

## Chapter 1

### Introduction

#### 1.1 Redox in biology

Reactions involving oxidation and reduction (redox) are a fundamental aspect of life. These reactions allow plants to photosynthesise, turning light into energy, and enable all organisms to transduce and use energy for the daily processes of living. There are a myriad of redox reactions involving a huge variety of substrates, but the main consequence of all these reactions is the oxidation of one species and the reduction of another. In the work for this thesis I focused on reactions occurring in the plasma membrane of cells, and more specifically redox systems that operate across the plasma membrane of the erythrocyte (RBC).

##### 1.1.1 Redox nomenclature

In order to discuss plasma membrane redox reactions an introduction to basic redox nomenclature is appropriate: Oxidation is the loss of electrons by a reactant and reduction is the gain of electrons by a reactant. In all redox reactions each compound can exist in a reduced ( $X^-$  or  $XH$ ) or an oxidised ( $X$ ) form; this is also known as a redox couple ( $X/X^-$ ), whose relationship is expressed in a redox half reaction (see Table 1.1). The electron transfer potential or reduction potential  $E_o'$  of a redox couple is a defining feature for a redox compound. It can be determined by measuring the electromotive force generated by a sample half-cell ( $1M X$ ,  $1M X^-$ ) connected to a standard reference half-cell ( $10^{-7} M H^+$ ,  $1 atm H_{2(g)}$ ), which has a reduction potential defined at 0 V at pH 7 and 25 °C for biochemical systems. If a redox couple has a negative potential relative to the reference then the substance has a lower affinity for electrons than  $H_{2(g)}$  and is on average a reducing agent. The

standard reduction potentials for a number of biologically significant redox compounds are shown in Table 1.1. Reactants at the top left of this table are strong oxidising agents; they readily accept electrons and have a positive reduction potential. Conversely reactants near the bottom right are strong reducing agents, they readily donate electrons and have a negative reduction potential.

**Table 1.1: Standard redox potentials (pH 7, 25 °C) and half reactions for molecules involved in membrane and cellular redox reactions.** Abbreviations are defined in the text and the abbreviations list.

Redox half reaction			
Oxidised form		Reduced form	$E_o'$ (mV)
$\frac{1}{2} O_2 + 2H^+ + 2e^-$	$\rightleftharpoons$	$H_2O$	815
$\alpha$ -tocopheroxyl $^{\cdot} + e^- + H^+$	$\rightleftharpoons$	$\alpha$ -tocopherol	500
$Fe(CN)_6^{3-}$ (ferricyanide) + $e^-$	$\rightleftharpoons$	$Fe(CN)_6^{4-}$ (ferrocyanide)	360
$O_2$ (g) + $2H^+ + 2e^-$	$\rightleftharpoons$	$H_2O_2$	295
AFR $^{\cdot} + H^+ + e^-$	$\rightleftharpoons$	Ascorbate	282
Cytochrome <i>c</i> ( $Fe^{3+}$ ) + $e^-$	$\rightleftharpoons$	Cytochrome <i>c</i> ( $Fe^{2+}$ )	235
DCIP (oxidised) + $e^-$	$\rightleftharpoons$	DCIPH $_2$ (reduced)	220
DHA + $2H^+ + 2e^-$	$\rightleftharpoons$	Ascorbate	60
Ubiquinone + $2H^+ + 2e^-$	$\rightleftharpoons$	$H_2O$ + ubiquinol	45
FAD + $2H^+ + 2e^-$	$\rightleftharpoons$	FADH $_2$ (in flavoprotein)	~ 0
Oxaloacetate $^- + 2H^+ + 2e^-$	$\rightleftharpoons$	Malate $^-$	-166
DHA + $H^+ + e^-$	$\rightleftharpoons$	AFR	-174
Pyruvate $^- + 2H^+ + 2e^-$	$\rightleftharpoons$	Lactate $^-$	-185
FAD + $2H^+ + 2e^-$	$\rightleftharpoons$	FADH $_2$ (free coenzyme)	-219
$\frac{1}{2}$ GSSG + $e^-$	$\rightleftharpoons$	GSH	-230
NAD $^+ + H^+ + 2e^-$	$\rightleftharpoons$	NADH	-320

Redox reactions, like any chemical reaction, are describable by the laws of thermodynamics; hence a redox reaction will only occur in a net sense if the free energy  $G$  of the system decreases. For a redox reaction the standard free energy change (pH 7, 25 °C)  $\Delta G^{o'}$  is related to the change in the standard reduction potential  $\Delta E_o'$  by

$$\Delta G^{o'} = -nF\Delta E_o'$$

where  $n$  is the number of electrons transferred,  $F$  is a proportionality constant called the Faraday constant ( $96.5 \text{ kJ V}^{-1} \text{ mol}^{-1}$ ) and  $\Delta E_o'$  is in volts.

Favourable redox potentials and free energies do not guarantee a redox reaction will occur *in vivo*. In biological systems the location of each reactant, i.e., their accessibility to other reactants and their local concentrations may play a significant role in determining the position of the (pseudo)equilibrium of the redox reaction. Similarly, the likelihood of reaction may also depend on whether an enzyme is involved. So the redox potential can only give a guide as to whether a redox reaction is likely to occur between pairs of substances and does not define whether it will occur or be biologically significant.

There are numerous enzymes involved in redox reactions in the cell cytosol and in the various membranes found inside and around it. Although all these enzymes perform the same fundamental roles, that is of catalysing a redox reaction, for historical and other reasons relating to their specific action a variety of names exist for these types of enzymes including: reductases, oxidoreductases, dehydrogenases, diaphorases and oxidases.

### **1.1.2 The redox status of the cell**

The plasma membrane separates the intracellular reducing environment from the extracellular oxidising one (Berridge and Tan, 2000b). Hence the plasma membrane and its constituent lipids and proteins are exposed to two very different environments, leading to a delicate balance of redox states. Compounds which disrupt the normal cellular balance, and require a response to return to homeostasis, are said to place stress on the system. Oxidants can perturb extracellular, cell membrane and intracellular redox states. This *oxidative stress* can lead to oxidation of reduced groups on membrane and cellular proteins, lipid peroxidation, and flow-on changes to intracellular metabolism.

The majority of membrane and intracellular proteins are maintained in a reduced state; making protection against the extracellular oxidising environment

advantageous for functionality (Berridge and Tan, 2000b). The intracellular redox status is maintained by a redox buffering system that can also assist in the control of extracellular oxidation (Kishi, *et al.*, 1999; Berridge and Tan, 2000b). This system consists of a combination of small molecules: the pyridine dinucleotides NAD(P)(H); the vitamins ascorbic acid (vitamin C; ascorbate) and  $\alpha$ -tocopherol (vitamin E); thiol containing compounds like glutathione; and antioxidant enzymes including glutathione peroxidase (GSHPx), catalase, superoxide dismutase (SOD) and membrane oxidoreductase complexes (Sandström and Marklund, 1990; May, *et al.*, 1996b; May and Qu, 1999; Washburn and Wells, 1999). The components of the cellular antioxidant system work synergistically; they are recycled and protected from stress by other members of the antioxidant family enabling the cell to constantly defend the functional and structural integrity of nucleic acids, proteins and phospholipids against oxidative stress (Chaudiere and Ferrari-Iliou, 1999).

Many compounds, both endogenous and those introduced to the cellular system, are capable of imposing oxidative stress and thus eliciting a homeostatic response in the cell. Reactive oxygen species (ROS) such as superoxide, hydroxyl radicals and peroxides, which are produced as by-products of basal metabolism, are known to induce oxidative stress that can lead to a number of alterations in the cell. Functional and morphological changes similar to those occurring in apoptosis, such as membrane blebbing, have been observed in endothelial cell membranes after exposure to peroxides (Van Gorp, *et al.*, 1999). Hydrogen peroxide is also known to trigger apoptosis in neutrophils (Aoshiba, *et al.*, 1999) suggesting a functional link between the effects of oxidative stress and apoptosis. Peroxides such as *tert*-butylhydroperoxide (tBHP) can cause changes in RBC membranes due to lipid peroxidation (Zavodnik, *et al.*, 1998; Ferreira, *et al.*, 1999), spectrin and ankyrin degradation and detachment of actin from the spectrin network, altering membrane fluidity (Zavodnik, *et al.*, 1998); and tBHP can increase ion permeability, making cells more susceptible to osmotically-induced shape changes (Spickett, *et al.*, 1998). Oxidative stress is also manifest in increased oxidation of protein thiol-groups (Snyder, *et al.*, 1988), proteolysis (Davies and Goldberg, 1987), and depletion of ATP and other adenine nucleotides resulting in an efflux of inosine, hypoxanthine, uric acid and xanthine from RBCs (Tavazzi, *et al.*, 2000).

Oxidative stress can increase flux through the human RBC oxidative pentose phosphate pathway (oPPP) by between 80 and 100% (Thorburn and Kuchel, 1985). The increased activity of the enzymes associated with the oPPP, such as glutathione S-transferase (GST), could therefore be used as plausible indicators of oxidative stress (Neefjes, *et al.*, 1999). Additionally, AMP deaminase in RBCs is activated by oxygen radicals leading to impairment of energy metabolism (Tavazzi, *et al.*, 2001). Oxidative stress can also activate and repress gene transcription in nucleated cell lines, while the antioxidant enzymes GSHPx and catalase prevent hydrogen peroxide-induced DNA single strand breakage (Sandström and Marklund, 1990), indicating that the response to ROS and oxidative stress occurs at all levels within the cell (Morel and Barouki, 1999).

The antioxidant defence system of some cells is stimulated by oxidative stress. Dierickx and co-workers (1999) showed that when rat hepatoma-derived Fa32 cells were exposed to peroxides generated by ozone therapy, oxidative stress was initially observed, but over the succeeding 24 hours concentrations of reduced glutathione (GSH) and GST phase II activity increased above original levels. The production of oxidants can also be turned to the advantage of the cell; neutrophils use oxidants as a cell surface defence mechanism against pathogens (del Castillo-Olivares, *et al.*, 2000).

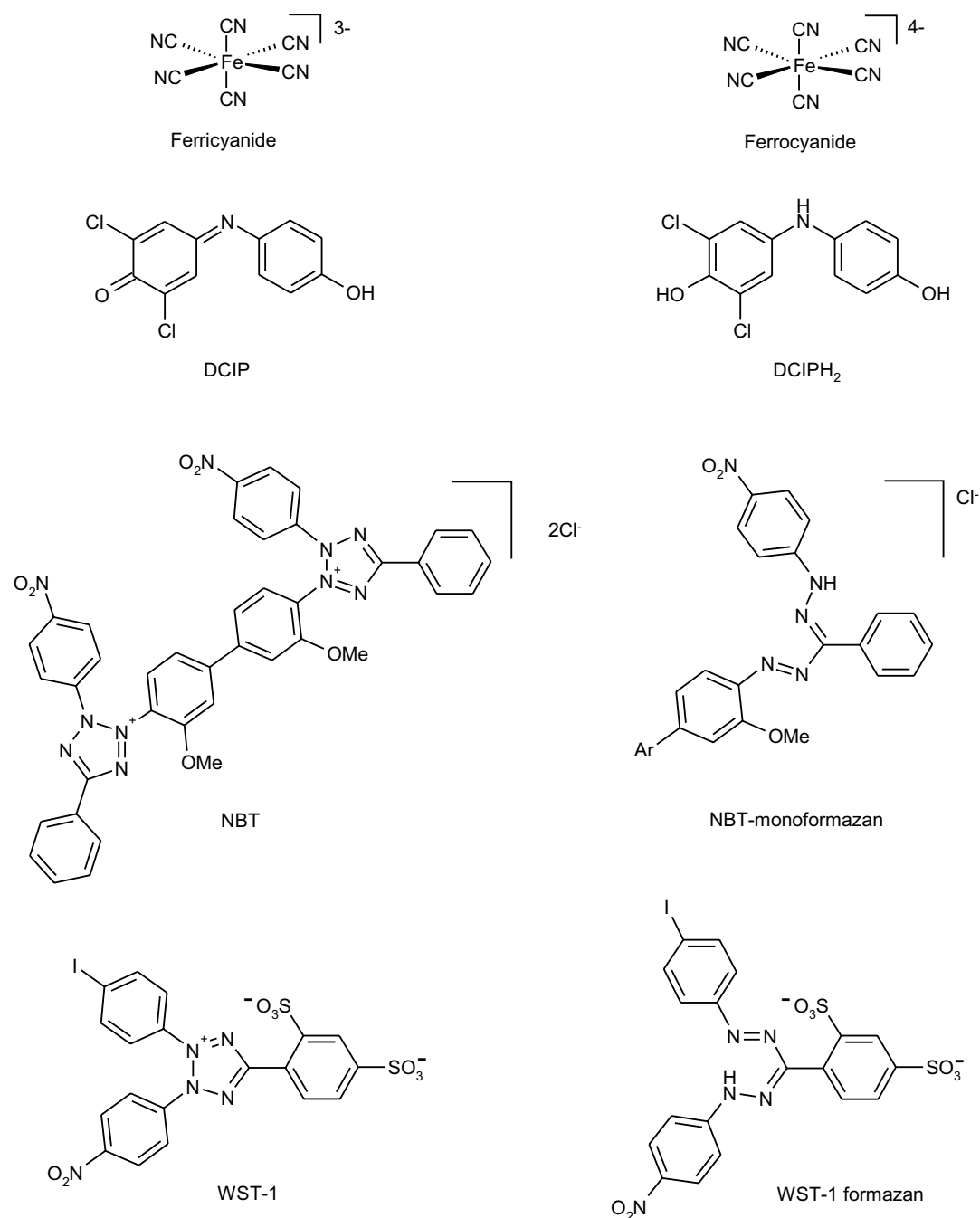
Understanding and controlling the effects of oxidative stress is not only important in the healthy individual, increased susceptibility to oxidative stress is apparent in a number of disease states. Diabetes mellitus patients are more susceptible than normal individuals to oxidative stress, possibly contributing to the vascular complications often observed as a secondary feature of the disease (Augustyniak, *et al.*, 1996). Preeclamptic toxemia, a common potentially fatal affliction of the third trimester of pregnancy, is thought to be caused by an antioxidant deficiency (Spickett, *et al.*, 1998). Additionally, some inflammatory diseases induce increased oxidant production by activated polymorphonuclear neutrophils, resulting in the acceleration of RBC aging (Lucchi, *et al.*, 2000).

