akinkugbe & Ette, 1983; Jain & Williams, 1985; Adelekan *et al.*, 1989), serum (Tangney *et al.*, 1989), and leukocytes (Chiu *et al.*, 1990). Some reports showed that sickle patients had significantly lower ascorbate levels in plasma or leukocytes than normal controls (Akinkugbe & Ette, 1983; Jain & Williams, 1985; Chiu *et al.*, 1990), while others reported that there were no significant differences (Adelekan *et al.*, 1989; Tangney *et al.*, 1989). Since the sickle patients appeared to have an adequate intake of dietary vitamin C, the cause of plasma ascorbate deficiency in sickle patients was attributed to increased ascorbate utilization (Chiu *et al.*, 1990). In the present study, the levels of ascorbate measured from some of the sickle patients' RBC appear to be higher than those from normal RBC. It could be speculated that more ascorbate partitioned into sickle cells than into normal ones, thereby giving less ascorbate in sickle blood plasma, as found by many investigators. It is also interesting to note that the storage of normal blood affected the distribution of ascorbate between plasma and cells. As reported, after a period of several hours the concentration of ascorbate in plasma decreased, while that in cells increased (Lowik *et al.*, 1991). Similarly, it was found that vitamin E levels in plasma were significantly lower for adult patients with sickle cell anemia than those of healthy normal black adults. However, the levels of vitamin E in sickle RBC of the same subjects were significantly higher than those in the normal RBC of control subjects (Tangney *et al.*, 1989).

A clear understanding of the physiology of ascorbate in blood is necessary for a more detailed analysis of our findings.

In summary, the activities of membrane bound antioxidants GPx and vitamin E are higher, while those of cytoplasm antioxidant enzymes SOD and CAT are lower, in sickle RBC than in normal RBC. In addition, the concentration of ascorbate in sickle RBC is higher than in normal RBC. The relationships among them and spin label reduction will be discussed in the following section and the overall radical reduction mechanism of the antioxidant system will be proposed in section 4.7.3.

4.7.2 Correlations between Spin Label Reduction and Antioxidant Activities

The purpose of measuring antioxidant activities or concentrations in this study is not only to compare the antioxidant levels of normal RBC with those of sickle RBC, but also to determine the effectiveness of these antioxidants on the spin label reduction.

Among these antioxidants, ascorbate displayed a strong positive correlation between its concentrations and spin label reduction rate constants (Figure 23A). In addition, at least 90 % of the reduction could be inhibited by adding an adequate amount of AO. Therefore, the high 5-DSA spin label reduction rate in some sickle cells can be attributed to the high ascorbate concentration in these sickle RBC.

On the average, sickle RBC samples that had higher reduction rate constants than normal RBC also had higher GPx activities and vitamin E concentrations, but lower SOD activities than those of normal RBC. It has been reported that RBC from sickle patients with moderate and severe symptoms gave low SOD activity (Schacter *et al.*, 1985; 1988). This is in agreement with our finding that the sickle RBC, which have higher reduction rates, also have lower SOD activities. It supports our speculation that sickle patients who have more severe symptoms may have higher reduction rates. Based on these findings, it may be suggested that the relationship among those antioxidants are fully expressed when the sickle

cell symptom is severe.

4.7.3 A Viewpoint on the Antioxidant System in Sickle RBC

A single mutation of the β6-Glu to Ala of Hb causes sickle disease. The existence of sickle disease is believed to be the result of evolution, driven by malaria, a fatal disease mainly present in the African continent. Besides the single mutation on the Hb, other alterations also occurred in sickle cells as consequences, proceedings, or parallel developments. Here, we do not intend to trace back when and why sickle disease evolved. However, the view of evolution may help us to understand the antioxidant system in RBC. Based on the results obtained in this study and other information about sickle disease available in the literature, we may be able to compose an hypothesis of the antioxidant system in sickle RBC and its alteration from normal RBC.

It has been reported that more superoxide radicals are generated in sickle cells than in normal ones due to the abnormalities in sickle Hb (Hebbel *et al.*, 1982). As a consequence, the sickle RBC are exposed to more severe free radical damage. Increased oxidation of lipid and protein constituents of the sickle membrane has been demonstrated (Chiu *et al.*, 1982; Rank *et al.*, 1985). For RBC to overcome this problem, the most economical way probably is to maintain a higher concentration of ascorbate in the cytoplasm, since ascorbate is a general reducing agent and ascorbate is partially responsible for the O_2^- reduction with the mechanism parallel to the SOD action (Nishikimi, 1975; Nandi & Chatterjee, 1987). This may explain why ascorbate concentration is lower in blood plasma and higher in cytoplasm of sickle RBC than that of normal controls. On the other hand, the lower activity of SOD in sickle cells than in normal cells could be due to the higher concentration of ascorbate in sickle cells.

While not actually a free radical, H_2O_2 is readily generating other free radicals and is capable of crossing membranes to initiate lipid peroxidation chain reactions. In the presence

of transition metals, H_2O_2 can be converted to ·OH, which is highly active and can damage almost any biological molecule it encounters. It is believed that ·OH is one of the most potent oxidants attacking red cell membrane and causing hemolysis (Chiu *et al.*, 1982). Therefore, membrane bound antioxidants are very important to protect membrane lipids and proteins.

In sickle cells, it is also known that there is an abnormal amount of membrane bound heme (Fischer *et al.*, 1975) and an increased susceptibility of membrane peroxidation (Das & Nair, 1980). The higher ascorbate concentration can accommodate most of free radicals in the cytoplasm. But those in the membrane must be taken care of by vitamin E, which is membrane bound, and GPx, which is weakly membrane bound (Duchon & Collier, 1971; Ursini & Bindoli, 1987). As observed in this study and by some other investigators (Beretta *et al.*, 1983; Lachant *et al.*, 1983; Tangney *et al.*, 1989), the vitamin E concentrations and GPx activities are higher in sickle cells than in normal cells.

Evolution is fascinating, and nature sometimes has its own way to overcome the disadvantages and to eliminate the defects that occurred during the process.

APPENDIX I

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Original Contribution

A METHOD TO EVALUATE THE ANTIOXIDANT SYSTEM FOR RADICALS IN ERYTHROCYTE MEMBRANES

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Abstract—The erythrocyte defense system against cellular oxidants is complex and efficient. Free radicals generated in cell membranes, however, are relatively sequestered from the cell's antioxidant mechanisms. When an oxidant challenge exceeds the capacity of the erythrocyte's antioxidant system, membrane damage may occur, causing red cell destruction and hemolytic anemia. In this study, we present a method for monitoring radical reduction in erythrocyte membranes, using fatty acid spin labels with nitroxide radicals on the hydrocarbon chains. About 50 μL of packed (about 5–6 × 10^8), carbon monoxide (CO)-gassed red blood cells are used. The electron paramagnetic resonance signals of the 5-doxylstearic acid spin labels in the intact cells are obtained as a function of time, at 37°C over a period of 2 h. The pseudo first-order rate constant for reduction of the spin label in normal adult intact cells under our experimental conditions is 4.3 ± 1.8 × 10^{-3}/min. The reproducibility and variability of the measurements are discussed. Since the measurements we describe reflect the extent of radical reductions occurring in cell membranes, we suggest that this method can be used to measure the ability to defend oxidants in membranes of erythrocytes with defective antioxidant systems. This method is particularly useful for measuring the modification of the antioxidant system toward radicals in membranes by drugs, chemicals, or environmental toxins.