While it is evident that cells have a wide array of intracellular antioxidative reactions in their arsenal for dealing with intracellular oxidative stress, they also neutralise attacks from the extracellular medium to preserve plasma membrane and intracellular redox homeostasis. Plasma membrane oxidoreductases (PMORs), that allow the reduction of extracellular oxidants by the reducing potential from intracellular reductants, provide the cell with an added layer of defence against oxidative stress. These systems are now discussed, from their discovery to current knowledge of their composition and mechanisms of action (see also Kennett and Kuchel, 2003).

## **1.2 Plasma membrane redox systems**

### **1.2.1 General introduction**

In 1925 Voegtlin and colleagues, through their investigation of the relation of redox states to cancer, provided the initial evidence for membrane redox activity. However, it was not until the 1960s, with the discovery that cells in isolation reduce extracellular cell-impermeable oxidants, such as ferricyanide (Dormandy and Zarday, 1965), that membrane-associated redox activity became a separate field of study. Over subsequent years, evidence for transmembrane electron transport, enabling the reduction of a variety of extracellular oxidants, was obtained from RBCs (Zamudio and Canessa, 1966; Mishra and Passow, 1969; Zamudio, *et al.*, 1969; Adachi, 1972; Wang and Alaupovic, 1978; Agutter, *et al.*, 1980) and mouse liver plasma membranes (Goldenberg, *et al.*, 1978, 1979). Since this time, PMORs have been identified in membranes from all plant and animal cell lines investigated (Baker and Lawen, 2000) and some membrane proteins involved in electron transport have been isolated and purified from a number of cell membranes (Wang and Alaupovic, 1978; Kitajima, *et al.*, 1981; Nisimoto, *et al.*, 1986; Zurbriggen and Dreyer, 1994; Villalba, *et al.*, 1995; del Castillo-Olivares, *et al.*, 1996; Bulliard, *et al.*, 1997; Kim, *et al.*, 2002; Baker, *et al.*, 2004a, 2004b).



**Figure 1.1: Non-physiological electron acceptors (and their reduced partners on right) for transplasma membrane redox systems.** Abbreviations: DCIP, 2,6-dichlorophenolindophenol; NBT, nitroblue tetrazolium; WST-1, water soluble tetrazolium-1 (Kennett and Kuchel, 2003).

The majority of PMORs have been studied using non-physiological electron acceptors (Figure 1.1); physiological acceptors are still largely unknown and may vary between cells (Baker and Lawen, 2000). Ferricyanide (Dormandy and Zarday, 1965; Mishra and Passow, 1969; Agutter, *et al.*, 1980; Crane, *et al.*, 1982; Grebing,

*et al.*, 1984; Papandreou and Rakitzis, 1989; Marques, *et al.*, 1995; Himmelreich and Kuchel, 1997; Fiorani, *et al.*, 2002; Baker, *et al.*, 2004b), 2,6-dichlorophenol-indophenol (DCIP) (Zamudio and Canessa, 1966; Dancey and Shapiro, 1976; Bulliard, *et al.*, 1997; Lenaz, *et al.*, 2002), nitroblue tetrazolium (NBT) (Zamudio, *et al.*, 1969; Mittler and Zilinskas, 1993; May, *et al.*, 1995b; Debnam and Shearer, 1997; Demehin, *et al.*, 2001) and water soluble tetrazolium-1 (WST-1) (Berridge and Tan, 1998; Tan and Berridge, 2000a, 2000b), can all accept electrons via transmembrane electron transport.

Some compounds which are present in biological systems have been shown to be electron acceptors *in vitro*. These include oxidised ascorbate derivatives (Navas, *et al.*, 1988), ubiquinone (Villalba, *et al.*, 1995), and lipid hydroperoxides (Agutter, *et al.*, 1980), but whether they are acceptors *in vivo* is yet to be determined. Oxygen can act as an electron acceptor for transmembrane redox activity in some, but not all, cell lines and may be a physiological electron acceptor (Baker and Lawen, 2000). Non-physiological electron acceptors are chosen for their experimental utility, based on their redox capability and the measurability of their redox states. It may seem surprising that such a variety of structurally diverse compounds can elicit a redox response from the cell. However, the variety of oxidants to which a cell can be exposed also suggests that a flexible oxidant reducing system, with a non-specific or indirect mechanism of action, is required. Information on how cells respond to oxidative stress, both at the substrate and whole cell metabolic level, has been gained from studying electron transport systems with these non-physiological acceptors. Some of the greatest challenges ahead lie in establishing the true physiological acceptors for these systems and under what *in vivo* conditions these systems are active.

### **1.2.2 Evidence for transplasma membrane electron transport**

Direct evidence for plasma membrane electron transport is hard to obtain, but sufficient evidence has been gathered by a range of techniques to establish that plasma membrane electron transport does occur. Nuclear magnetic resonance

(NMR) spectroscopy enables real-time, non-invasive, and simultaneous investigation of extra- and intracellular events, hence providing direct insight into the metabolic effects of extracellular oxidative stress and the reduction of extracellular oxidants (Ciriolo, *et al.*, 1993; Himmelreich and Kuchel, 1997; Himmelreich, *et al.*, 1998). Membrane potential measurements (Van Duijn, *et al.*, 2001b) and observations of free radicals with electron paramagnetic resonance spectroscopy (EPR) (May, *et al.*, 2000a, 2000b; Van Duijn, *et al.*, 2000) also provide strong evidence for electron transfer. These techniques coupled to biochemical assays of redox reactions and metabolites have enabled the elucidation of membrane electron-transfer events.

**Table 1.2: Electron transport activities in open isolated membranes (NADH is the electron donor; number of experiments if known is given in brackets).**

Membrane source	Electron acceptor	Activity (nmol reduced / min / mg protein)	Temp (°C)	Reference
Human RBC	Ferricyanide	568 ± 46 (24)	25	(Zamudio and Canessa, 1966)
Human RBC	Ferricyanide	457	37	(Zamudio, <i>et al.</i> , 1969)
Human RBC	Ferricyanide	241 ± 33 (4)	37	(Miner, <i>et al.</i> , 1983)
Human RBC	Ferricyanide	455 ± 32 (5)	37	(Crane, <i>et al.</i> , 1982)
Pig RBC	Ferricyanide	250	37	(Crane, <i>et al.</i> , 1982)
Guinea Pig RBC	Ferricyanide	76 ± 5 (4)	37	(Miner, <i>et al.</i> , 1983)
Rabbit RBC	Ferricyanide	80 ± 12 (4)	37	(Miner, <i>et al.</i> , 1983)
Sheep RBC	Ferricyanide	132 ± 27 (4)	37	(Miner, <i>et al.</i> , 1983)
Cat RBC	Ferricyanide	489 ± 45 (4)	37	(Miner, <i>et al.</i> , 1983)
Dog RBC	Ferricyanide	15 ± 2 (4)	37	(Miner, <i>et al.</i> , 1983)
Mouse liver	Ferricyanide	600	25	(Goldenberg, <i>et al.</i> , 1979)
Human RBC	Cytochrome <i>c</i>	13 ± 3 (5)	37	(Crane, <i>et al.</i> , 1982)
Human RBC	Cytochrome <i>c</i>	7.9 ± 0.72 (13)	25	(Zamudio and Canessa, 1966)
Mouse liver	Cytochrome <i>c</i>	70	25	(Goldenberg, <i>et al.</i> , 1979)
Human RBC	Indophenol	37 ± 8 (4)	37	(Crane, <i>et al.</i> , 1982)
Human RBC	DCIP	17.5 (2)	25	(Zamudio and Canessa, 1966)
Mouse liver	DCIP	40	25	(Goldenberg, <i>et al.</i> , 1979)
Mouse liver	Oxygen	60	25	(Goldenberg, <i>et al.</i> , 1979)

The localisation of redox activity to the plasma membrane was assisted by the discovery of NADH dehydrogenase activity in isolated RBC membranes (ghosts)

(Zamudio and Canessa, 1966). The NADH:(acceptor) oxidoreductase identified was capable of reducing ferricyanide, cytochrome *c*, and DCIP with specific activities of 568, 7.9 and 17.5 IU/mg protein, respectively (Table 1.2). The enzyme could not be released by membrane lysis or ethylenediaminetetraacetic acid (EDTA) treatment and so was thought to be transmembranous. Subsequent histochemical and membrane permeability results provided an argument for an endofacial protein and reactive site (Zamudio, *et al.*, 1969). However, the purification of a 40 kDa glycoprotein with NADH:(acceptor) oxidoreductase activity from human RBC membranes suggested transmembranous redox activity with catalytic sites on either side of the membrane (Wang and Alaupovic, 1978).

Manipulation of RBC membranes produces a variety of surface topologies: open ghosts (sheets), sealed inside-out vesicles, and right-side-out (resealed) vesicles (Steck and Kant, 1974). Using open and closed-oriented ghosts the orientation of NADH:ferricyanide oxidoreductase activity was clarified (Crane, *et al.*, 1982; Grebing, *et al.*, 1984). Maximal ferricyanide reductase activity was observed when assayed in open sheets. This result suggested that catalytic sites were present on both sides of the membrane, and consequently, provided evidence for transmembrane activity (Löw and Crane, 1995). Incubation of whole cells with diazobenzene sulfonate (DABS, which binds to exposed exofacial proteins) prior to membrane isolation, produced open ghosts with 35% less ferricyanide reductase activity than untreated controls, indicating that at least some of the observed activity is transmembranous (Grebing, *et al.*, 1984). All membrane orientations have been shown to exhibit ferricyanide reductase activity; high affinity sites for NADH,  $K_m = 90 \mu\text{M}$ , and ferricyanide,  $K_m = 125 \mu\text{M}$  (Crane, *et al.*, 1982; Grebing, *et al.*, 1984) are associated with transmembrane activity, while low affinity sites for NADH and ferricyanide, found on each face of the membrane, and an additional high affinity NADH site situated on the inside face may represent endo- and exofacial activities. The high affinity endofacial activity, attributed to NADH:cytochrome *b*<sub>5</sub> (cyt *b*<sub>5</sub>) reductase, may contribute to the 65% uninhibited activity observed with DABS (Crane, *et al.*, 1982; Grebing, *et al.*, 1984).

Within the field of PMORs, there has been much conjecture as to the mechanism of transmembrane reduction. Orringer and Roer (1979) observed that increasing RBC ascorbate concentrations led to increased rates of extracellular ferricyanide reduction. They concluded that ascorbate efflux from the cell and non-enzymic reaction of ascorbate with ferricyanide explained the observed reduction, rather than transmembrane electron transport. The ferricyanide reduction was saturable for dehydroascorbate (DHA) and appeared to need NADH formed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to reduce intracellular DHA to ascorbate. Investigations into the rate of ascorbate efflux and reaction with ferricyanide (Schipfer, *et al.*, 1985; Himmelreich, *et al.*, 1998) refuted these claims and an increasing body of evidence shows that RBC plasma membranes do contain transmembrane electron transport activity.

It was also suggested that redox activity in isolated plasma membranes was associated with contamination of the membrane preparation by sub-cellular membranes (Löw and Crane, 1978). Plasma membranes, endoplasmic reticulum (ER), Golgi and secretory vesicles appear to have a common origin, but differences in cholesterol content and enzymes such as glucose-6-phosphatase suggest that differentiation occurs during their formation (Löw and Crane, 1978). Sub-cellular membranes such as the mitochondrial and ER membranes are known to contain redox systems. Some ER redox systems are involved in fatty acid saturation (NADH:cyt *b*<sub>5</sub> reductase), desaturation and elongation. Others perform ROS detoxification roles (NADPH oxidation) (Jansson and Schenkman, 1977). Goldenberg and co-workers (1979) showed that an NADH-oxidising system, specific to the plasma membrane, exists in mouse liver. The activity is inhibited by atebtrin (quinacrine), insulin, and *para*-chloromercuribenzenesulfate (pCMBS) and stimulated by glucagon. ER and outer-mitochondrial NADH-oxidising systems are not inhibited and in some cases they are stimulated by atebtrin, inhibited to a greater extent by pCMBS and are unresponsive to glucagon. A differential sensitivity to azide (0.1 M) was found for ferricyanide reductase activities, with inhibition of 73% in plasma membrane, 10% in Golgi, 7% in the outer-mitochondrial membrane and no inhibition of ER activity. Plasma membrane ferricyanide reductase activity can also be found in isolated membranes cytochemically using Hatchett's 'brown' method

(Goldenberg, *et al.*, 1979). Similarities exist between PMORs and the well-characterised mitochondrial electron transport chain, reduction of extracellular oxidants is concomitant with proton efflux (Dormandy and Zarday, 1965; Medina, *et al.*, 1988; Sun, *et al.*, 1992). Oxygen consumption, however, does not increase and activity is not affected by the mitochondrial electron transport inhibitor cyanide and the uncoupler of oxidative phosphorylation dinitrophenol, although ferricyanide-induced ATP formation is affected (Mishra and Passow, 1969).

A number of different electron transport processes are present in cell membranes. These processes appear to be sensitive to their immediate ionic environment and cellular redox state as large variations in oxidoreductase activities have been reported (Table 1.2). This may reflect variations in donor source and therefore redox capacity. Differences for systems from the same cell type and species may also come from inadequate accounting for endofacial membrane redox activities such as NADH:cytochrome *c* oxidoreductase and NADH:cyt *b*<sub>5</sub> reductase (in RBCs related to NADH:methaemoglobin reductase (NADH-MR) (Choury, *et al.*, 1981), see Chapter 5). The different conditions used to obtain membranes and perform the redox assays may also be reflected in the disparity of the results.

### **1.2.3 NADH:(acceptor) oxidoreductases**

PMORs are generally named for the putative electron donor followed by the electron acceptor added to the system. Unfortunately this can lead to some confusion as in isolated plasma membranes the donor is added to the system and is hence known, but in whole cell systems must be inferred from consequent metabolic changes. It is also difficult to distinguish whether differences in activity with different acceptors is the result of these acceptors having different affinity for the PMOR or the product of the presence of multiple types of PMOR (Table 1.2). Although various activities have been separated in the following sections, this does not necessarily mean (except where clearly stated) that these activities are associated with different membrane protein complexes.