Keywords—Fatty acid spin labels, Erythrocyte membranes, Antioxidants, Radical reduction, Free radicals

INTRODUCTION

Free radicals are formed in biological systems by various endogenic or exogenic compounds, and play an important role in diseases and aging. Erythrocytes have been extensively studied as a target for oxidative damage.[1–3] Hemoglobin (Hb) oxidation produces radicals,[4,5] which could enhance intracellular and membrane protein oxidation and lipid peroxidation,[6] but probably by different mechanisms, since the two processes can occur independently.[7] Drugs or xenobiotics that can undergo oxidation–reduction reactions in erythrocytes may also generate radicals. When an oxidant challenge exceeds the capacity of the erythrocyte's antioxidant system, membrane damage may occur to cause red cell destruction and hemolytic anemia.[2]

The erythrocyte defense system against cellular oxidants is complex and efficient, and consists of a series of interrelated processes. The defense system against oxidants in the cell membrane is less effective since radicals in the membrane would be relatively sequestered from the cell's antioxidant mechanisms, which, with the exception of vitamin E, are largely cytoplasmic.[8,9] Thus, even mildly defective antioxidant systems may cause extensive damage in erythrocyte membranes. For example, in sickle cell anemia (SCA), some of the antioxidant systems appear to be abnormal in sickle erythrocytes,[10–13] and erythrocyte oxidation might underlie the development of some of the known abnormal membrane properties.[8] Generally, individual enzyme activities were measured in the SCA studies. For example, it has been reported that SCA patients have increased glutathione peroxidase activities,[11] but decreased superoxide dismutase activities,[13] or decreased glutathione peroxidase activities and increased superoxide dismutase activities.[12] However, it is difficult to assess the effectiveness of the concerted actions of the intracellular free radical scavenging enzyme systems toward radicals in membranes. Yet an understanding of the actions of the erythrocyte antioxidant system toward radicals in membranes, or lipophilic oxidants, is essential for the study of red blood cell physiology and pathophysiology.

Radicals are generally difficult to study. For example, peroxyl radicals may participate in chain reactions, and are thus difficult to study experimentally. Paramag-

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netic nitroxide radicals (> NO•) resemble ground state oxygen (•O-O•), and may thus compete with oxygen in free radical addition reactions. The nitroxide spin-pairing products are expected to be less reactive, and thus easier to study. Therefore, the nitroxides are potentially useful in studying oxygen involvement in free radical reactions.[14]

The reductions of various nitroxide spin labels, to hydroxylamines, which have no electron paramagnetic resonance (ESR) signal, have been reported in various biological systems. For example, the reductions of fatty acid spin labels in membranes which consist of electron transfer systems have been studied.[14–18] The intracellular reduction of water-soluble spin labels in erythrocytes have also been observed.[19–24] However, fatty acid spin labels have been considered stable in erythrocytes, for example in erythrocytes from normal mice over time periods of 30 min.[23]

We have monitored the ESR signals from fatty acid spin labels in human erythrocyte membranes for a longer period of time under somewhat different conditions than the above-mentioned work, and observed reduction with a pseudo first-order initial rate constant of about $4.3 \pm 1.8 \times 10^{-3}$/min. In this report, we show that nitroxide radicals on fatty acid spin labels located in the membranes of intact erythrocyte are reduced by intracellular antioxidant systems, and present a method to evaluate the efficiency of cellular antioxidant systems for radicals in erythrocyte membranes.

MATERIALS AND METHODS

Red blood cells

Fresh, normal, adult human red blood cells (RBCs) were obtained from a local blood bank. The RBCs were washed, at 4°C, with an isotonic buffer solution of either 5 mM sodium phosphate buffer with 150 mM NaCl at pH 8.0 (PBS), or sodium phosphate buffer (1.4 mM NaH_2PO_4 and 8.6 mM Na_2HPO_4) with 135 mM NaCl, 5 mM KCl, and 11 mM glucose at pH 7.4 and osmolality 290–300 mosmol/kg (BSKG). The buffer solutions were gassed with carbon monoxide (CO) at 4°C before use. The blood cells were assessed optically, using optical cells with very narrow light paths (0.01 mm), at wavelengths of 540 and 569 nm to ensure that the Hb molecules inside the cells were fully CO-liganded.[25] Generally, 30 min of bubbling CO gas into the cold buffer immediately before use provided sufficient CO gas in the buffer to fully ligate the Hb molecules with CO. For oxygenated cells, O_2-gassed buffers, instead of CO-gassed buffers, were used to wash the RBCs. Optical spectra of these cells at 541 and 577 nm were obtained to ensure that all Hb molecules were O_2-liganded.[25]

Spin-labeled RBC

Fatty acid nitroxide spin probes, 5-doxylstearic acid or 16-doxylstearic acid, were purchased from either Syva (Palo Alto, CA) or Aldrich (Milwaukee, WI) and used without further purification. The spin labels were stored in ethanol solution at a concentration of about 0.5 mg/mL. Spin labels in ethanol (1 mL) were introduced into a small container, and ethanol was evaporated to dryness with nitrogen, at room temperature, in the dark, to give a thin film of spin labels on the container walls. About 10 mL of buffered, fatty-acid-free bovine serum albumin (BSA) (2.2 mg/mL) was added to the container. The solution was stirred for at least 4 h at room temperature to give a spin-labeled BSA solution, with a spin label concentration of about 5×10^{-5} M, as determined by ESR measurements. 50 μL packed RBC with hematocrit greater than 95% (about $5–6 \times 10^8$ cells) were mixed with 1 mL of the spin-labeled BSA solution for about 30 min at 4°C, and the mixture was washed with cold buffer to remove excess spin-labeled BSA. To avoid echinocyte formation,[26,27] the fatty acid spin labels were not mixed directly with red blood cells.

The spin-labeled cells (about 20 μL) were packed in small 50 μL hematocrit capillaries, which were used as ESR sample tubes, by a microhematocrit centrifuge for 3 min at 4°C. The heights of the packed cells in the hematocrit tubes were at least 2 cm to ensure proper ESR measurements. About 30–50 μL packed red blood cells are needed to give about 20 μL spin-labeled cells. If the ESR spectrometer is equipped with a loop-gap resonator[28] to give better signal-to-noise ratios, about 10 μL packed cells will be needed to prepare sample for a single ESR measurement. The total sample preparation time was about 2 h.

Hemolysates

Centrifuge tubes containing washed, CO-gassed RBCs with hematocrit values greater than 95% were immersed in liquid nitrogen for about 30 s to allow complete freezing of the cells, followed by immersion in a water bath at room temperature for about 2 min. The thawed samples were then centrifuged for about 10 min at 30,000 g and 4°C. The supernatant in each tube was removed, stored on ice, and used within 5 min.

Membranes and spin-labeled membranes

White membrane ghosts were prepared from washed cells. Cells were lysed and washed with 5 mM sodium phosphate buffer at pH 8.[29] Membrane concentrations were represented by protein concentrations, which were determined by modified Lowry method.[30]

Spin-labeled membranes were obtained by labeling

white membranes (4.3 mg/mL) with 5-doxylstearic acid labels to give a spin label concentration of about 1.4×10^{-4}M.[31] The membranes were not labeled with spin-labeled BSA solution, as was done for the RBC samples, since spin-labeled BSA strongly adsorbs to isolated ghosts, but not to intact red cells.[32]

NADPH, vitamin C and vitamin E-treated samples

Beta-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), purchased from Sigma (St. Louis, MO), was dissolved in CO-gassed PBS buffer to give a concentration of 2.15 mM. Vitamin C (L-ascorbic acid) solution at a concentration of 20 mM, from Sigma, was freshly prepared with CO-gassed, high phosphate concentration buffer (0.155 M NaH_2PO_4 and 0.103 M Na_2HPO_4) to maintain the pH value of the vitamin C solution at about 8.0. Vitamin C solution was highly acidic in PBS solution. About 30.5 mg of vitamin E (*d*-α-tocopherol), from Kodak (Rochester, NY), was dissolved in dehydrated ethyl alcohol, dried to a thin film with nitrogen gas, and suspended in 1 mL of CO-gassed PBS by swirling with glass beads followed by sonication for about 10 min immediately before use. About 20 μL of NADPH solution, 10 μL of vitamin C or vitamin E with equal amounts of buffer, or 10 μL of vitamin C plus 10 μL of vitamin E were mixed with 20 μL hemolysate and 20 μL spin-labeled membrane and used for ESR measurements immediately. Control samples consisted of 20 μL of PBS instead of NADPH, vitamin C, or vitamin E solution. The final NADPH concentration in the sample containing hemolysate was about 0.74 mM, assuming that the hemolysate contained about 0.02 mM NADPH.[33] The final concentration of the vitamin C in the sample was 3.3 mM, assuming that the hemolysate contained about 0.01 mM vitamin C,[34] and of the added vitamin E was 11.8 mM, assuming that the vitamin E droplet was homogeneously suspended in the sonicated solution. The concentrations of NADPH, vitamin E and vitamin C, and the solution conditions were chosen for experimental convenience. Another set of control samples which consisted of membranes and NADPH, vitamin C, or vitamin E at concentrations equivalent to those stated above, but included no hemolysate was also prepared.

ESR measurements and data analysis

All ESR measurements were done on a Varian E-109 ESR spectrometer with an IBM temperature controller. A scientific program, ASYST (MacMillan Software), modified for ESR operation, was used for data acquisitions and data analysis on a Zenith personal computer. The ESR measurements were made immediately after the spin-labeled samples were prepared. The ESR cavity was prewarmed to 37 ± 0.5°C. The samples were kept at 4°C. Little spin label reduction was observed at 4°C over periods of several hours. About 1 min after the cold sample was placed in the warm cavity, the sample temperature reached 37°C, as indicated by a thermocouple inserted in the sample, and the ESR signals were recorded as a function of time for about 2 h at 37°C, with spectra taken about every 20 to 30 min. Some ESR measurements covered a period as long as 22–24 h. For a few samples, we also monitored the central peak signal continuously with the following procedures. First an ESR spectrum was obtained, then the spectrometer was switched to nonscanning mode to continuously follow the peak height of the central peak. At a later time, the spectrometer was switched to the scanning mode so that a full ESR spectrum was obtained. Then the spectrometer was returned to the nonscanning mode to monitor the central peak. Since the full ESR spectrum was obtained in about 2 min, no noticeable interruption was detected for the continuous recording of the central peak height (Fig. 1). The full ESR spectra obtained in this manner allowed us to check the line-shape and line-width of the ESR signals during the time period of the ESR experiments. This approach provided more accurate measurements of signal reduction, but more laborious than the one with spectra taken at different time intervals.

From the ESR spectra, the values of the central peak height at time t (h_t, see Fig. 1) were obtained. Since the ESR spectra had equivalent line-shapes and line-widths at different times, the h_t values were proportional to the spin concentrations at time t. Double integrations of the ESR spectra at different times verified that the spin concentrations were directly proportional to the peak height values.

Pseudo first-order rate constants for spin label reduction reactions were obtained from the slope of a linear regression fit of $\ln (h_t/h_o)$ versus time plot, where h_o was the initial peak height of ESR spectrum obtained at $t = 0$ (see Fig. 1). The SYSTAT (SYSTAT, Inc.) statistical personal computer program was used for the linear regression analysis.

RESULTS AND DISCUSSION

Amounts of spin labels

In this study, the ESR signal reduction was followed as a function of time. At the end of 24 h, only about 5–10% of the signal remained in the spin-labeled red blood cells. In principle, the higher the initial concentrations of spin label in the erythrocyte membranes, the more accurately one can follow the decrease in signal intensities of the spin label at later times. However, spin–spin broadening posed an upper limit to the spin

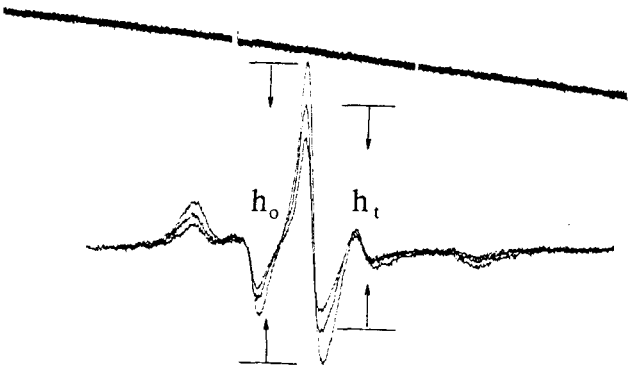

Fig. 1. A typical signal reduction data set for intact red blood cells spin labeled with 5-doxylstearic acid spin label. At time 0 (left-hand side of the figure), the full spectrum of the spin label was recorded with a central peak height of h_o. Then the central peak height was followed over a period of 2 h (as shown on the top of the figure). The tracing was interrupted occasionally during the 2 h time period to obtain full ESR spectra to check the line-shape and line-width of the signals. The central peak height at time t was designated as h_t. The ESR experiment was done at 37°C.

label concentration per cell that could be used for this study. The spin labels may cluster at higher concentrations to distort the ESR signal. In studies where fatty acid spin labels were introduced directly into red blood cell membrane ghosts, the spin labels clustered in membranes at a spin label-to-lipid weight ratio of about 1/400, resulting in spin–spin exchange induced broadening in the ESR spectra.[35] In our study, the line-widths of the ESR signals were carefully checked, and no spin broadening was observed in the ESR spectra. The spin label concentrations were about 1.5×10^{-4} M, or about 8.7×10^6 spin labels per cell, measured at 4°C, where reduction was insignificant.

Reduction of 5-doxylstearic acid

In an earlier study of parasitized erythrocytes, spin label 5-doxylstearic acid was reduced by the electron transport chain in the parasite, but it was stated that no reduction was observed in the control, nonparasitized cells.[23] However, in our studies, we observed consistent reductions in the ESR signals of CO-gassed, intact erythrocytes over a period up to 24 h after labeling. Figure 1 contains a typical set of spectra showing signal reduction of the central peak of 5-doxylstearic acid spin labels in intact cells, in buffer PBS, over a period of 2 h. As shown in Fig. 1, the three full ESR spectra of the sample obtained at different time intervals during the reduction experiment exhibit no change in the ESR signal line-shape or line-width. Similar reduction was observed when cells were placed in BSKG buffer.

Reduction of 5-doxylstearic acid spin labels by intracellular components

Figure 2 shows a plot of a typical set of data for ESR signal reduction. At the end of 22 h, for this particular blood sample, about 95% of the original signal of 5-doxylstearic acid spin labels intercalated in the membranes of CO-gassed intact erythrocytes, in PBS at 37°C, disappeared, with only about 5% signal remaining. Also shown are the retention of signal for the same spin labels when intercalated in white membrane ghosts isolated from the same batch of erythrocytes, under the same experimental conditions as used for intact erythrocytes. No signal reduction was observed over a period of 22 h. When we added about 40 μL hemolysate, prepared from the same batch of blood sample used for intact cell studies, to the spin-labeled membrane ghosts samples (about 40 μL at about 4 mg/mL concentration), we observed signal reductions of about 30% over a period of 2 h, similar to those observed in intact cells, shown in Fig. 2 inset for clarity. These data suggest that intracellular components, which were not present in the white membrane ghost sample, were responsible for the signal reductions observed in intact cells.

Reduction rate constants

In order to obtain rate constants from the signal reduction data presented above, we need to demonstrate that the spin label translocation rate is relatively faster than the spin reduction rate in the erythrocytes. When the fatty acid spin labels are initially introduced to the erythrocyte membranes, they reside in the outer leaflet of the membrane bilayer. In order for them to be reduced by intracellular components, the labels must translocate to the inner leaflet. We monitored the spin reduction for a system which consisted of labeled intact cells plus hemolysate and obtained a reduction rate

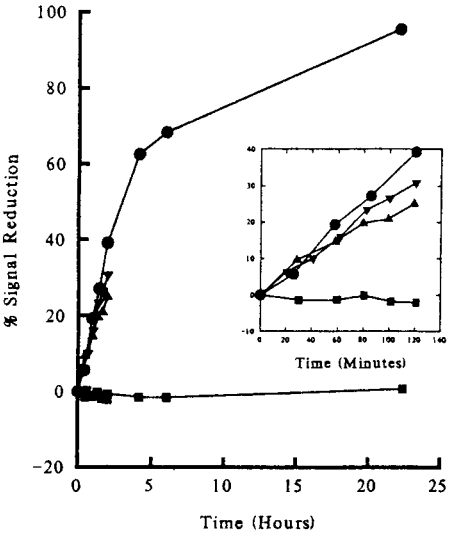

Fig. 2. Percent ESR signal reduction ($1-h_t/h_o$) over a period of 22 h for spin-labeled intact cells (●), white membrane ghosts (■), white membrane ghosts plus hemolysate (▲), and intact cells plus hemolysate (▼). The same batch of blood was used to prepare the samples. The samples were in 5 mM phosphate buffer with 150 mM NaCl at pH 8.0. The buffer was gassed with carbon monoxide at 4°C before use. For clarity, the data obtained within the first 2 h were shown in the inset.