### 1.2.3.1 NADH:ferricyanide oxidoreductase

Ferricyanide (Figure 1.1) has commonly been used to study PMORs as it, and its reduced form ferrocyanide, is membrane impermeable, so any observed reduction is extracellular. Ferricyanide reduction is readily measured spectrophotometrically either directly by light absorption at 420 nm ( $\epsilon = 1 \text{ mM}^{-1}\text{cm}^{-1}$ ) or through conjugated assay systems with phenanthroline derivatives, increasing the extinction coefficient to  $\sim 20 \text{ mM}^{-1}\text{cm}^{-1}$  and hence giving higher sensitivity to detection (Avron and Shavit, 1963).

The timecourse of the NADH:ferricyanide oxidoreductase reaction is biphasic in RBCs (Schipfer, *et al.*, 1985) and Ehrlich ascites cells (Medina and Nunez de Castro, 1994), with a poorly reproducible fast phase, which can be removed by diluting the cell suspension, followed by a slower linear phase. Navas and co-workers (1986) showed that HeLa cell reduction of extracellular ferricyanide and diferric transferrin produced a decrease in intracellular NADH and a concomitant increase in intracellular  $\text{NAD}^+$ . They concluded that NADH is the electron donor for plasma membrane electron transport to ferricyanide.

The effects of NADH:ferricyanide oxidoreductase activity on RBC metabolism in near *in vivo* conditions can be monitored by using  $^{13}\text{C}$ ,  $^1\text{H}$  and  $^{31}\text{P}$  NMR (Himmelreich and Kuchel, 1997). During ferricyanide reduction, the ratio of reduced to oxidised glutathione (GSH-to-GSSG) remains stable. Himmelreich and Kuchel (1997) interpreted this to mean that reducing equivalents did not derive from NADPH or the oPPP, a view supported by a lack of NADPH:ferricyanide oxidoreductase activity identified in RBC membranes (Crane, *et al.*, 1982). May and co-workers (1996b) also found that GSH concentrations do not change, but attributed this to GSH recycling as  $\text{CO}_2$  production through the oPPP was increased 4-fold above controls when cells were treated with 1 mM ferricyanide, an effect doubled by the presence of DHA.

NADH does not appear to be the sole source of electrons for ferricyanide reduction. Himmelreich and Kuchel (1997) observed a minimal decrease in lactate concentration and a 10% increase in pyruvate above controls; this is in agreement

with other investigators who obtained minimal variation in NADH concentrations in glucose-supplied RBCs exposed to ferricyanide (May, *et al.*, 1996b). Glucose-starved cells exposed to ferricyanide exhibited a reversal of the NAD<sup>+</sup>-to-NADH ratio suggesting that although NADH may not be the major electron donor it is necessary, especially under starvation conditions, for coping with oxidative stress.

Ferricyanide reductase activity in RBCs has been observed to vary between donors, and relates to the intracellular ascorbate concentration (Himmelreich and Kuchel, 1997). Ascorbate supplementation increases a donor's ferricyanide reductase capacity, clear evidence that ascorbate plays a role in the electron transport system (Himmelreich and Kuchel, 1997). Ferricyanide reductase activity also varies between cell lines. Reduction of ferricyanide by pulmonary arterial endothelial cells is slow compared to other cell lines and other electron acceptors (Merker, *et al.*, 1998), but increasing the intracellular ascorbate concentration, by incubation with DHA, also leads to the stimulation of ferricyanide reductase activity. The accumulation of evidence for the involvement of ascorbate in ferricyanide reduction has led to the suggestion that it might be the major electron donor for trans-membrane ferricyanide reduction (May, *et al.*, 1995b). Hence, the nomenclature "NADH:ferricyanide reductase" is somewhat misleading. The effects of ascorbate and its role in PMORs are covered in more detail in §1.2.4.

Various inhibitors of ferricyanide and other NADH:(acceptor) oxidoreductases have been identified. The effects of which are summarised in Table 1.3 and are covered in more detail in §1.2.5 (chemical structures are shown in Figure 1.5 within this section).

### **1.2.3.2 NADH:DCIP oxidoreductase**

Many studies have used multiple electron acceptors to explore membrane redox activity and most systems appear to be able to reduce ferricyanide, DCIP, and cytochrome *c*, though all to different extents. DCIP reductase activity has often been compared to ferricyanide reductase activity. In most cases DCIP reductase activity is less than that of ferricyanide (Table 1.2). This may indicate separate systems for

reduction or may simply reflect the affinity of the oxidoreductase for the two different donors.

**Table 1.3: Effects of inhibitors on PMOR activity in isolated membranes.**

Membrane source	Electron acceptor	Inhibitor	% inhibition	reference
Mouse liver	O <sub>2</sub>	7 mM atebtrin	~60	(Goldenberg, <i>et al.</i> , 1979)
Mouse liver	DCIP	7 mM atebtrin	~60	(Goldenberg, <i>et al.</i> , 1979)
Human Jurkat <sup>a</sup>	WST-1	200 µM capsaicin	81 ± 6	(Berridge and Tan, 1998)
Rat liver	O <sub>2</sub>	200 µM capsaicin	50 ± 4	(Vaillant, <i>et al.</i> , 1996)
Human Jurkat <sup>a</sup>	WST-1	200 µM DHC <sup>b</sup>	93 ± 6	(Berridge and Tan, 1998)
Rat liver	O <sub>2</sub>	200 µM DHC <sup>b</sup>	80 ± 6	(Vaillant, <i>et al.</i> , 1996)
Purified from RBC	Ferricyanide	1 mM DTNB <sup>c</sup>	97	(Wang and Alaupovic, 1978)
Human RBC	Ferricyanide	30-70 µU/mL insulin	23.8 ± 8.5	(Crane, <i>et al.</i> , 1982)
Bovine endothelial <sup>a</sup>	TBOP <sup>d</sup>	0.4 mM iodoacetate	~50	(Merker, <i>et al.</i> , 2002)
Human RBC <sup>a</sup>	Ferricyanide	1 mM iodoacetate	~50	(Marques, <i>et al.</i> , 1995)
Human RBC <sup>a</sup>	Ferricyanide	1 mM iodoacetate	100	(Schipfer, <i>et al.</i> , 1985)
Human RBC <sup>a</sup>	Ferricyanide	10 mM iodoacetate	90	(Himmelreich and Kuchel, 1997)
Human Jurkat <sup>a</sup>	WST1	5 mM iodoacetate	82	(Berridge and Tan, 2000b)
Human RBC	Ferricyanide	10 µM pCMB <sup>e</sup>	100	(Zamudio and Canessa, 1966)
Purified from RBC	Ferricyanide	1 mM pCMB	100	(Wang and Alaupovic, 1978)
Mouse liver	O <sub>2</sub>	80 µM pCMB	~80	(Goldenberg, <i>et al.</i> , 1979)
Mouse liver	DCIP	80 µM pCMB	~90	(Goldenberg, <i>et al.</i> , 1979)
Human Jurkat <sup>a</sup>	WST-1	25 µM pCMBS	18 ± 9	(Berridge and Tan, 1998)
Rat liver	O <sub>2</sub>	20 µM pCMBS	0	(Vaillant, <i>et al.</i> , 1996)
Rat liver	Ferricyanide	20 µM pCMBS	94 ± 1	(Vaillant, <i>et al.</i> , 1996)
Namalwa p <sup>+</sup> cells <sup>a</sup>	Ferricyanide	20 µM pCMBS	88 ± 5	(Vaillant, <i>et al.</i> , 1996)
Human Jurkat <sup>a</sup>	WST-1	32 µM resiniferatoxin	90 ± 5	(Berridge and Tan, 1998)
Rat liver	O <sub>2</sub>	32 µM resiniferatoxin	98 ± 2	(Vaillant, <i>et al.</i> , 1996)
Human Jurkat <sup>a</sup>	WST-1	20 µg/mL SOD	80 ± 6	(Berridge and Tan, 1998)

<sup>a</sup> Whole cells

<sup>d</sup> TBOP denotes toluidine blue O polyacrylamide

<sup>b</sup> DHC denotes dihydrocapsaicin

<sup>e</sup> pCMB denotes *para*-chloromercuribenzoate

<sup>c</sup> DTNB denotes 2,2'-dinitro-5,5'-dithiodibenzoic acid

Isolated lymphocyte membranes contain an NADH:DCIP oxidoreductase activity, which shows high affinity for NADH (1  $\mu$ M) and has higher activity in lymphocyte membranes from diabetic individuals than non-diabetic controls (Lenaz, *et al.*, 2002). The low  $K_m$  value for NADH suggests that the enzyme is saturated with NADH under normal conditions, suggesting that increased activity in membranes from diabetics is due to increased expression of the enzyme and therefore increased copy number in the membranes of these patients (Lenaz, *et al.*, 2002).

DCIP reductase activity has also been investigated in isolated mouse liver (Goldenberg, *et al.*, 1978) and bovine RBC membranes (Adachi, 1972). The mouse activity is hormone sensitive, stimulated by 50 nM glucagon and inhibited up to 90% by insulin at physiological concentrations (8  $\mu$ U/mL). At higher concentrations of both hormones the effects were reversed (Goldenberg, *et al.*, 1978). Bovine RBC membranes appear to have two DCIP reductases, each with differing sensitivities to NADH and NADPH, but an almost identical capacity to reduce DCIP (Adachi, 1972).

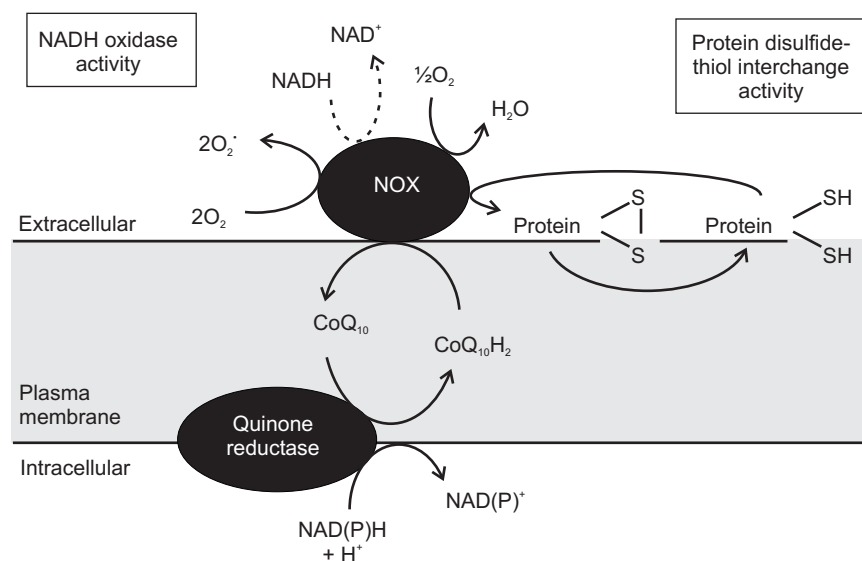
### 1.2.3.3 NAD(P)H oxidases

NADH oxidase activity accounts for less than 10% of the residual oxygen consumption in Namalwa cells treated with cyanide and azide by  $\rho^0$  cells (Larm, *et al.*, 1995). It couples the oxidation of NADH with the reduction of molecular oxygen (Vaillant, *et al.*, 1996). It has generally been studied in isolated membranes by measuring rates of NADH oxidation or oxygen consumption, but more recently it has been investigated indirectly in whole cells by measuring WST-1 reduction (Berridge and Tan, 1998). NADH is unlikely to be the physiological electron donor for cell surface NADH oxidase as it is not present at significant concentrations in the surrounding plasma.

Cell surface NADH oxidase activity has been suggested to be a terminal oxidase for transplasma membrane electron transport systems. Constitutive (cNOX) and tumour associated (tNOX) cell surface NADH oxidase activities have been described

extensively by Morr  and colleagues (Morr , 1998; Kishi, *et al.*, 1999; Morr , *et al.*, 2000; Chueh, *et al.*, 2002; Morr , *et al.*, 2002; Hedges, *et al.*, 2003; Morr  and Morr , 2003a, 2003b). They report that NADH oxidase proteins have oscillating NADH or hydroquinone oxidase activity with protein-disulfide interchange (see Figure 1.2) and may be involved in entrainment of a biological clock (Chueh, *et al.*, 2002; Morr , *et al.*, 2002). The quinone analogue capsaicin strongly inhibits tNOX activity suggesting the involvement of quinones. The cNOX and tNOX activities are differentiated by their different periodicity, 24 and 22 min, respectively, and their sensitivity to anti-tumour drugs (Chueh, *et al.*, 2002). The claim for oscillatory behaviour is based on small changes in each of the activities, with a 2 + 3 oscillatory pattern, two major maxima followed by three minor maxima for the NADH oxidase activity and the inverse for the protein disulfide-thiol interchange activity (although in some reported data these maxima are not clearly discernable) occurring at 24 or 22 min intervals (Morr  and Morr , 2003b). This apparent periodicity is not affected by temperature, and two samples with out-of-phase periodicity will entrain to a common period over time. This led to the suggestion that the NADH oxidase protein may function as a circadian clock driver (Morr , *et al.*, 2002). The periodicity of tNOX is suggested to arise from small changes in secondary structure. Fourier transform infrared spectroscopy and circular dichroism studies indicate a cyclical  $\alpha$ -helix to  $\beta$ -sheet transformation for approximately 1% of the protein. Mutation of a putative copper binding site in tNOX eliminates the oscillatory pattern (Morr  and Morr , 2003b).

A tNOX protein has been cloned and characterised from HeLa cell membranes. The cloned and bacterially expressed protein retains the proposed 22 minute periodicity and is inhibited by capsaicin, a feature that is characteristic of the *in situ* tNOX protein (Chueh, *et al.*, 2002). Functional motifs were identified by sequence comparison and site-directed mutagenesis. Motifs including putative quinone, adenine dinucleotide (NADH) and copper binding domains were identified as well as potential active-site cysteines, and N and O-linked glycosylation sites.



**Figure 1.2: Proposed NADH oxidase (NOX) activity and its association with other membrane redox components.** Adapted from Morr  and colleagues (2000); Morr  and Morr  (2003a).

HeLa cell membranes contain a cell-surface NADH oxidase which can be eluted from the membrane by decreasing the pH from 7 to 5 (Kishi, *et al.*, 1999). This eluted NADH oxidase activity can oxidise reduced coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>H<sub>2</sub>), even in the absence of NADH, at a rate similar to when NADH is present. The oxidation of CoQ<sub>10</sub>H<sub>2</sub> is not affected by SOD or catalase. Oxygen consumption is not stoichiometric with NADH or CoQ<sub>10</sub>H<sub>2</sub> oxidation; hence the investigators suggest that protein disulfides act as the electron acceptors for the rest of the NADH or CoQ<sub>10</sub>H<sub>2</sub> oxidation (Kishi, *et al.*, 1999). NADH oxidase activity has been identified in the sera of cancer patients (Morre, *et al.*, 1997; Berridge and Tan, 2000a), presumably released from the cell surface. Serum NADH oxidase activity could therefore potentially be used diagnostically as a cancer indicator.

Vijaya and colleagues (1984) showed that RBC membranes have very low (2–3 nmol min<sup>-1</sup> mg protein<sup>-1</sup>) basal NADH oxidase activity, which may explain why RBCs have been reported to possess no such activity (Sun, *et al.*, 1992). The NADH oxidase described by Vijaya and colleagues (1984) can be stimulated 50 fold in the presence of vanadate and phosphate. NADH oxidation is found to be stoichiometric with oxygen consumption and to produce hydrogen peroxide. The oxidation shows biphasic kinetics suggesting the presence of low (133 μM) and high (3 μM) affinity

binding sites for NADH. The oxidase is inhibited by SOD, implicating the involvement of superoxide anions in the oxidation reaction.

NADH oxidase activity in whole cells has been studied using WST-1 as the extracellular electron acceptor (Berridge and Tan, 1998; Berridge and Tan, 2000a, 2000b). Cell surface and transmembrane NADH oxidase activity are differentiated by the electron acceptor/donor system used. WST-1 reduction in the presence of phenazine methosulfate (PMS) is thought to represent transmembrane NADH oxidase activity (Berridge and Tan, 1998, 2000b), while WST-1 reduction in the presence of NADH is attributed to cell-surface NADH oxidase activity (Berridge and Tan, 2000b). The two activities show differing sensitivities to many inhibitors (Table 1.4), but both are inhibited by SOD, suggesting that superoxide is involved to some extent in the reduction of WST-1. Whole cell activities also display different susceptibilities to inhibition to those characterised in isolated plasma membranes.

**Table 1.4: Comparison of whole cell surface and transmembrane NADH:WST-1 oxidoreductase activity with NADH oxidase from isolated membranes (Berridge and Tan, 2000b).**

	Transmembrane NADH:WST-1 oxidoreductase	Cell surface NADH:WST-1 oxidase	Isolated membrane NADH oxidases
Substrate	0.45 mM WST1 + 18 $\mu$ M PMS	0.45 mM WST1 + 0.2 mM NADH	NADH
Initial rate	$\sim 40$ mA <sub>450</sub> / min / cells	$\sim 5$ mA <sub>450</sub> / min / cells	
20 $\mu$ M pCMBS	No inhibition	Inhibits 70-80%	No inhibition
20 $\mu$ M NEM <sup>a</sup>	97% inhibition	Stimulates	
5 mM iodoacetate	Inhibits		
100 $\mu$ M capsaicin	Inhibits	Stimulates 25-50%	Inhibits
20 $\mu$ M retinoic acid	Weakly inhibits	Stimulates $\sim 40\%$	Inhibits
5 mM deoxyglucose	Inhibits 29-36%		
1 mM cyanide	Stimulates 60-70%		
20 $\mu$ g/mL SOD	Inhibits 70%	Inhibits 70%	
20 $\mu$ M arachidonic acid		Stimulates	

<sup>a</sup> NEM denotes *N*-ethylmaleimide

Intracellular NADH concentrations were found to be important for the transmembrane reduction of extracellular WST-1 (Berridge and Tan, 1998). Respiratory inhibitors, which would increase intracellular NADH in nucleated cells, stimulated WST-1 reduction. Conversely, glycolytic inhibitors, which decrease intracellular NADH concentrations, inhibited WST-1 reduction. The involvement of membrane quinones has also been suggested. Dicoumarol, which inhibits DT-diaphorase at its quinone acceptor site, inhibits WST-1 reduction in a concentration-dependent manner (Berridge and Tan, 1998). Also, ubiquinone (coenzyme Q<sub>10</sub>; CoQ<sub>10</sub>) has been shown to stimulate NADH-oxidase activity in isolated rat plasma membranes (Brightman, *et al.*, 1992; Vaillant, *et al.*, 1996).

The respiratory burst of inflammatory cells produces superoxide that is postulated to be involved in killing invading micro-organisms. This burst is associated with a membrane NADPH oxidase that transfers electrons from intracellular NADPH to extracellular molecular oxygen (Tan and Berridge, 2000). Phorbol ester-activated neutrophils reduce WST-1 in the absence of an intermediate electron acceptor, with the reduction almost completely inhibited by 20 µg/mL SOD. The NADH oxidase inhibitor resiniferatoxin fails to affect WST-1 reduction. As with NADH oxidase activity, inhibition of glycolysis inhibits WST-1 reduction (Berridge and Tan, 1998).

### **1.2.4 Ascorbate:(acceptor) oxidoreductases**

Vitamin C is an essential requirement for all human cells. It is involved in a number of different physiological functions and is not synthesised by the human body. Ascorbate can undergo either one or two electron oxidation to monodehydroascorbate free radical (AFR), or DHA, respectively. Two molecules of AFR can disproportionate to give one ascorbate and one DHA. DHA is unstable in aqueous media undergoing irreversible ring opening, but can be recycled back to ascorbate and avoid degradation. This recycling appears to play a role in the protection of RBCs from oxidative stress. DHA and ascorbate enter the RBC via the GLUT1 glucose transporter, however the rate of entry of ascorbate is substantially less than that of DHA (Himmelreich, *et al.*, 1998; May, *et al.*, 2001a). Most nucleated cells also contain sodium-dependent ascorbate transporters thus providing

another means of entry into the cell against a concentration gradient (Goldenberg, *et al.*, 2000).

#### 1.2.4.1 Ascorbate:ferricyanide oxidoreductase

Ascorbate reduction of ferricyanide is thermodynamically favourable, based on redox potentials (Table 1.1). The compounds can react non-enzymatically and through plasma membrane electron transport systems. Ascorbate, and DHA (which is reduced to ascorbate inside the cell), both stimulate ferricyanide reductase activity (Himmelreich and Kuchel, 1997; Van Duijn, *et al.*, 1998). The uptake of ascorbate by HL-60 cells is enhanced by the presence of ferricyanide. This occurs via oxidation of ascorbate to DHA by ferricyanide, followed by the uptake of DHA by GLUT1. The increase in ascorbate in HL-60 cells is inhibited by the GLUT-1 inhibitor cytochalasin (Van Duijn, *et al.*, 1998). Ascorbate mediation of ferricyanide reductase activity by RBCs may be distinct from NADH:ferricyanide oxidoreductase; each show differing sensitivities to exofacial inhibitors, such as the sulfhydryl agent pCMBS (Van Duijn, *et al.*, 1998; May and Qu, 1999), and proteolytic enzymes (May and Qu, 1999). Inhibition of the endofacial ascorbate- but not NADH-oxidation reactions by sulfhydryl agents indicates an endofacial oxidation activity that is independent of NADH:cyt *b*<sub>5</sub> oxidoreductase (May and Qu, 1999).

The reduction of ferricyanide by RBCs is accompanied by oxidation of intracellular ascorbate in a one-electron oxidation, producing AFR (May and Qu, 1999) and causes efflux of <sup>14</sup>C-labelled DHA through GLUT1 from <sup>14</sup>C-ascorbate loaded cells (May, *et al.*, 1995a). Similarly, DHA efflux is observed with hydrogen peroxide as the oxidant. The loss of DHA from cells is attenuated by increasing the cell concentration which may explain the lack of DHA efflux reported by Himmelreich and co-workers (1998) in their whole-cell, high haematocrit (Hc) NMR experiments. Ascorbate appears to be the main electron donor as removal of ascorbate from cells by oxidation to DHA with Tempol (2,2,6,6,-tetramethyl-1-oxy-4-hydroxypiperidine), followed by washout, results in a 66% decrease in ferricyanide reductase activity. This suggests that less than a third of basal enzymic activity uses NADH (May, *et al.*, 1995a).

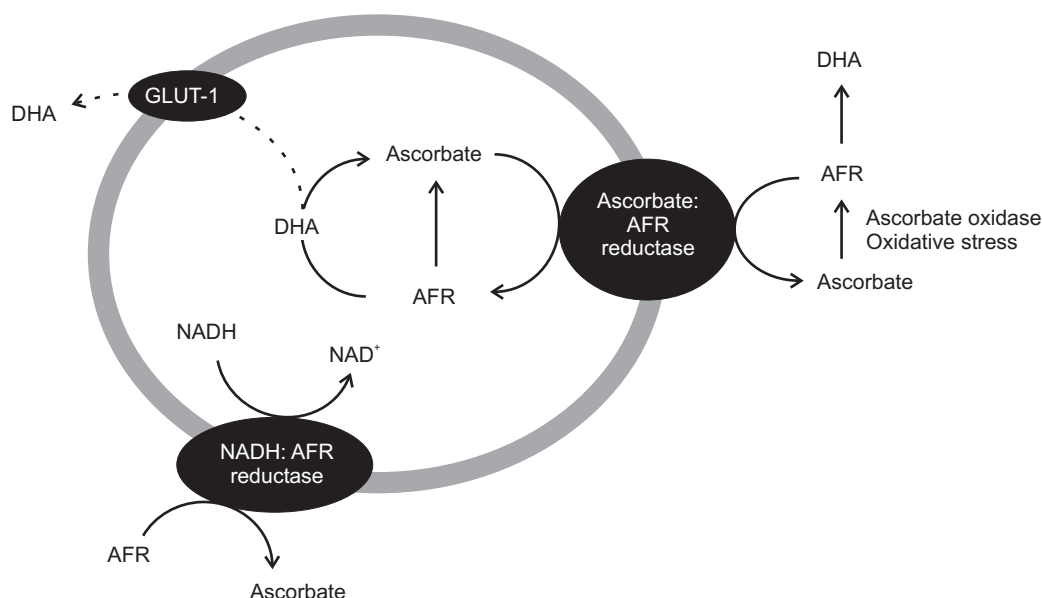
Enhancement of ferricyanide reduction by ascorbate is reliant upon ascorbate recycling. That is, the amount of ferricyanide reduced in an hour is greater than the ascorbate content of HL-60 cells could reduce were it not recycled, even if each ascorbate molecule were reducing two ferricyanide molecules (Van Duijn, *et al.*, 1998). May (1999) posits that RBC transmembrane reduction is rate limiting for ferricyanide, and not ascorbate, as the half-maximal ferricyanide reduction rate is at extracellular DHA concentrations that are less than 100  $\mu\text{M}$ , while the rate of ascorbate accumulation is half-maximal at 400  $\mu\text{M}$  DHA. Ascorbate recycling is discussed in greater detail in §1.2.7.1.

#### **1.2.4.2 Ascorbate:ascorbate-free-radical oxidoreductase**

RBCs can preserve extracellular ascorbate concentrations by reduction of AFR (May, *et al.*, 2000a; Van Duijn, *et al.*, 2000, 2001b). This reduction may represent the physiological action of at least one PMOR (Figure 1.3). AFR reduction, like ferricyanide reduction, depolarises the membrane; the more hyperpolarised the membrane, the faster the AFR reduction (Van Duijn, *et al.*, 2001b). Enhancement of AFR reduction can also be achieved by increasing the intracellular ascorbate concentration (Van Duijn, *et al.*, 2000). The reductase activity is inhibited by *N*-ethylmaleimide (NEM) that brings about a loss of available GSH. Presumably this is the basis of decreased intracellular ascorbate recycling (May, *et al.*, 2000a). Unlike its effect on ascorbate:ferricyanide oxidoreductase activity, pCMBS does not affect the preservation of extracellular ascorbate.

Van Duijn and colleagues (2000) found that low NADH concentrations result in higher rates of ascorbate oxidation to AFR, while the opposite was found for high NADH concentrations, indicating that NADH is a necessary requirement for ascorbate regeneration from AFR. These authors speculate on the functional components of the electron transport system claiming that an  $\alpha$ -tocopherol shuttle is an unlikely candidate given the redox potential (Table 1.1) and electrogenic nature of the transport (Van Duijn, *et al.*, 2000; Van Duijn, *et al.*, 2001b). CoQ<sub>10</sub> involvement is also thought to be unlikely as ubiquinone inhibitors (capsaicin and dicoumarol) fail

to perturb the effect. Involvement of a cytochrome in a protein or protein complex is suggested to be the most likely candidate for the electron transport pathway (discussed below in §1.2.5.5).



**Figure 1.3: Model of putative AFR recycling mechanisms in the RBC plasma membrane.** Adapted from Van Duijn and colleagues (2000).

### 1.2.4.3 Ascorbate:NBT oxidoreductase

The redox dye NBT forms an insoluble monoformazan on 2-electron reduction (Figure 1.1). Whole cells and ghosts reduce extracellular NBT in the presence of intracellular ascorbate, with the resultant monoformazan deposited at the RBC membrane (Zamudio, *et al.*, 1969; May, *et al.*, 1995b). Oxidised NBT has limited solubility in aqueous media, while reduced NBT is insoluble. Hence dissolution or concentration of oxidised NBT in the membrane presumably occurs prior to reduction (Demehin, *et al.*, 2001). Unlike ferricyanide, NBT reacts slowly with ascorbate so that background reduction of NBT by ascorbate is minimal. The sulfhydryl reagents *para*-chloromercuribenzoate (pCMB), pCMBS and NEM (Figure 1.5), along with ferricyanide, inhibit NBT reduction, the latter possibly through substrate competition for the same transmembrane enzyme (May, *et al.*, 1995b).