similar to that for intact cells (Fig. 2 and inset). If the translocation movements of the labels in membranes are slow, relative to ESR measurement time, some labels would locate in the outer leaflet during the ESR experiments, and we would expect that the addition of hemolysate to the intact cells would result in a faster reduction than for intact cells without added hemolysate, since in the sample of intact cells with added hemolysate, both sides of the bilayer leaflet are in contact with the "intracellular" components. We found that the reductions for intact cells and for intact cells plus hemolysate are similar. We conclude that the translocation movements of 5-doxylstearic acid spin labels in erythrocyte membranes are relatively faster than the spin reduction, and the signal reduction data can be use to provide reduction rate constants. The 5-doxylstearic acid spin label reduction in many biological systems follows a first-order reaction mechanism.[15,18] Our results in intact cells, over time periods of 2 h, exhibit linear fits in semilogarithmic plots, with correlation coefficients generally > 0.99. A semilogarithmic plot of data for intact RBCs shown in Fig. 1 is illustrated in Fig. 3. The correlation coefficient for the linear fit was 0.996. Since the spin label translocation rate is relatively faster than the spin reduction rate, the excellent linear fit for signal reduction data exhibited in Fig. 3 represents a pseudo first-order reduction reaction, with a rate constant (k) of about 5.1×10^{-3}/min. As discussed below, the k values vary from batch to batch (each batch was defined as the blood sample from one unit of blood obtained from the blood bank) of blood samples.

Reproducibility and variability

To check the reliability and reproducibility of the ESR measurements, we repeated the ESR measurements of multiple ESR samples prepared from the same spin-labeled samples (group 1), and stored at 4°C until the ESR measurements, which were done at 37°C. We also prepared multiple spin-labeled samples from the same batch of blood (group 2) and obtained ESR measurements from these samples. In addition, we obtained blood samples from different batches (group 3) and prepared ESR samples in parallel for ESR measurements. We found that the coefficient of variability (CV)[36] of reduction rate constants, defined as the standard deviations divided by the averaged value of a set of individual k values, for multiple samples prepared from one batch of blood (group 1 or 2) was about the same. Data obtained from 15 batches of blood show that the CV of individual batches of blood range from 2.1 to

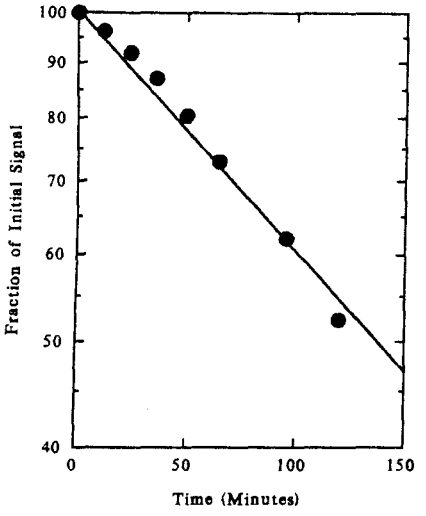

Fig. 3. A semilogarithmic plot of the fraction of initial signal ($h/h_o \times 100$) versus time for CO-gassed, spin-labeled red blood cells in PBS. The data were from Fig. 1. A linear fit gave a pseudo first-order rate constant for reduction of about 5.1 $\times 10^{-3}$/min, with a correlation coefficient of 0.996.

19.7%, with an average CV of about 9.2 ± 5.9%. This indicates that the reproducibility of these measurements are quite good with only about 10% uncertainty in the k values.

The variation from batch to batch (group 3) was much larger. The k values from 42 batches of blood range from 1.5 $\times$ 10^{-3}/min to 8.4 $\times$ 10^{-3}/min, with an average value of 4.3 ± 1.8 $\times$ 10^{-3}/min. The CV for batch to batch was about 41.9%.

Table 1 presents a selection of the data from experiments of groups 1 and 2 that exhibit the smallest and the largest variability coefficients and the data with the lowest and highest rate constants for samples in groups 1, 2, and 3.

Sensitivity to changes in antioxidant system

The antioxidant system involves enzymes that scavenge reduced products of oxygen, such as superoxide dismutase, catalase, glutathione peroxidase, and reduced nicotinamide-adenine dinucleotide (NADH) methemoglobin reductase as well as membrane-associated vitamin E, vitamin C, intracellular glutathione, and the reduced pyridine nucleotides (NADH and NADPH).[37-39] To test the sensitivity of this method, we have chosen to alter the concentrations of NADPH, vitamin C, or vitamin E as examples to modify the antioxidant system. NADPH is important in providing reducing equivalents in reactions that are critical in protecting against oxidant damage.[37,38,40] Vitamin C is an aqueous, intracellular component, and is known to reduce spin labels in solution. Vitamin E is membrane-associated, and thus was also tested.

Figure 4 shows the signal reductions as a function of time in these treated systems. For the particular batch of

Table 1. Reproducibility and Variability of the Pseudo First-Order Rate Constatns for the Reduction of 5-Doxylstearic Acid Spin Labels in CO-Gassed Intact Red Blood Cells, at 37°C

Blood[a]	Sample[b]	k[c] (10^{-3}/min)	Averaged k (10^{-3}/min)	Coefficient of Variability
1	a	1.5	1.5	—
2	a	1.6		
	b	1.7		
	c	1.7	1.7	5.9
3	a	4.6		
	b	4.7	4.7	2.1
4	a	5.2		
	b	6.9	6.1	19.7
5	a	8.2		
	b	7.9	8.1	2.5
6	a	8.4	8.4	—

The averaged coefficient of variability (CV) for k of samples in groups 1 & 2[d] from 15 blood batches was 9.2 ± 5.9. The CV for samples in group 3[e] from 42 blood batches was 41.9. The averaged rate constant for 42 blood batches was 4.3 ± 1.8 $\times$ 10^{-3}/min.

[a]Each entry under *Blood* represents one batch of blood from the blood bank.

[b]Each entry under *Sample* represents and ESR sample.

[c]k is the pseudo first order rate constant for spin label reduction.

[d]Samples in groups 1 and 2 represent multiple samples prepared from the same batch of blood.

[e]Samples in grouip 3 represent samples prepared from different batches of blood.

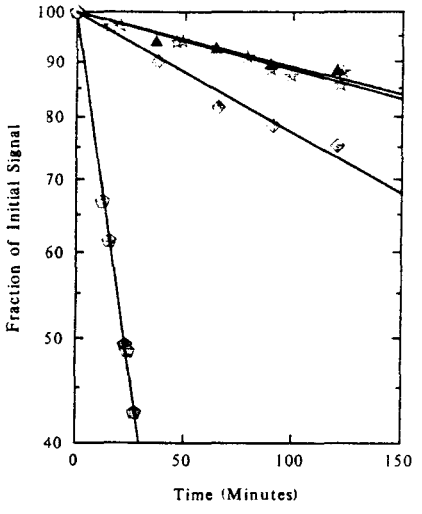

Fig. 4. A semilogarithmic plot of the fraction of initial signal versus time for 5-doxylstearic acid spin labels in samples containing 20 μL of white membrane ghosts (4.3 mg/mL), 20 μL CO-gassed hemolysate and 20 μL of 5mM phosphate buffer with 150 mM NaCl at pH 8.0 (▲), 10 μL of vitamin E solution (about 70.8 mM) with 10 μL of buffer (shaded star), 20 μL of NADPH solution (2.15 mM) (shaded diamond), or 10 μL of vitamin C solution (20 mM) with 10 μL of buffer (shaded pentagon). The rate constant obtained for the hemolysate sample was 1.17×10^{-3}/min with a correlation coefficient of 0.989 for the linear fit. The rate constant for the same blood sample but with vitamin E was 1.24×10^{-3}/min, with a correlation coefficient of 0.993. The rate constant for sample with NADPH was 2.57×10^{-3}/min, with a correlation coefficient of 0.994, and with viatmin C was 2.76×10^{-2}/min, with a correlation coefficient of 0.997. The rate constant for sample with vitamin C was determined over a time periods of 1h.

blood used, the rate constant for the membrane with hemolysate was 1.17×10^{-3}/min, and the addition of vitamin E, at a concentration of about 11.8 mM, appeared to have no effect on the reduction of spin labels in membranes. The rate constant was 1.24×10^{-3}/min, very similar to that without the added vitamin E.