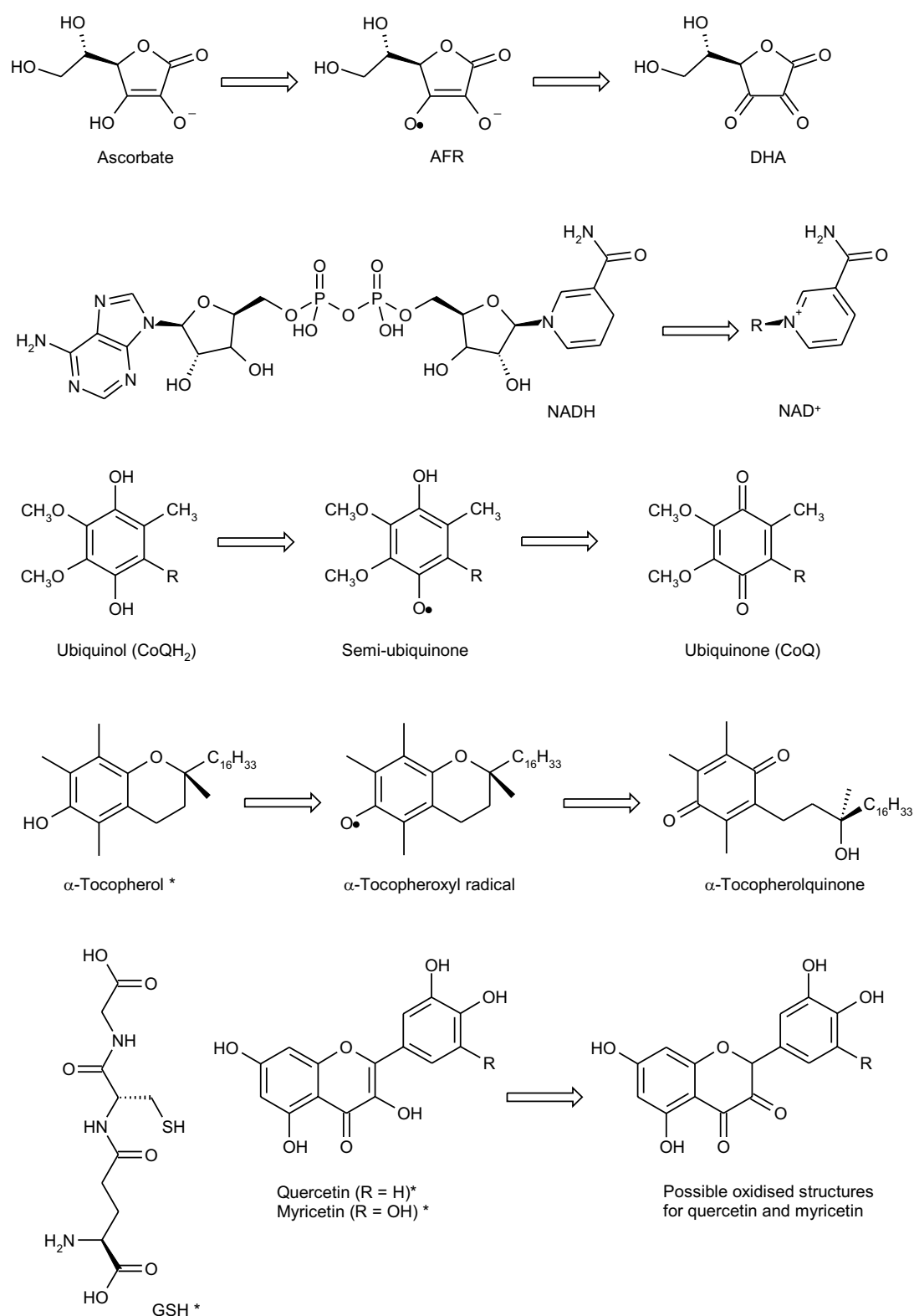
Reduction of NBT correlates with intracellular ascorbate content and resulting DHA efflux, although to a lesser extent than with ferricyanide reduction (May, *et al.*, 1995b). Demehin and co-workers (2001) found that like ferricyanide, NBT reduction is biphasic. The initial rapid reduction is attributed to reduced species within the membrane such as  $\alpha$ -tocopherol and membrane accessible species like ascorbate. Contrary to electron transport theory, they attribute the slower phase of NBT reduction to its interaction with membrane bound haemoglobin (Hb) that can react directly with NBT dissolved in the membrane. These authors also suggest that increased oxidative stress correlates with increased membrane-Hb interaction.

## **1.2.5 What is the mechanism of electron transport?**

### **1.2.5.1 Electron donors**

Electron donation appears to be reasonably specific; two main electron donors have been identified compared to the long and growing list of electron acceptors. As discussed above, both NAD(P)H and ascorbate act as electron donors to membrane redox systems, with ascorbate as the main reducing agent. More recently, other electron donors have been identified (Figure 1.4). Compounds such as  $\alpha$ -tocopherol and quinone most probably act as transmembrane shuttles and are more likely to be intermediates in the pathway (see Figure 1.2) than pure intracellular donors.

Flavonoids, which have iron chelating and antioxidant properties, inhibit DHA transport. Contrary to expectation, the flavonoids quercetin and myricetin, but not other flavonoids that have less electron-donating ability, enhance rather than attenuate ferricyanide reductase activity. RBCs readily take up both of these flavonoids. The degree of enhancement cannot be explained by direct reaction with extracellular ferricyanide (Fiorani, *et al.*, 2002). Therefore, these flavonoids may also act as intracellular donors for this redox activity.



**Figure 1.4: Electron donors and possible electron donors (\*) for transplasma membrane redox systems (with their oxidised partners on the right) (Kennett and Kuchel, 2003).**

### 1.2.5.2 The involvement of coenzyme Q<sub>10</sub>

CoQ<sub>10</sub> is present in mitochondrial membranes and is a major component of the mitochondrial electron transport chain. It is also present in plasma membranes (Sun, *et al.*, 1992). NADH:ferricyanide reductase activity of intact Namalwa p<sup>0</sup> cells is stimulated 1.2–2.2 fold by CoQ<sub>10</sub> and shorter chain analogues (Vaillant, *et al.*, 1996). Extraction of CoQ<sub>10</sub> from RBC membranes decreases NADH:ferricyanide oxidoreductase activity by 80%. This can be substantially restored by addition of 10 μM CoQ<sub>10</sub>, and moderately restored by the addition of α-tocopherolquinone (Sun, *et al.*, 1992). CoQ<sub>10</sub> can also re-activate ferricyanide reductase after inhibition by CoQ<sub>10</sub> analogues. NADH oxidase activity in rat liver cells requires CoQ<sub>10</sub> for full activity (Sun, *et al.*, 1992). RBC membranes contain less CoQ<sub>10</sub> (0.01 nmol/mg total membrane protein) than α-tocopherolquinone (1.2 nmol/mg total membrane protein). As stimulation of activity is proportional to CoQ<sub>10</sub> and α-tocopherolquinone content, this may reflect the low NADH oxidase activity observed in these membranes. An NADH:quinone reductase which also shows ferricyanide reductase activity and sensitivity to the anti-tumour agent adriamycin has been purified from rat liver plasma membranes (Sun, *et al.*, 1992). Thus, CoQ<sub>10</sub> may act in a manner similar to that in the mitochondrial electron transport chain; that is, as an electron shuttle in the plasma membrane as part of a PMOR (see Figure 1.2). Inhibition of ferricyanide reduction in HeLa cell membranes by ubiquinone analogues also inhibits proton release from the cells while CoQ<sub>10</sub> restores proton release (Sun, *et al.*, 1992).

Villalba and co-workers (1995) purified a CoQ<sub>10</sub> reductase from pig-liver plasma membranes. The protein, p34, catalyses NADH-dependent reduction of CoQ<sub>10</sub> and is sensitive to quinone-analogue inhibitors. Increasing CoQ<sub>10</sub> and p34 content in the membrane increases transmembrane NADH:AFR reductase activity. p34 has internal sequences that are identical to cyt *b*<sub>5</sub> reductase and may represent an endoplasmic-facing component of an electron transport chain involving CoQ<sub>10</sub> as the transmembrane component (Gomez-Diaz, *et al.*, 1997).

### 1.2.5.3 Proteinaceous components

The isolation and purification of dehydrogenase proteins from cell membranes indicates that at least some component of the redox activity is protein associated. An NADH:(acceptor) oxidoreductase, distinct from NADH:cyt *b*<sub>5</sub> reductase, has been isolated and purified from RBC membranes. It is a glycoprotein of 40 kDa apparent subunit molecular weight and represents 1% of the total RBC membrane protein mass (Wang and Alaupovic, 1978). It is a transmembrane protein, and has intracellular oxidation and extracellular reduction sites. The enzyme is specific for NADH and is able to reduce ferricyanide. Further evidence for glycoprotein involvement in ferricyanide reductase activity is the removal of such activity from isolated membranes and whole Ehrlich ascites cells by the glycosidases  $\beta$ -galactosidase and fucosidase (del Castillo-Olivares, *et al.*, 1994). NADH:AFR reductase activity in rat liver plasma membranes is also inhibited by lectins (Navas, *et al.*, 1988), again suggesting a role for cell surface glycoproteins in PMOR activity. NADH:DCIP oxidoreductases isolated from bovine and rat synaptic membranes are complexes of at least three different proteins, some of which are peripheral (Bulliard, *et al.*, 1997). The purification of these and other PMORs is discussed in more detail in Chapter 7.

Trypsin treatment (0.1% (w/v), 15 min, 37 °C) of Ehrlich ascites cells, and isolated plasma membranes, results in inhibition of NADH:ferricyanide reductase activity by 60 and 15%, respectively (del Castillo-Olivares, *et al.*, 1994). Trypsin can digest extrinsic proteins and domains by cleaving exposed polypeptides on the carboxyl side of arginine and lysine residues. The inhibition of ferricyanide reductase activity by trypsin suggests that its external hydrophilic domains are important for activity. Trypsin also inhibits NADH:AFR reductase activity in isolated rat liver plasma membranes (Navas, *et al.*, 1988).

### 1.2.5.4 The importance of sulfhydryl groups

NADH- (Zamudio and Canessa, 1966; Grebing, *et al.*, 1984; Schipfer, *et al.*, 1985; Vaillant, *et al.*, 1996) and ascorbate-dependent ferricyanide reduction (May and Qu, 1999) are inhibited by pCMBS in RBCs and other cell lines (see Table 1.3),

suggesting the involvement of sulfhydryl groups in membrane redox reactions. The situation appears to be more complex in isolated membranes. Resealed ghosts made from pCMBS-treated whole RBCs have inhibited ascorbate-dependent ferricyanide reduction (May and Qu, 1999). In contrast, “leaky” ghosts made from pCMBS-treated whole RBCs show no signs of inhibition of NADH-dependent ferricyanide reduction or ascorbate:ferric citrate oxidoreductase activities (May and Qu, 1999). The latter observation is more likely to reflect endofacial NADH reductase activity or direct reduction of ferricyanide by NADH, rather than a lack of inhibition of NADH-dependent ferricyanide reductase activity. pCMBS inhibits flavonoid-enhanced ferricyanide reduction (Fiorani, *et al.*, 2002) but not the fast phase of NADH-dependent ferricyanide reductase activity in RBCs (Schipfer, *et al.*, 1985). In contrast, the fast phase of ferricyanide reduction in Ehrlich ascites cells is inhibited by pCMBS (Medina and Nunez de Castro, 1994). Thus, it appears that these reported fast phases are of different origin and that the RBC fast phase is mediated by membrane reductant(s) other than membrane protein sulfhydryls. The variability in sensitivity to sulfhydryl agents is a good indication of the existence of a number of different membrane electron transport systems.

Other cellular thiols also appear to be involved in membrane redox reactions. Glutathione, the most abundant thiol-containing molecule in the RBC, is a major antioxidant through both the direct reduction of intracellular oxidised species and as a cofactor for detoxifying enzymes such as GSHPx and GST. Under conditions of oxidative stress, flux through the oPPP is increased. This increase in flux, originally suggested to be controlled by glucose-6-phosphate dehydrogenase (G6PDH), is actually controlled by hexokinase activity (Thorburn and Kuchel, 1985). Hence, changes in the redox status of RBC glutathione reflect the cell's response to oxidative stress and an increase in flux through the oPPP.

#### **1.2.5.5 Involvement of prosthetic groups and cytochromes**

Inhibition of ferricyanide reductase activity by the lipid-soluble iron reagent *o*-phenanthroline (Figure 1.5; Schipfer, *et al.*, 1985), but not by bathophenanthroline sulfonate (a non-haem-iron protein inhibitor; Grebing, *et al.*, 1984), suggests the

involvement of a haem or metal centre in the electron transfer. Calcium may also be involved in redox activity, as chlorpromazine (Figure 1.5), which affects  $\text{Ca}^{2+}$  function through calmodulin and membrane structure, completely inhibits transmembrane ferricyanide-reductase activity in open and inside-out ghosts, but not right-side-out ghosts (Grebing, *et al.*, 1984).

Cytochrome  $b_{561}$  (cyt  $b_{561}$ ) facilitates ascorbate:AFR oxidoreductase activity in chromaffin granules. To test the hypothesised involvement of this and other cytochromes in ascorbate:AFR oxidoreductase activity Van Duijn and colleagues (2001a) investigated the presence of cytochromes in the RBC membrane. Neither reverse transcriptase polymerase chain reaction (RT-PCR) on reticulocyte mRNA transcripts nor Western blotting of RBC membrane protein extracts provided evidence for either cyt  $b_{561}$  mRNA or protein. Cytochrome  $P-420$ , a possible breakdown product of cytochrome  $P-450$ , had previously been reported to be present in RBC membranes (Bruder, *et al.*, 1980). However, Van Duijn and colleagues (2001a) found no evidence for either of these cytochromes. Spectrophotometric studies of RBC membranes do, however, suggest the presence of unknown cytochromes in RBC membranes which could oxidise ascorbate and may be involved in this or other oxidoreductase activities (Van Duijn, *et al.*, 2001a).

Flavin nucleotides, such as FAD, may also be a component of transmembrane electron transport systems. Non-covalently bound FAD has been found in cat, dog, guinea pig, human, rabbit, and sheep RBC membranes, where the FAD content roughly correlates with NADH-ferricyanide reductase activity (Miner, *et al.*, 1983).

## **1.2.6 How does redox activity relate to the metabolic state?**

### **1.2.6.1 Metabolism and energy generation**

ATP levels can be maintained in RBCs by extracellular ferricyanide, even in the presence of the ATP regeneration inhibitor iodoacetate. This protection is enhanced by the provision of adenosine as a substrate (Mishra and Passow, 1969) and is likely

to be the result of an increase in the amount of ATP made per glucose molecule consumed under oxidative conditions. This is probably due to decreased flux through the Rapoport-Luebering 2,3-bisphosphoglycerate (2,3BPG) shunt (Himmelreich and Kuchel, 1997). The NAD(P)H oxidase of kidney podocyte membranes is activated by extracellular ATP in a time- and dose-dependent manner, producing superoxide (Greiber, *et al.*, 1998). It is hypothesised that ATP modulates enzyme activity at both transcription and translation levels; RT-PCR show that p67phox, a proposed subunit of NADPH oxidase, is transiently increased by ATP. In contrast, NADH oxidase activity of soybean plasma membranes is inhibited by 1 nM ATP via an increase in the enzyme's  $K_m$  for NADH from 60  $\mu$ M to greater than 100  $\mu$ M. ATP also increases the  $V_{max}$  nearly 2-fold (Morré, 1998). In perfused liver cells, however, ATP concentrations are unaffected by ferricyanide reduction (Clark, *et al.*, 1982).

In HeLa cells, extracellular regeneration of ascorbate from AFR is enhanced by forskolin and dibutyryl cAMP, agents that increase cAMP (Rodriguez-Aguilera, *et al.*, 1993). Agonists and antagonists of protein kinase A stimulate and inhibit AFR reduction, respectively. These links of PMORs to cAMP and protein kinase activity suggest that there may be some functional link between redox activity and cell growth and signalling (see §1.2.8.2).

The conversion of human Namalwa  $\rho^+$  cells to  $\rho^0$  cells, which no longer contain a functional mitochondrial respiratory system, results in a 4-fold increase in plasma membrane ferricyanide reductase activity (Larm, *et al.*, 1994). The up-regulation of ferricyanide reductase activity appears to be compensating for the loss of mitochondrial respiratory activity, enabling these cells to recycle intracellular redox compounds such as NADH. Treatment of  $\rho^+$  and  $\rho^0$  cells with the NADH: ferricyanide reductase inhibitor pCMBS results in a 45% inhibition of growth in the former and is lethal for the latter (Larm, *et al.*, 1994).

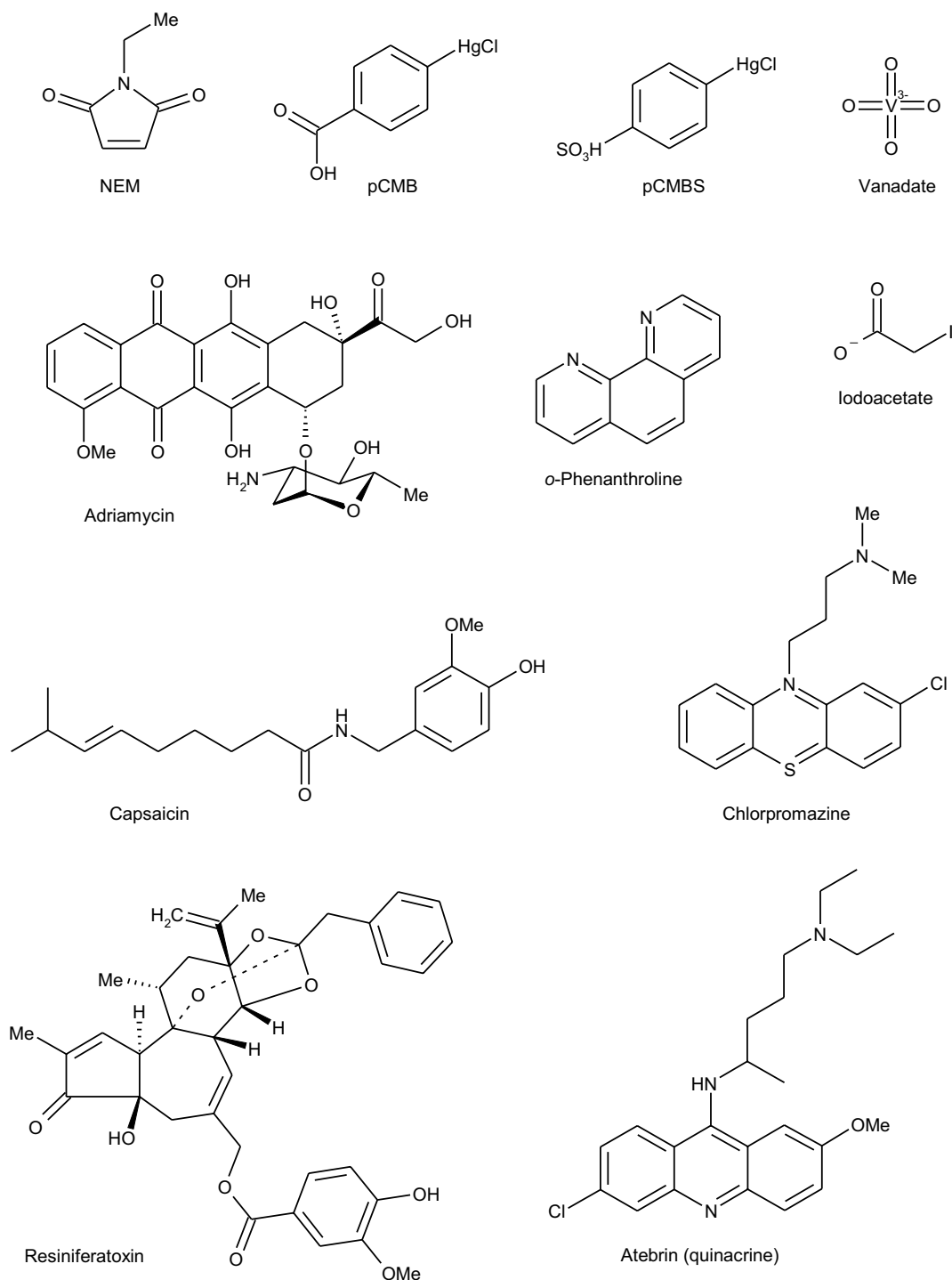


Figure 1.5: Inhibitors of transmembrane redox systems (Kennett and Kuchel, 2003).

### 1.2.6.2 Glycolytic enzymes

Reduction of ferricyanide by RBCs is strongly attenuated by the GAPDH inhibitor iodoacetate (Figure 1.5) (Mishra and Passow, 1969; Schipfer, *et al.*, 1985; Marques,

*et al.*, 1995; Himmelreich and Kuchel, 1997). This inhibition is countered with 6.4 mM DHA (Himmelreich and Kuchel, 1997). Vanadate (Figure 1.5), which inhibits GAPDH and other glycolytic enzymes, also inhibits ferricyanide reductase activity (Schipfer, *et al.*, 1985), but in the presence of phosphate it is reported to stimulate NADH oxidase activity in RBCs (Vijaya, *et al.*, 1984). These results suggest the existence of a functional link between glycolysis, NADH formation and transmembrane redox activity.

The enolase inhibitor fluoride has a variable effect; Himmelreich and Kuchel (1997) observed that fluoride and iodoacetate exhibited similar levels of inhibition of the transmembrane ferricyanide reductase activity and the recovery of activity with ascorbate, respectively, while Schipfer and co-workers (1985) saw no inhibition with fluoride. From these results it is evident that electron transport depends on the metabolic state of the cell, and that both NADH and ascorbate are electron donors for the system. Merker and colleagues (2002) have shown that in pulmonary arterial endothelial cells the intracellular NADH-to-NAD<sup>+</sup> ratio is important for plasma membrane electron transport capacity. Inhibition of glycolysis by iodoacetate or 2-deoxyglucose decreases membrane redox activity by 47 and 53%, respectively, and results in concomitant decreases in the NADH-to-NAD<sup>+</sup> and NADPH-to-NADP<sup>+</sup> (in the case of 2-deoxyglucose) ratios. The more pronounced effect on NADH than NADPH suggests a major role for NADH in endothelial cell protection against extracellular oxidative stress.

### 1.2.6.3 Hormone responses

Hormone-receptor binding appears to modulate NADH dehydrogenase activity. When supplied at higher than physiological concentrations, insulin (Dormandy and Zarday, 1965; Marques, *et al.*, 1995) and somatotrophin (Marques, *et al.*, 1995), are found to stimulate ferricyanide reductase activity in whole RBCs and ghosts. This stimulation is attenuated by NEM, iodoacetate, and vanadate (Marques, *et al.*, 1995). In contrast, other researchers have found that physiological concentrations of insulin inhibit membrane redox activity in human RBCs (Crane, *et al.*, 1982), and mouse liver plasma membranes (Goldenberg, *et al.*, 1978). Insulin also inhibits adenylate

cyclase activity to a similar extent as its inhibition of PMOR activity (Goldenberg, *et al.*, 1978), perhaps suggesting that the two actions are related. Interestingly, insulin, as well as inhibiting ferricyanide reductase activity, is found to alter both the membrane potential and the acidification of the extracellular fluid in unwashed RBC suspensions (Dormandy and Zarday, 1965). Glucagon is found to inhibit ferricyanide reductase activity in perfused liver, with half maximal inhibition at 0.1 nM (Clark, *et al.*, 1982), but to stimulate activity in mouse liver plasma membranes studied in isolation (Goldenberg, *et al.*, 1978, 1979). Hence, it seems likely that there are multiple effectors, direct or otherwise, of hormone response to redox signals.

The  $\beta$ -adrenergic agonists adrenaline and ritodrine stimulate RBC membrane redox activity in a pH- and concentration-dependent manner, while  $\beta$ - but not  $\alpha$ -adrenergic antagonists are inhibitory (Marques and Bicho, 1997). Hormone-enhanced redox activity is susceptible to inhibition by pCMB(S) and NEM. The NEM inhibition is reversible by GTP, most likely through a G-protein receptor coupling to the oxidoreductase (Marques and Bicho, 1997).

#### 1.2.6.4 Ion channels

Links between the hormone activation of ferricyanide reductase activity and  $\text{Na}^+/\text{H}^+$  antiport activation are also likely to exist, as the antiport inhibitor amiloride prevents redox stimulation by insulin and somatotrophin (Marques, *et al.*, 1995). Amiloride inhibits ferricyanide reductase activity by ~38% and proton extrusion by ~88% in Ehrlich ascites cells. Similar results have been reported in HeLa cells and isolated hepatocytes (Medina, *et al.*, 1988). However, amiloride is not a specific inhibitor of the  $\text{Na}^+/\text{H}^+$  antiporter and may also inhibit  $\text{Na}^+/\text{K}^+$  ATPase activity and alanine uptake by hepatocytes (Renner, *et al.*, 1988). Hence, interpretations of the affect of amiloride should be treated with caution.

A functional link between the cellular redox responses and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels has also been suggested (Miner, *et al.*, 1983; Fehlau, *et al.*, 1989). Miner and co-workers (1983) studied both activities in RBCs from a number of different

mammalian species (Table 1.2). However, no correlation between NADH:ferricyanide oxidoreductase activity and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel activity was found in any of the species. Interestingly, both membrane proteins displayed a similar sensitivity to  $\text{Pb}^{2+}$ , atebirin (a known NADH:ferricyanide oxidoreductase inhibitor and flavin antagonist) and menadione, and both have activity contingent on the metabolic state of the cell. However, the redox activity appeared not to be sensitive to the ion-channel activator propranolol (Fehlau, *et al.*, 1989). The enzymes thus appear to be functionally distinct, but the influence of redox activity on channel gating may yet be shown to be important.

The involvement of metal ions in PMOR activity has also been investigated, but again with mixed results. Sodium ions at millimolar concentrations and the divalent cations  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Mg}^{2+}$  at micromolar concentrations stimulate ferricyanide reductase activity in Ehrlich ascites cells (del Castillo-Olivares, *et al.*, 1994). In contrast,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  ions inhibit NADH:(acceptor) oxidoreductase activity in soybean hypocotyls (Sandelius, *et al.*, 1987), and  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions inhibit ferricyanide reductase activity in RBC ghosts (Zamudio, *et al.*, 1969).

#### **1.2.6.5 Apoptosis**

Apoptosis can be induced in many mammalian cell lines by inhibitors of mitochondrial respiration (Lawen, *et al.*, 1994). Inhibitors of plasma membrane NADH oxidase activity, such as the ubiquinone analogues capsaicin and resiniferatoxin, can also induce apoptosis in human B-cell and mouse myeloid cell lines (Wolvetang, *et al.*, 1996). Apoptosis induction is through a bcl-2-sensitive pathway which involves signalling through the phosphatase calcineurin (Lawen, *et al.*, 1994; Wolvetang, *et al.*, 1996) and can be inhibited by bcl-2, but not with inhibitors of mitochondrial respiration. These results suggest that NADH oxidase may be a redox-sensing protein which, depending on conditions, can stimulate apoptosis in the cell. In addition, capsaicin-induced apoptosis is minimal in transformed cell lines which have low PMOR activity (Macho, *et al.*, 1999).

## **1.2.7 Preservation of intracellular and membrane redox components in an active state**

### **1.2.7.1 Ascorbate**

Himmelreich and co-workers (1998) used  $^{13}\text{C}$  NMR to determine the site of DHA reduction. Both ascorbate and DHA give rise to separate intra- and extracellular  $^{13}\text{C}$  resonances due to differences in their interaction with solvent in the two environments, the so-called “split-peak” phenomenon (Kirk and Kuchel, 1988a). The data suggest that DHA is reduced extracellularly, with reducing equivalents coming from the intracellular oxidation of ascorbate. The fate of this extracellular ascorbate, however, was not explained. The authors found that cytochalasin B, a GLUT1 inhibitor, did not inhibit the transport of high concentrations of DHA into metabolically active cells. Hence, they suggested the existence of an alternative DHA transport pathway that may reduce DHA during transport. However, the physiological relevance of this hypothesis could be challenged due to the long time scale and high concentrations of DHA employed.