The addition of NADPH to give a final concentration of 0.74 mM enhances the reduction of spin labels in membranes. The rate constant increased to 2.57×10^{-3}/min, an increase of about 120%. There was no ESR signal reduction for membrane samples with or without similar amounts of NADPH.

Vitamin C, at concentrations of about 3.3 mM, increased the rate constant dramatically, to about 2.76×10^{-2}/min. The rate constant was determined over a time periods of only 1 h, due to the very rapid reduction in this system. Sample containing only vitamin C and membrane also exhibited spin label reduction, with a rate constant of about 1.5×10^{-2}/min (data not shown). The vitamin C and hemolysate effects on spin label reduction appear to be additive. The addition of vitamin E to samples treated with vitamin C did not further increase the rate constant (data not shown). These data demonstrated that some of the changes in antioxidant system affect the reductions of radicals in membranes, and can be detected by ESR.

Reduction efficiency

We have used another spin label with the nitroxide radicals at the 16th carbon positions on the hydrocarbon chain of the fatty acid (16-doxylstearic acid spin label) as well as 5-doxylstearic acid spin label to demonstrate that the reduction efficiency of radicals in membranes by intracellular components is inversely proportional to the distance of the radical from the surface of the inner leaflet of the membranes. The same blood samples were used to prepare the ESR samples needed for this comparison study. Figure 5 shows a typical semilogarithmic plot of the reductions of 5-doxylstearic acid and 16-doxylstearic acid spin labels in samples obtained from the same blood sample, as a function of time. The 16-doxylstearic acid has a slower reduction rate (3.6×10^{-3}/min) than the 5-doxylstearic acid (6.6×10^{-3}/min). The rate constant for 16-doxylstearic acid reduction was almost half the value for 5-doxylstearic acid. This finding demonstrates that it is less efficient to reduce the more buried radicals in the membranes of intact erythrocytes.

Re-oxidation of 5-doxylstearic acid spin label by oxygen

Our samples were saturated with CO gas to avoid reoxidation of the spin label by oxygen. If samples were prepared from O_2-gassed buffer rather than CO-gassed buffer, we observed a slower reduction, in part due to reoxidation of the spin label from the hydroxylamine (ESR silent) form back to the nitroxide (ESR active) form.[41]

In parallel runs, with the same batch of blood sample, the spin reductions for O_2-gassed samples were always lower than the ones for CO-gassed samples. Figure 6 shows the semilogarithmic plot of data obtained from a typical run of CO- and O_2-gassed samples. The ESR signal reduction data of the CO-gassed sample followed a linear fit with a coefficient of 1.000. The data for the O_2-gassed sample did not give a good linear fit when data points beyond 90 min were included, since the reduction rate began to decrease due to reoxidation. If we do not include the data points beyond 90 min, where the reaction can no longer be described by pseudo

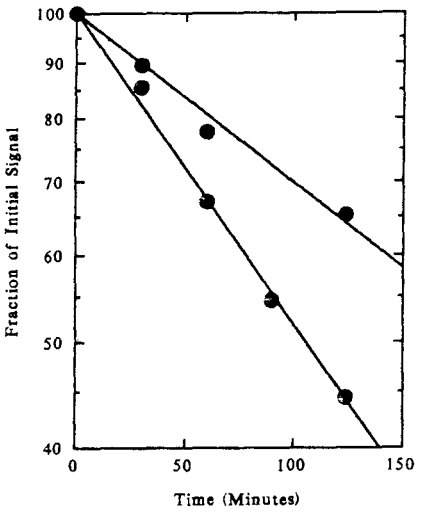

Fig. 5. A semilogarithmic plot of the fraction of initial signal versus time for 5-doxylstearic acid spin label (shaded circle), and 16-doxylstearic acid spin label (●) in CO-gassed intact red blood cells. The rate constant for reduction is 6.6×10^{-3}/min from the linear fit, with a correlation coefficient of 0.999, for 5-doxylstearic acid spin label, and 3.6×10^{-3}/min, with a correlation coefficient of 0.997, for 16-doxylstearic acid spin label.

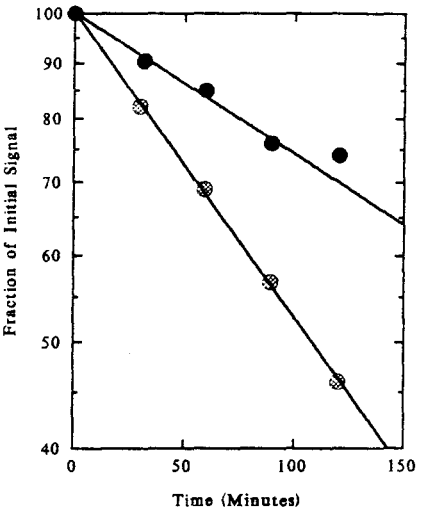

Fig. 6. A semilogarithmic plot of the fraction of initial signal versus time for 5-doxylstearic acid spin label in intact red blood cells with the red blood cells in CO-gassed buffer (shaded circle), or in O_2-gassed buffer (●). The linear fit gives a rate constant for reduction of 6.4×10^{-3}/min for CO-gassed samples, with a correlation coefficient of 1.000, and 3.0×10^{-3}/min for O_2-gassed samples during the first 90-min, with a correlation coefficient of 0.996.

first-order kinetics, the data followed a relatively good linear fit, with a correlation coefficient of 0.996. From these linear fits, we obtained a rate constant of 6.4×10^{-3}/min for the CO-gassed system and 3.0×10^{-3}/min for system under O_2 atmosphere during the initial time period of 0–90 min. The average ratio of $k(CO)/k(O_2)$ was 2.4 ± 0.7 for five different paired experimental runs.

It is important to have erythrocytes fully CO-gassed, as indicated by the conversion of HbO_2 to HbCO in intact spin-labeled erythrocytes, before the introduction of the spin labels to cells. Otherwise, the extent of the spin label reduction in cells depends on the amount of oxygen in the system.

CONCLUSION

We have described a simple spin-labeling method to measure the antioxidation capacity in membranes of erythrocytes. This method involves spin labeling about 30 to 50 μL of packed intact erythrocytes in fully CO-gassed PBS buffer with the commercially available fatty acid spin label, 5-doxylstearic acid spin label, at 4°C, and monitoring the ESR signal reduction at 37°C for about 2 h. Pseudo first-order reduction rate constants are obtained from linear fits of semilogarithmic plots of fraction of initial signal versus time.

When we modified the antioxidant system by adding excess NADPH, the rate constants were more than doubled from the values without excess amounts of NADPH. When fatty acid spin labels with nitroxide groups at different positions of the hydrocarbon chains are used, a gradient of the efficiency of the reduction of radicals at different positions in the hydrocarbon core of the bilayer can also be determined. The rate constant for the reduction of spin label radicals at the C16 position from the head group of the fatty acid molecule was only half as high as that at the C5 position. Thus, the pseudo first-order radical reduction rate constants can be used as an index for the effectiveness of the antioxidant system toward radicals in membranes in the erythrocyte.

This method can be applied to RBCs with defective antioxidant systems, such as those from patients with blood diseases. This method is particularly useful for measuring the modification of the antioxidant system toward radicals in membranes by drugs, chemicals, or environmental toxins, since the same batch of blood sample can be used in this case as controls to provide only about 10% uncertainty in the findings.

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APPENDIX II

Superoxide Dismutase

SOD was first discovered by McCord and Fridovich in 1969. The enzyme contains metal and is distributed throughout the various tissues of aerobic organisms. The metalloforms of SOD could be CuZnSOD, MnSOD and FeSOD. Human erythrocytes only contain the CuZnSOD form (Imadaya *et al.*, 1988), which consists of a dimer with one copper and one zinc per each subunit and has a molecular weight approximately 32,000 daltons. The role of the zinc (Zn^{+2}) in SOD is most likely to maintain the stability and organization of the protein. The copper (Cu^{+2}) serves as the catalytic center for this enzyme dismutating two molecules of O_2^- to form H_2O_2 and O_2. A mechanism involving the copper center is thought to be as follows (Allen & Bilski, 1982):

$$Cu^{+2} + O_2^- \longrightarrow CuO_2^+ \longrightarrow Cu^+ + O_2$$

$$Cu^+ + O_2^- + 2H^+ \longrightarrow Cu^{+2} + H_2O_2$$

Since about 3 % of total circulating hemoglobin is converted to methemoglobin per day (Carrel *et al.*, 1975), and then reduced again by methemoglobin reductase, O_2^- is continuously generated in this cycle. A major function of SOD in RBC has been proposed to prevent the formation of methemoglobin (Lynch *et al.*, 1977). Nevertheless, O_2^- poses the greatest danger when it interacts with peroxides to form highly reactive intermediates which can then attack red cell membrane and cause hemolysis. Therefore, the dismutation of O_2^- must be very important to red cells.

Catalase

CAT was named by Loew in 1901 (Percy, 1984). It is one of the oldest known enzymes and present in most aerobic cells, especially concentrated in liver tissues and erythrocytes. The usual form of CAT has a molecular weight of 240,000 daltons and consists

of four subunits, each containing a heme [Fe(III)-protoporphyrin] group bound to its active site. CAT was thought to mediate the detoxification of H_2O_2 via the following mechanism (Deisseroth & Dounce, 1970):

$$P\text{-}Fe^{+3}\text{-}OH + H_2O_2 \longrightarrow P\text{-}Fe^{+3}\text{-}OOH + H_2O$$

$$P\text{-}Fe^{+3}\text{-}OOH + H_2O_2 \longrightarrow P\text{-}Fe^{+3}\text{-}OH + H_2O + O_2$$

It has been shown that CAT binds four molecules of NADPH (Kirkman & Gaetani, 1984), which is important for the maintenance of its catalytic activity (Eaton *et al.*, 1972; Gaetani *et al.*, 1989). The reason for existence of high levels of CAT in erythrocyte is not clear. The argument is based largely on the action of GPx for dispersal of H_2O_2 (Cohen & Hochstein, 1963). It is now generally considered that CAT and GPx are both active in detoxification of H_2O_2 in human erythrocytes (Nicholls, 1972; Gaetani *et al.*, 1989).

Glutathione Peroxidase

GPx was first described as an erythrocyte enzyme by Mills in 1957. The enzyme catalyzes the oxidation of GSH to GSSG at the expense of H_2O_2. GPx containing selenium (Se) is a tetramer with one residue of selenocysteine per mole at each of the active sites and shows very high activity toward H_2O_2 as well as organic hydroperoxides. It has a molecular weight of 84,000 dalton. There is evidence suggesting that the GPx molecule has a hydrophobic region which enables it to gain access to LOOH in the membrane and to reduce them to the corresponding harmless hydroxy fatty acids (LOH) (Chiu *et al.*, 1976; Stults *et al.*, 1977). Therefore, GPx is weakly membrane bound and can prevent membrane lipid peroxidation by catalyzing the reaction (Duchon & Collier, 1971; Ursini & Bindoli, 1987):

$$LOOH + GSH \longrightarrow LOH + GSSG$$

GSH, a tripeptide (γ-L-glutamyl-L-cysteinylglycine), is required by GPx for the decomposition of H_2O_2. GSH participates in intracellular redox reactions and also serves as effective protection against free radicals (Eaton & Brewer, 1974; Barclar, 1988).

The physiological function of GPx in the erythrocyte seems clear that it protects hemoglobin from oxidative damage by H_2O_2. In addition, GPx also protects hemoglobin from oxidative damage by a wide variety of hydroperoxides other than H_2O_2.

Vitamin E

Vitamin E is a lipid-soluble molecule composed of four tocopherols (α-, β-, γ-, and δ-). It has been shown that α-tocopherol (αTH) is the best antioxidant of the four isomers (Burton & Ingold, 1981). The mode of vitamin E, αTH, action as a free radical scavenger was first proposed by Tappel (1962). It converts lipid peroxyl radicals to the less reactive form as follows:

$$LOO\cdot + \alpha TH \longrightarrow LOOH + \alpha T\cdot$$

$$LOO\cdot + \alpha T\cdot \longrightarrow LOOH + \alpha TQ$$

There is a consensus hypothesis that vitamin E can be regenerated from the tocopheroxyl radicals ($\alpha T\cdot$) by physiological electron donors such as ascorbic acid and GSH (Tappel, 1962; Hill & Burk, 1984; Wefers & Sies, 1988; Niki, 1991). However, there remains some doubts whether the vitamin E regeneration through ascorbic acid or GSH occurs *in vivo* (McCay, 1985; Murphy & Kehrer, 1989; Burton *et al.*, 1990).

The major physiological role of vitamin E in human erythrocytes is probably as a biological antioxidant protecting red cell membranes from peroxidative damage (Chiu *et al.*, 1982).

Ascorbate

Ascorbate has versatile functions in biological systems such as stimulation of enzymes, activation of hormones, and facilitation of biosynthesis (Padh, 1990). It has been known that ascorbate also functions as an antioxidant and radical scavenger to protect oxidative damage (Machlin & Bendich, 1987).

Ascorbate is a weak dibasic acid (AH_2), with pKa values of 4.2 and 11.8. Therefore, under physiological conditions, ascorbate anion (AH^-) is present predominantly. AH^- undergoes a two-step oxidation process to form DHA (A), with the formation of ascorbate free radical A^-, as an intermediate (Niki, 1991).

$$AH_2 \longrightarrow AH^- + H^+$$

$$AH^- \longrightarrow A^- + H^+$$

$$A^- \longrightarrow A$$

The formation of ascorbate free radical may proceed via a hydrogen atom abstraction or by electron transfer followed by rapid deprotonation, while the former mechanism is believed to be more favorable (Njus & Kelley, 1991).

Ascorbate functions as an antioxidant by stabilizing active oxygen species that induce and carry the chain oxidations of biological molecules. Probably the most important reaction in the inhibition of oxidation by ascorbate may be the scavenging of oxygen radicals such as superoxide, hydroxyl, hydroperoxyl, lipid peroxyl, and lipid alkoxyl radicals. Under many types of oxidative stress, ascorbate forms the first line of antioxidant defense (Block *et al.*, 1991). However, the role of ascorbate in red cells is not completely understood. In erythrocytes, ascorbate is considered as a synergist in sparing and maintaining the concentration of vitamin E to prevent lipid peroxidation (Niki, 1991). On the other hand, ascorbate has the potential to function as a prooxidant in the presence of ferric ion by reducing ferric ion to ferrous ion which decomposes peroxides to generate hydroxyl radicals (Hebbel, 1986).

APPENDIX III

It is known that red cells from sickle cell anemia patients consist of heterogeneous cell population and the heterogeneity contributes to cell-to-cell variability in sickling. Due to different degrees of dehydration, sickle cells have heterogeneous intracellular hemoglobin concentrations and thus different cell densities. The most dense cells is enriched with irreversible sickle cells. Therefore, density gradient separation was used to get cells with more homogeneous density. The spin label reductions in these density separated cells were studied.

Density Separation of RBC

Washed RBC in BSKG buffer (pH 7.4, osmolality 290 - 300 mmol/kg) were adjusted to a hematocrit of about 30 %, layered onto a discontinuous stractan density gradient, and centrifuged at 36,000 rpm (154,000 g) in a swinging bucket rotor for 45 min with ultracentrifuge. Stractan solution was prepared according to the method of Corash *et al.* (1974), as modified by Clark *et al.* (1976). Briefly, stractan (arabinogalactan) powder was dissolved in a certain amount of distilled water and the solution was deionized with cationic and anionic Amberlite to remove impurities and to reduce the osmolality to less than 100 mmol/kg. Then, stractan solution was adjusted to 290 - 300 mmol/kg by the addition of BSA (3 g/100 ml solution), 0.15 M potassium phosphate butter, pH 7.4 (10 ml/90 ml solution), $MgCl_2 \cdot 6H_2O$ (116 mg/100 ml available water), glucose (200 mg/100 ml available water), and variable amounts of NaCl. The pH of the stractan solution was adjusted to 7.4 with 3 N NaOH. The solutions of desired densities were made by diluting the stock stractan solution with BSKG, and the densities of each solution were measured by the use of a digital densitometer.

After density separation, the two or three fractions of sickle cells were collected by using transfer pipettes and then washed with CO-gassed PBS buffer three times. The

unseparated sickle cells which went through stractan were also washed simultaneously. The same cell labeling procedure and EPR measurement were followed as previously described.