RBCs, unlike many other cells, do not store appreciable amounts of ascorbate. Intracellular ascorbate concentrations are similar to those found in plasma, at around  $60\ \mu\text{M}$  (Goldenberg, *et al.*, 2000), but can be increased markedly by incubation with extracellular DHA. Thus, twenty-fold increases in intracellular ascorbate concentrations have been identified in cells incubated with  $10\ \mu\text{M}$  DHA (May, *et al.*, 2001b). Ascorbate recycling can also occur through the direct reaction of DHA with GSH in a two-electron reduction which bypasses AFR formation (May, *et al.*, 1996b). GSH-independent reduction of DHA may also be mediated by the enzymes glutaredoxin, protein disulfide isomerase (Wells, *et al.*, 1995) and the seleno-enzyme NADPH-dependent thioredoxin reductase (May, *et al.*, 1997; Mendiratta, *et al.*, 1998). DHA reduction by GSH requires glucose to maintain GSH in its reduced form. However, in the absence of glucose, reduction of DHA can occur via intracellular NADH (May, *et al.*, 2001b).

AFR can also be recycled at the RBC membrane. NADH:AFR oxidoreductase activity, identified by May and colleagues (2000a), has both endofacial and

transmembrane orientations, and enables RBCs to maintain their ascorbate concentrations. The endofacial enzyme has a high affinity for both AFR and NADH ( $K_m < 2 \mu\text{M}$ ). The transmembrane activity, which reduces extracellular AFR, accounts for ~12% of the total NADH-dependent AFR reductase activity (May, *et al.*, 2001a). This AFR reductase activity is distinguishable from ascorbate enhanced NADH:ferricyanide reductase activity through its sensitivity to Triton X-100 and insensitivity to digestion by cathepsin D. The authors suggest that AFR reduction at the membrane may help facilitate recycling of  $\alpha$ -tocopherol in the plasma membrane.

Ascorbate may also be recycled by a so-called “bystander” effect. During the respiratory burst activated neutrophils produce superoxide which can oxidise plasma ascorbate to DHA. This DHA can be taken up by neighbouring non-activated cells and be reduced intracellularly to ascorbate (Nualart, *et al.*, 2003). This preservation of ascorbate will also increase the neighbouring cell’s capacity to withstand the oxidants produced during the respiratory burst.

### 1.2.7.2 Membrane components

Ferricyanide, as well as inducing intracellular oxidation, causes peroxidation of membrane lipids. The concentrations of  $F_2$ -isoprostanes (markers of lipid peroxidation formed by free-radical oxidation of arachidonic acid) are observed to double in sealed ghosts treated with ferricyanide (May, *et al.*, 1996a). Ruptured ghosts that are resealed to contain ascorbate largely prevent  $F_2$ -isoprostane formation, but oxidise intravesicular ascorbate, presumably through transmembrane electron transfer. A correlation between this ascorbate oxidation and low membrane  $\alpha$ -tocopherol concentration is found. The findings that  $\alpha$ -tocopherol oxidation by ferricyanide decreases in the presence of ascorbate, and that increasing  $\alpha$ -tocopherol concentration enhances ferricyanide electron transfer, strongly suggest a role for  $\alpha$ -tocopherol in transmembrane electron transport (May, *et al.*, 1996a).

Ascorbate has been shown to play a protective role against peroxidation of RBC membrane lipids and tocopherols by tBHP, preserving lipids by up to 92%, and  $\alpha$ - and  $\gamma$ -tocopherol by 50% and 65%, respectively (Mawatari and Murakami, 2001).

Ascorbate can also limit  $\alpha$ -tocopherol depletion in membranes treated with DABS (May, *et al.*, 2000b), hence protecting against lipid peroxidation (which occurs when  $\alpha$ -tocopherol levels are depleted by 20%). This protection is concomitant with transmembrane electron transfer from ascorbate as shown by EPR and the formation of intracellular AFR (May, *et al.*, 2000b).

$\alpha$ -Tocopherol in human RBC membranes appears to be recycled by two distinct methods, an enzymatic method involving the endofacial membrane NADH:cyt  $b_5$  reductase mediated reduction of membrane tocopheroxyl radicals, and a non-enzymatic mechanism involving ascorbate and dihydrolipoic acid (Constantinescu, *et al.*, 1993).

## **1.2.8 Role of membrane redox systems**

### **1.2.8.1 Protection of membrane components**

Agutter and co-workers (1979, 1980) suggest that PMORs might be present in plasma membranes to protect against lipid peroxidation. In fact they found an NADH:lipid hydroperoxide reductase in RBC membranes which, in contrast to other reported NADH:(acceptor) oxidoreductases, is stimulated rather than inhibited by pCMB (Agutter, *et al.*, 1980).

### **1.2.8.2 Growth and development**

A role for PMORs in cell growth has been suggested by a number of researchers (Ellem and Kay, 1983; Navas, *et al.*, 1992; Sun, *et al.*, 1994; Thomas, *et al.*, 1996; Zurbriggen and Dreyer, 1996) and the role of membrane redox systems in cell transformation has been reviewed recently (Chueh, 2000). Ellem and Kay (1983) found that 3–100  $\mu$ M ferricyanide can replace 2-oxycarboxylates in serum-deficient media to maintain cell attachment and division of a human melanoma cell line. This presumably operates through supplying electrons to intracellular redox-sensitive species, allowing normal metabolic processes to continue. At higher concentrations, ferricyanide inhibits cell growth (Ellem and Kay, 1983). Ferricyanide and CoQ<sub>10</sub> in the absence of other growth factors can also stimulate the growth of many other cell

types (Crane, *et al.*, 1994) but not K562 cells (Inman, *et al.*, 1994). Conversely, natural serum components that stimulate growth, such as epidermal growth factor and platelet derived growth factor, also activate cell-surface NADH oxidase activity (Crane, *et al.*, 1994). An increase in cytoplasmic pH is apparently associated with cell growth in mammalian cell lines (Navas, *et al.*, 1986). Hence, the proton extrusion initiated by PMOR activity may explain, in part, the stimulation of cell growth by electron acceptors such as ferricyanide and transferrin (Navas, *et al.*, 1986). The growth of PC12 cells in the presence of ferricyanide is accompanied by an acidification of the intracellular compartment as well as stimulation of DNA synthesis via a p42/p44 mitogen activated protein (MAP) kinase pathway, rather than via activation of protein kinase C, which occurs in the absence of intracellular acidification (Thomas, *et al.*, 1996).

Zurbriggen and Dreyer (1996) show that an NADH:DCIP oxidoreductase is differentially active throughout the cell cycle of the mouse neuroblastoma NB41A3 cell line. It is most active during the G1 and G2/M-phases. Addition of inhibitors of G1 and M phase to cells increases DCIP reductase activity in these arrested cell states. Additionally, a human neuroblastoma cell line NTera-2 exhibited a 50% decrease in redox activity during G0-phase upon differentiation. These authors clearly show that membrane redox activity is modulated at various phases of the cell cycle and that it plays a role in cell-growth and differentiation.

Drugs that inhibit cancer-cell growth also inhibit ferricyanide reductase activity in a number of cell lines. Ehrlich ascites cell ferricyanide reductase activity is inhibited by acivicin (5  $\mu$ M;  $66 \pm 3\%$ ), azaserine (10  $\mu$ M;  $60 \pm 5\%$ ), daunomycin (1  $\mu$ M;  $48 \pm 7\%$ ), and mitoxantrone (20  $\mu$ M;  $48 \pm 3\%$ ) (Medina, *et al.*, 1988). Ferricyanide reductase activity in virus transformed liver cells is inhibited by adriamycin (Sun, *et al.*, 1983) and in HL-60 cells inhibition of ferricyanide reductase activity strongly correlates with susceptibility of the cells to adriamycin inhibition of their cell growth (Morré, *et al.*, 1994).

Amino acid transport into Ehrlich ascites tumour cells via the Na<sup>+</sup>-dependent transport system A requires a trans-membrane Na<sup>+</sup> gradient and is stimulated by

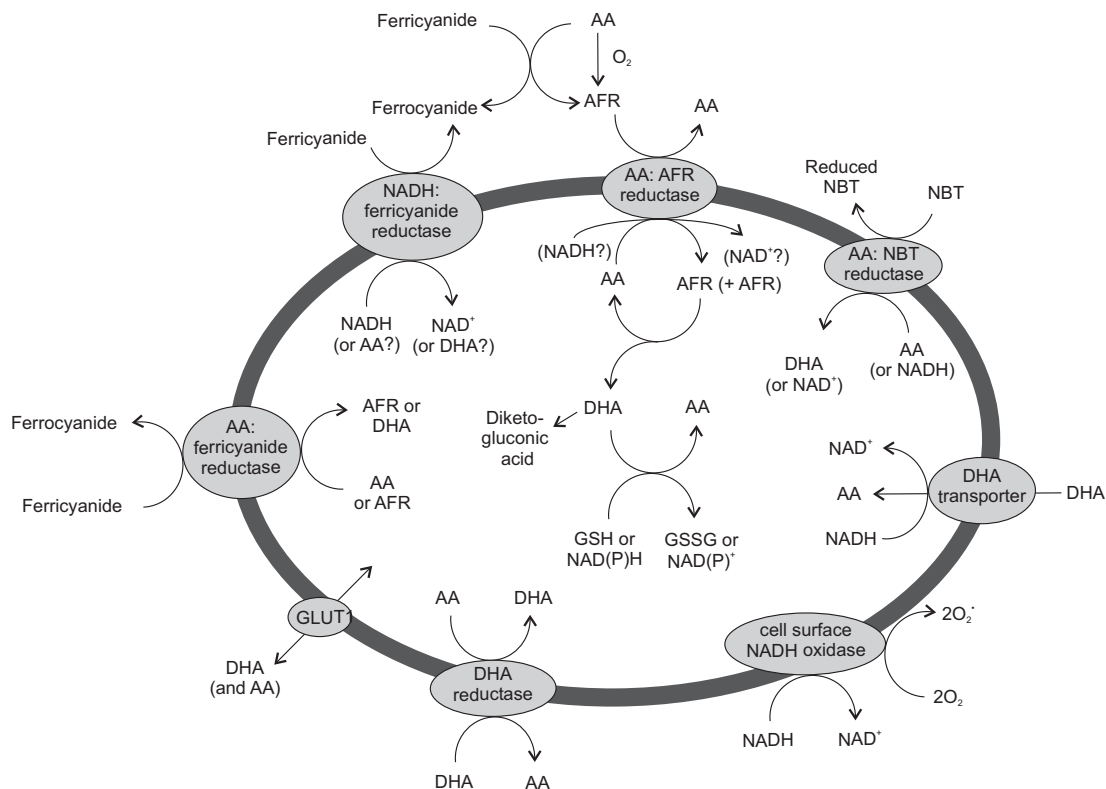
NADH or ascorbate in the presence of PMS. The uptake of 2-aminoisobutyric acid is inhibited by atebirin, a known inhibitor of PMOR activity. Neither ferricyanide nor DCIP can replace PMS as the electron acceptor (Yamamoto and Kawasaki, 1981).

### 1.2.8.3 Iron transport

Investigations of non-transferrin mediated iron uptake by K562 cells suggests that plasma membrane ferricyanide reductase activity plays a role in iron transport (Inman and Wessling-Resnick, 1993). As it appears that  $\text{Fe}^{2+}$  might be the preferred substrate for non-transferrin iron transport, the ability to reduce  $\text{Fe}^{3+}$  by a membrane ferrireductase should aid iron transport. A potential ferrireductase in K562 cells has been studied although it does not appear to be regulated by intracellular iron content. Cadmium ions inhibit both ferricyanide reductase activity and iron transport. This common inhibition, together with ferricyanide inhibition of transferrin-independent iron-uptake suggests that these two activities are coupled and may in fact be performed by the same molecular complex (Inman, *et al.*, 1994).

### 1.2.9 Electron import systems

The majority of studies on the PMORs that have been reported are electron export systems (Figure 1.6). Specifically, they transport electrons from *intracellular* electron donors to *extracellular* electron acceptors. These systems, as already discussed (§1.2.3–§1.2.8), are important for protecting cells from extracellularly derived oxidative stresses. The occurrence of such systems implies that cells have a way of redressing their redox “balance”. The extrusion of electrons from the cell is often accompanied by the release of protons (Dormandy and Zarday, 1965; Medina, *et al.*, 1988; Sun, *et al.*, 1992) ensuring that the redox balance is maintained. Links of PMOR with ion-channel activity (Miner, *et al.*, 1983; Medina, *et al.*, 1988; Fehlau, *et al.*, 1989; Marques, *et al.*, 1995) also suggest that cells can return to their original membrane potential after dealing with an oxidative stress. Can reduced species outside the cell transfer electrons to intracellular acceptors, especially when the intracellular environment is under oxidative stress?



**Figure 1.6: Proposed electron export systems in the human RBC.**

Extracellular NADH has been shown to have a hyperpolarising effect on mouse skeletal muscle plasma membranes (Zemkova, *et al.*, 1984). This hyperpolarising effect is inhibited by ouabain, an inhibitor of  $\text{Na}^+/\text{K}^+$  ATPase, and PMOR inhibitors including adriamycin, pCMBS and atebtrin. In the process, NADH is oxidised and there is an apparent increase in  $\text{K}^+$  membrane-permeability. Depolarisation of the membrane is observed in the presence of extracellular AFR (Van Duijn, *et al.*, 2001b). These effects are similar to those observed with electron export NADH:(acceptor) oxidoreductases and may imply that the action of extracellular NADH also involves a PMOR, and potentially an electron import system.