SEM and Cell Dimension Measurements

A drop of washed RBC was fixed by adding a few drops of 2 % glutaraldehyde. Specimens were then post-fixed on glass slides with two drops of 1 % osmium tetroxide (O_8O_4) for 3 min, and followed by dehydration in a graded series of aqueous acetone (30:50:75:95:100 %) and by CO_2 critical point drying with a Polaron E300 Series II critical point drying apparatus. Specimens were sputtered with gold-palladium using SPI-MODULE Sputter Coater. The RBC on the slides were examined and selected for picture taking using ISI-SX-30 Scanning Electron Microscope (SEM). About five fields per sample were analyzed at magnification of 2,000 times. Approximately 20 to 60 cells were counted for each sample. The longest diameters of the cells were measured from the SEM pictures with the aid of a computer light-pen and a computer program (Bioquant II).

Examination of Sample Treatment Effect on Spin Label Reduction

Both PBS (pH 8.0) and BSKG (pH 7.4) isotonic buffers are usually recommended for erythrocyte suspensions. PBS was used in this study. BSKG, stractan, and ultra-centrifugation were used particularly for the separation of cells according to their densities. The effects of these treatments of cells on the spin label reduction were examined using the same batch of normal blood with the following sample treatments: (1) RBC was washed with PBS; (2) RBC was washed with BSKG and maintained in BSKG for 1 - 2 hr, and washed with PBS again; (3) RBC was washed with BSKG, mixed with stractan (1.120 g/ml[1]), maintained in stractan-BSKG medium for 1 - 2 hr, and finally washed with PBS; (4) RBC was washed

[1]a density higher than the normal cells such that cells stayed on the top of stractan.

with BSKG, then gone through stractan (1.065 g/ml[2]) by ultracentrifugation, and washed with PBS afterwards. The labeling and EPR measurements for above samples were carried out under the same conditions.

The rate constants of spin label reduction from the above four samples were 3.6, 3.9, 4.3 and 3.7 x 10^{-3}/min, respectively. The average value of the rate constants from these samples was 3.9 ± 0.3 x 10^{-3}/min, and the CV value was 7.7 %. This CV value is within the variation range for the same batch of blood sample shown in Table 1. Therefore, it can be considered that the spin label reduction rates in RBC were not affected by PBS or BSKG buffers, stractan, and ultracentrifugation.

Cell Dimension of Density Separated Sickle RBC

In general, volume percentages of different density components varied from patient to patient, with 70 - 90 % of light (d < 1.120 g/ml), 10 - 15 % of medium (d = 1.120 - 1.135 g/ml), and 5 - 15 % of dense (d > 1.135 g/ml) cells. Typical SEM pictures of light, medium, and dense sickle RBC from the same patient are shown in Figure 25. Unseparated sickle RBC from this patient as well as normal RBC are also shown for comparison. Figure 26 presents the average values of cell long diameters for unseparated normal and sickle RBC, and separated sickle RBC of 7 sets of samples. The dense sickle cells had the longest (9.93 ± 1.75 μm) and normal cells had the shortest (6.64 ± 0.30 μm) cell long diameters. The light sickle cells were a little longer (7.52 ± 0.44 μm) than normal cells in cell dimension. In addition, the long diameters of unseparated sickle cells (7.90 ± 0.47 μm) were in between light (7.52 ± 0.44 μm) and medium (8.61 ± 0.98 μm) sickle cells.

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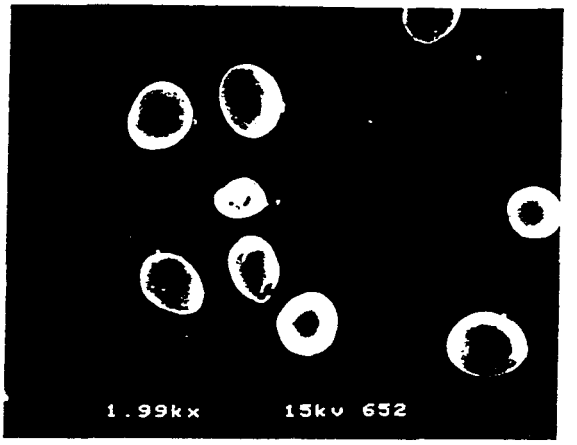
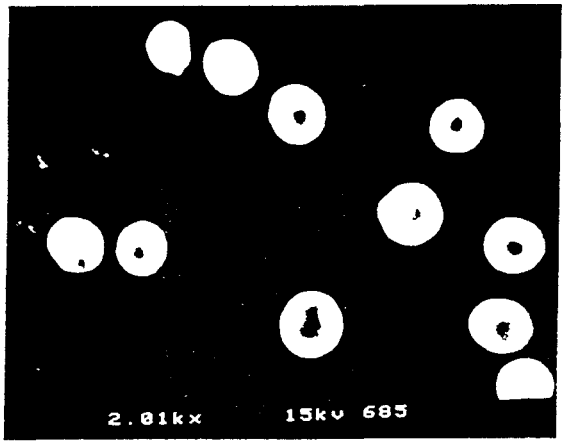
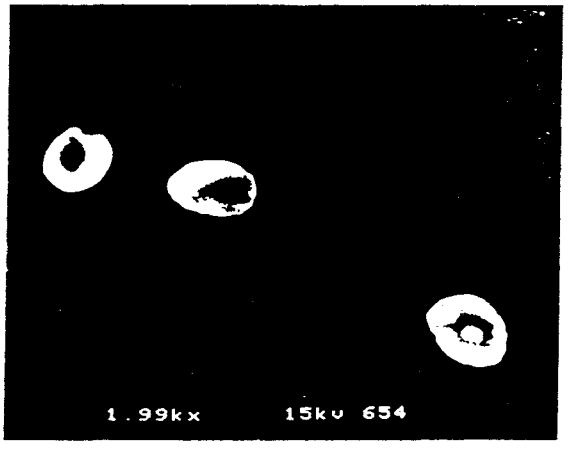
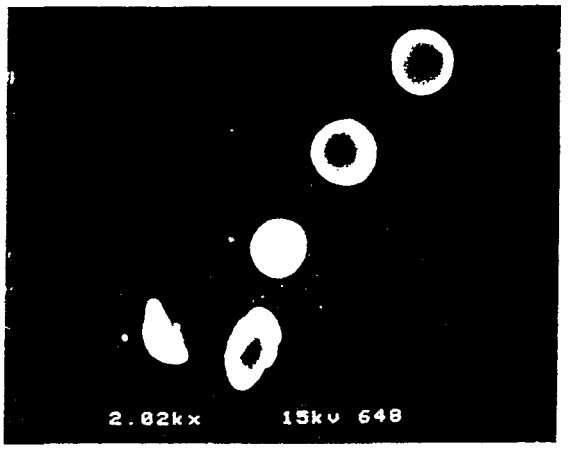
[2]a density lower than the normal cells such that cells could go through the stractan layer by ultracentrifugation.

Figure 25 **SEM pictures of unseparated normal and sickle RBC and density separated sickle RBC.** Left upper is unseparated normal cells; Left down is unseparated sickle cells; Right upper is light sickle cells ($d < 1.120$ g/ml); Right middle is medium sickle cells ($d = 1.120 \sim 1.135$ g/ml); Right down is dense sickle cells ($d > 1.135$ g/ml) cells. Unseparated and separated sickle RBC were from the same patient. Magnification is about 2,000 times.


1.99kx 15kv 652


2.01kx 15kv 685


1.99kx 15kv 654


2.02kx 15kv 648

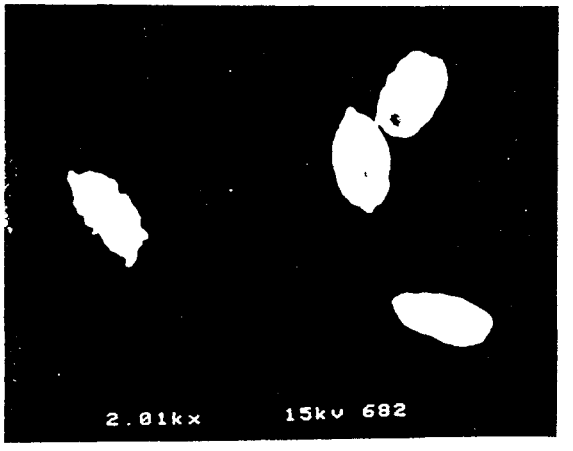
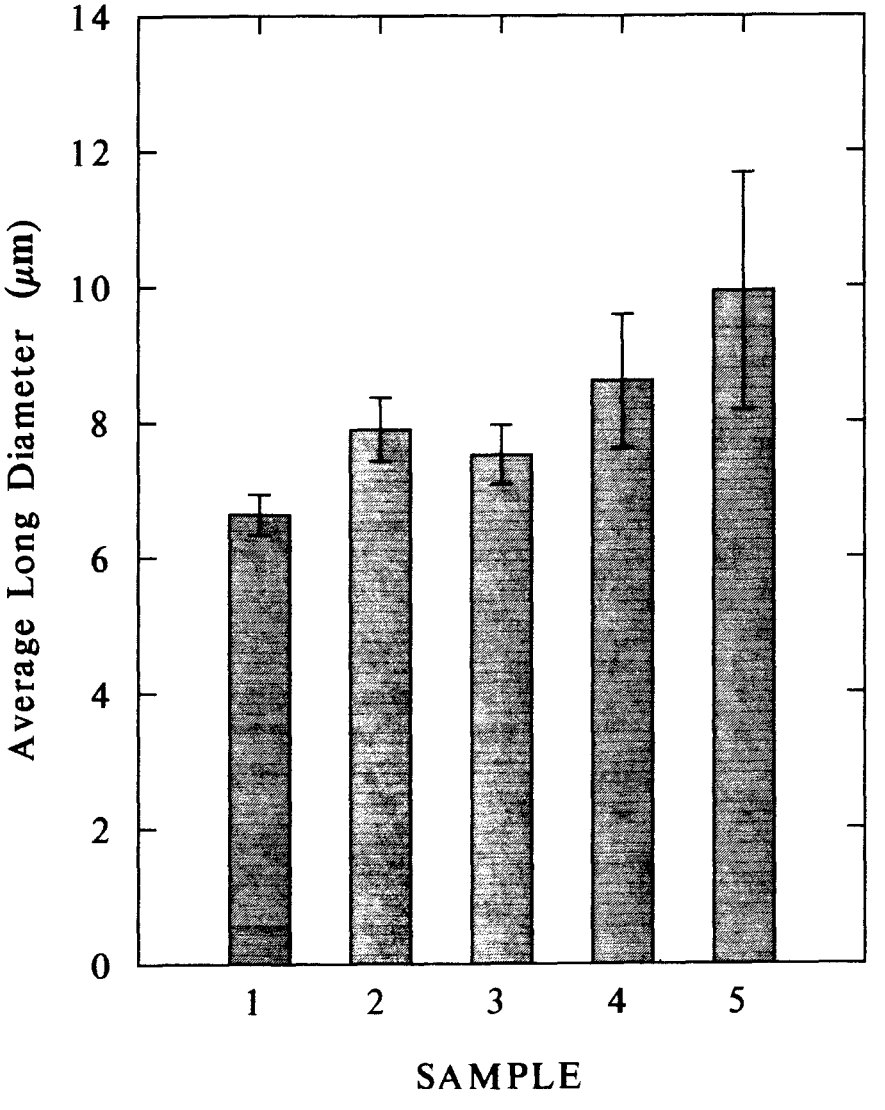

2.01kx 15kv 682

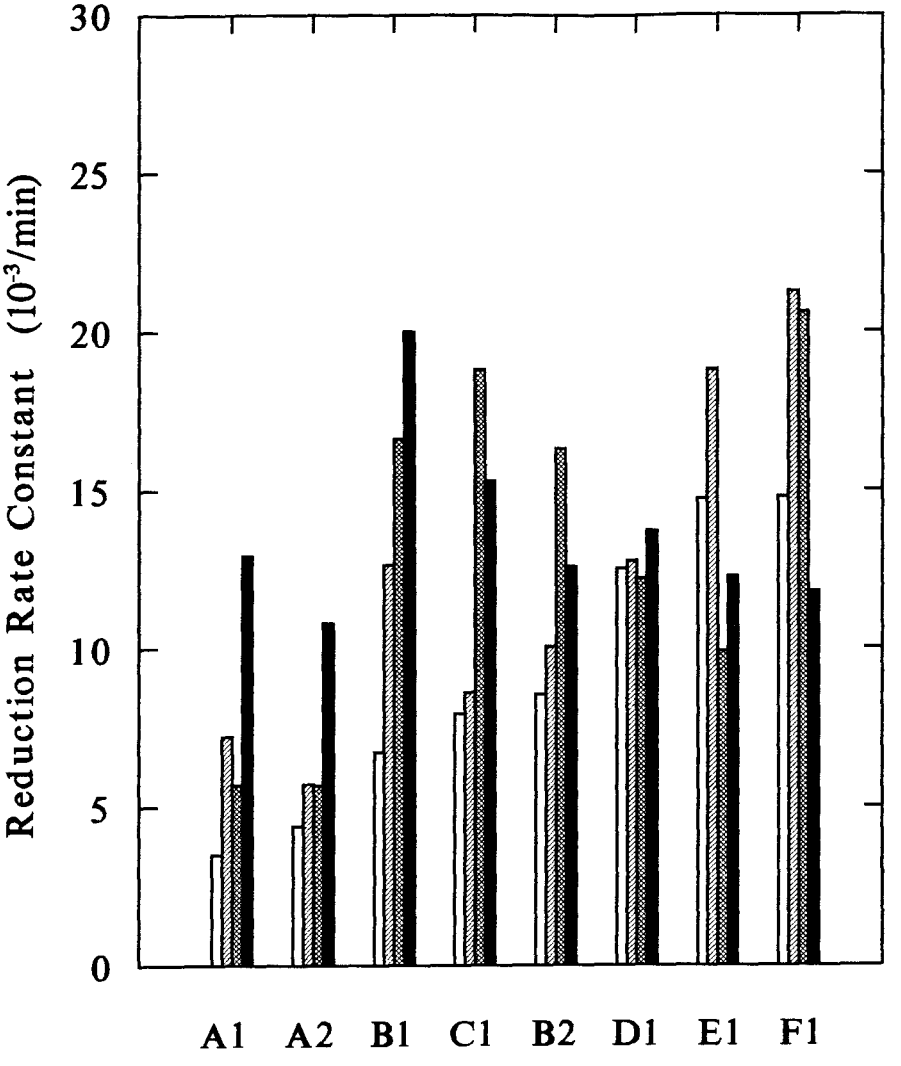
Figure 26 **Average cell long diameters for unseparated normal and sickle RBC and separated sickle RBC.** 1. unseparated normal cells; 2. unseparated sickle cells; 3. light sickle cells (d < 1.120 g/ml); 4. medium sickle cells (d = 1.120 ~ 1.135 g/ml); 5. dense sickle cells (d > 1.135 g/ml) cells. Data were from seven sets of samples.


14
12
10
8
6
4
2
0
Average Long Diameter (μm)
1
2
3
4
5
SAMPLE

Reduction Rates of Density Separated Sickle RBC

The spin label reduction rates for light, medium, and dense cells from 6 patients (8 samples) are shown in Figure 27. No clear patterns were observed for the reduction rates of these light, medium, and dense cells. For the two patients with repeat runs, patient A showed reproducibility in rate constant measurements and patient B showed large variations within a set of dense cells. For all 8 samples, the unseparated cells had lower rate constants than the density separated cells. The rate constants of the light cells were more similar to those of the unseparated cells. The rate constants of the dense cells were often higher than those of the light cells (Samples A1, A2, B1, B2, C1, and D1).

Figure 27 **Spin label reduction rate constants of unseparated and density separated sickle RBC.** Unseparated sickle cells (□); Light sickle cells (d < 1.120 g/ml) (▨); Medium sickle cells (d = 1.120 ~ 1.135 g/ml) (▩); Dense sickle cells (d > 1.135 g/ml) cells (■). The different letter labels represent different sickle patients, and the number represents the times of sample run from the same patient.


Reduction Rate Constant (10-3/min)
30
25
20
15
10
5
0
A1
A2
B1
C1
B2
D1
E1
F1

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VITA

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

This dissertation is therefore accepted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

Feb. 11, 1992
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