More recently, the effect of extracellular GSH on intracellular glutathione has been investigated using various NMR methods (Ciriolo, *et al.*, 1990; Ciriolo, *et al.*, 1993). Initial results show that adding an NMR-undetectable amount of GSH, but not GSSG, to a cell suspension leads to the appearance of peaks which are thought to be from intracellular GSH (Ciriolo, *et al.*, 1990). Intra- and extracellular glutathione peaks are indistinguishable under normal conditions, making identification of

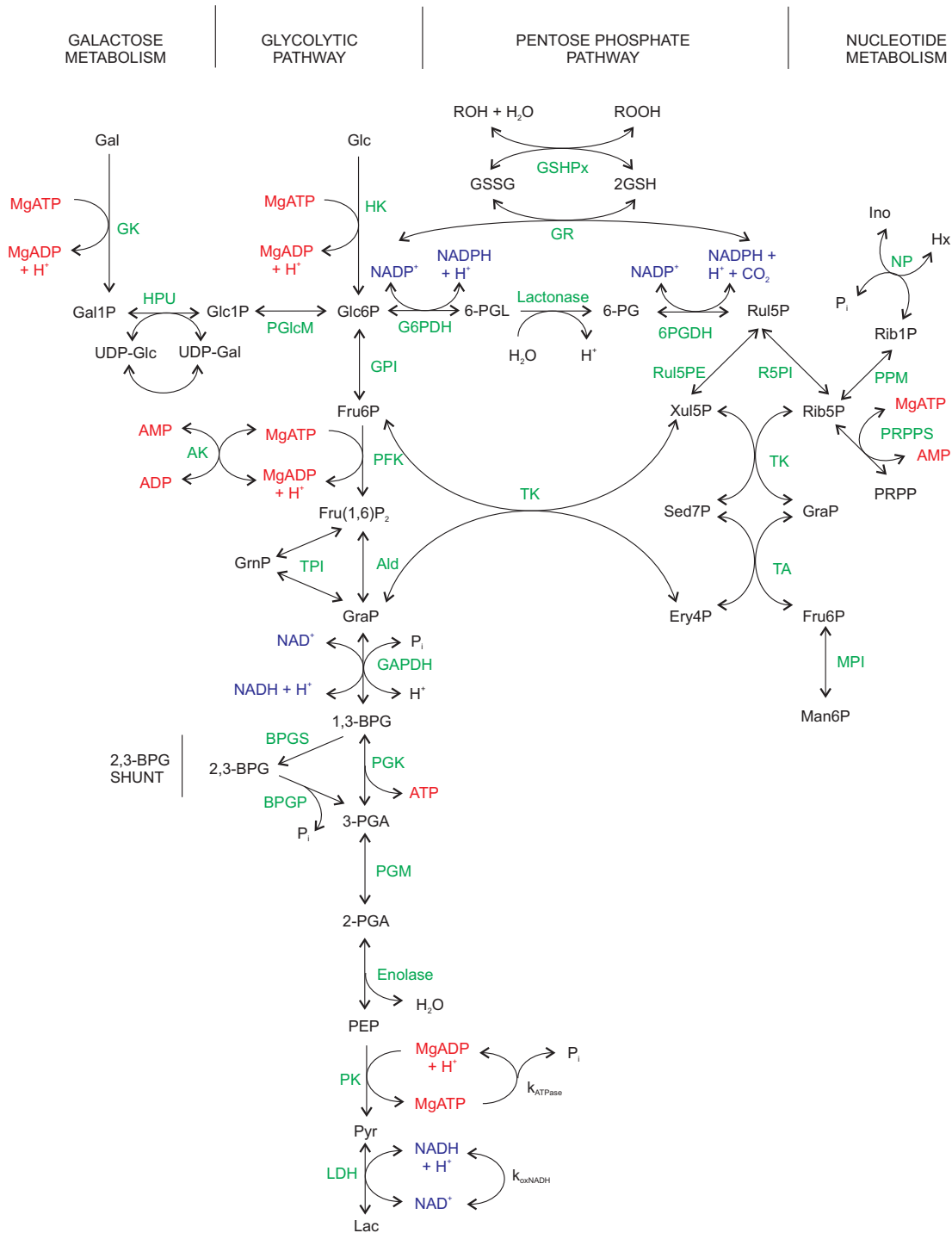
changes in the appropriate glutathione population difficult. Only upon dilution of the cell suspension Hc and the introduction of chemical shift reagents were the signals reported to be separable. Under such conditions Ciriolo and colleagues (1993) observe an apparent increase in intracellular GSH when GSH is added extracellularly. They attribute this to the release of GSH from mixed GSH-protein disulfides from the cytosolic face of the membrane, through transmembrane thiol/disulfide exchange from extracellular GSH, and thus electron import.

## **1.3 RBCs as a model system**

### **1.3.1 RBCs and the effects of oxygen**

The RBC, through its role as the body's oxygen and carbon dioxide transporter, is under constant exposure to possible sources of ROS, making it an ideal system for studying the effects of oxidative stress. As has been discussed, RBCs are able to respond to and reduce extracellular oxidants. The mobility of the RBC makes it an ideal antioxidant not only for its own membrane and local environment, but also as an oxidant scavenger throughout plasma-accessible parts of the body.

RBCs possess many advantages as a model system and hence have been the cell of choice for study of PMORs in many laboratories. Firstly, a great deal is known about their intermediary and secondary metabolism (Figure 1.7). The absence of organelles means that metabolic pathways are simpler than those found in more structurally complex cells. The plasma membrane is readily isolated free of other contaminating membranes, facilitating membrane studies (L6w and Crane, 1995; May and Qu, 1999). Additionally, a comprehensive computer model of RBC metabolism has been developed from the assembly of quantitative information on enzyme activities, kinetic parameters, and responses to various effectors, protons and metal ions (Mulquiney, *et al.*, 1999; Mulquiney and Kuchel, 1999a, 1999b). This model can help predict the effect of a specific metabolite on this complex system, which often does not lend itself to intuitive explanations. The inclusion of the effects of oxidative stress in the RBC model would enable a better understanding of redox control and its link to the metabolic status of the cell.



**Figure 1.7: Main metabolic pathways in human RBC, based on diagram from Mulquiney and Kuchel (1999b).** Abbreviations: Ald, aldolase; AK, adenosine kinase; 1,3-BPG, 1,3-bisphosphoglycerate; 2,3-BPG, 2,3-bisphosphoglycerate; BPGS, bisphosphoglycerate mutase; Ery4P, erythrose 4-phosphate; Fru(1,6)P<sub>2</sub>, fructose 1,6-bisphosphate; Fru6P, fructose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gal, galactose; Gal1P, galactose 1-phosphate; GK, galactose kinase; Glc, glucose; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; GraP, glyceraldehyde 3-phosphate; GrnP, dihydroxyacetone phosphate; GPI, phosphoglucose isomerase; GR, glutathione reductase; GSH, reduced glutathione;

GSHPx, glutathione peroxidase; GSSG, oxidised glutathione; HK, hexokinase; HPU, uridine diphosphogalactose-4-epimerase; Hx, hypoxanthine; Ino, inosine;  $k_{ATPase}$ , non-glycolytic energy consumption;  $k_{ox\ NADH}$ , reduction processes requiring NADH; Lac, lactate; LDH, lactate dehydrogenase; Man6P, manose 6-phosphate; MPI, phosphomanose isomerase; NP, nucleotide phosphorylase; PEP, phosphoenol pyruvate;  $P_i$ , inorganic phosphate; PFK, phosphofructokinase; 6-PG, 6-phosphogluconate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; 6PGDH, 6-phosphogluconate dehydrogenase; PGK, phosphoglycerate kinase; 6-PGL, 6-phosphoglucono- $\gamma$ -lactone; PGlcM, phosphoglucomutase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PPM, phosphopentose mutase; PRPP, 5-phosphoribosyl 1-pyrophosphate; PRPPS, 5-phosphoribosyl-1-pyrophosphate synthase; Pyr, pyruvate; Rib1P, ribose 1-phosphate; Rib5P, ribose 5-phosphate; R5PI, ribose-5-phosphate isomerase; Rul5P, ribulose 5-phosphate; Rul5PE, ribulose-5-phosphate epimerase; Sed7P, sedoheptulose 7-phosphate; TA, transaldolase; TK, transketolase; TPI, triose phosphate isomerase; UDP-Gal, uridine diphosphogalactose; UDP-Glc, uridine diphosphoglucose; Xul5P, xylulose 5-phosphate.

The RBC, with its well developed oxidative-defence mechanism and ability to traverse many different metabolic and oxidative environments, may play a role in protection against oxidative stress at the whole body level. It is known to scavenge (reduce) both intra- and extracellular oxidants (Thom, *et al.*, 1997). RBCs are able to protect neutrophils from apoptosis by scavenging extracellular  $H_2O_2$  (Aoshiba, *et al.*, 1999), consequently increasing their own susceptibility to peroxide-induced oxidative damage (Augustyniak, *et al.*, 1996). The oxidant-induced haemolysis (RBC rupture) characteristic of G6PDH deficiency (Thorburn and Kuchel, 1985) is caused by an inability of the RBC to fully recycle redox intermediates after oxidative stress (Bozzi, *et al.*, 1996), highlighting the importance of a well maintained redox balance. For stored blood to be viable for clinical and experimental use it is important that it retains its antioxidant capacity. Bank blood monitored over 25 days exhibits a 30% decrease in GSH concentration, decreases in antioxidant enzyme activity and total plasma antioxidant activity (Jozwik, *et al.*, 1997), suggesting that blood stored for long periods will be unable to endure oxidative stress.

### **1.3.2 NMR spectroscopy for redox monitoring**

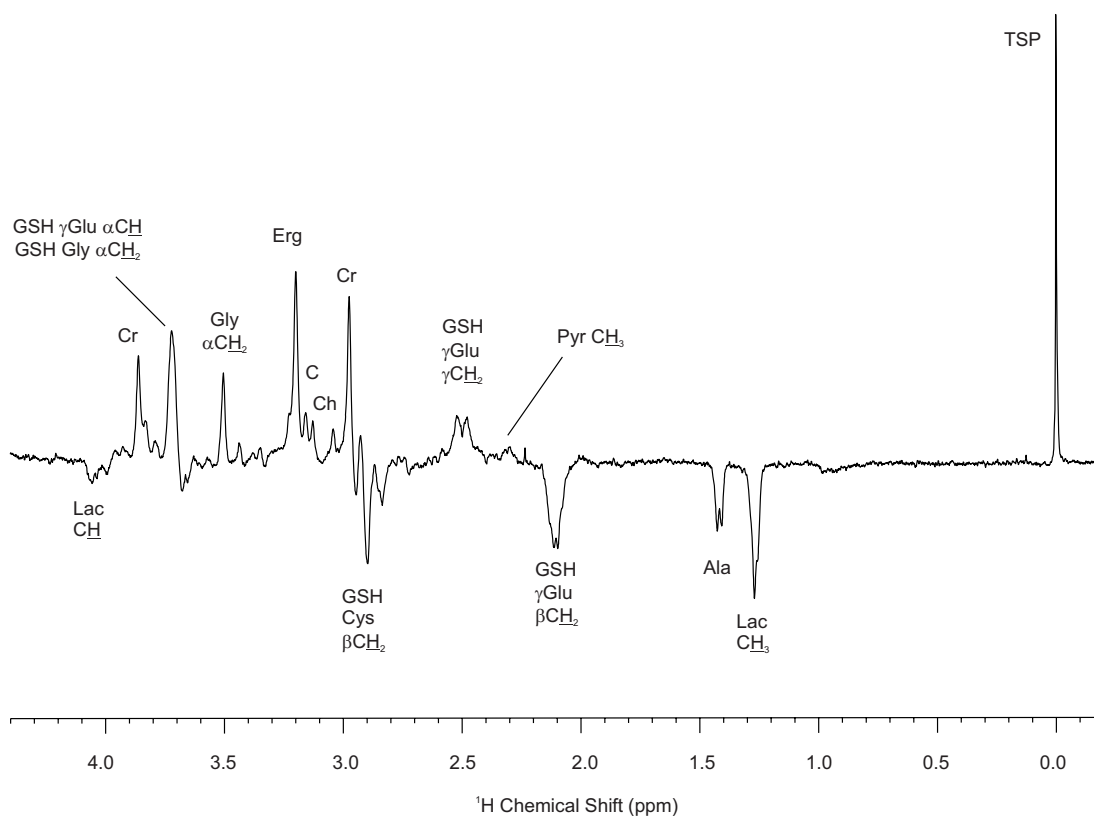
One of the main techniques employed throughout the work described in this thesis has been NMR spectroscopy. NMR experiments allow non-invasive, real-time

measurements of delicate metabolic systems under near *in vivo* environments (Brown, *et al.*, 1977). Application of radio frequency radiation to a sample in the magnetic field of an NMR spectrometer results in excitation of NMR receptive atomic nuclei, altering the equilibrium distribution of spin states. NMR experiments can be designed to obtain different information from a sample by altering the sequence, phase, length, and separation between radio frequency pulses. Application of the Hahn spin-echo NMR pulse sequence ( $\pi/2-\tau-\pi-\tau$ -acquisition;  $\tau = 60$  ms) is appropriate for RBC studies as only highly mobile, low molecular-weight compounds give rise to a signal (the broad  $^1\text{H}$  spectral resonances from Hb and membrane components are selectively eliminated through relaxation; (Kuchel, 1989). Protons in different chemical and magnetic environments give rise to different signals in a  $^1\text{H}$  NMR spectrum. The position of the signal in the spectrum reflects the immediate chemical and magnetic environment of the proton, while the signal intensity is dependent on the number of protons giving rise to the signal.

The application of the spin-echo pulse sequence leads to phase modulation of some signals. By convention a singlet and the central signal of a triplet are upright in a phased spin-echo spectrum. The phase of the outer components of a multiplet depends on the signal multiplicity and the value of the coupling constant ( $J$ ). In  $^1\text{H}$  spectra of RBCs an important example is the signal from the lactate methyl group. In protonated solvents (i.e.,  $^1\text{H}_2\text{O}$ ) this resonance is inverted in spin-echo spectra, but in deuterated solvents (i.e.,  $^2\text{H}_2\text{O}$ ) the peak is upright as the spins are effectively decoupled through enzyme-catalysed solvent exchange of the proton at C2 of the lactate (Kuchel, 1989).

An RBC suspension in physiological saline or phosphate buffered saline (PBS), gives a characteristic spin-echo spectrum (Figure 1.8). The peak integrals in spin-echo spectra are influenced by the  $J$ -modulation effect noted above (Ciriolo, *et al.*, 1993), and possible changes in the  $T_2$  relaxation time during the experiment. Hence, peak amplitudes rather than peak integrals are often measured (Kuchel, 1989). Furthermore, as partial saturation of the magnetization of spin-populations can occur with rapid pulsing in an NMR experiment, it is often difficult to obtain reliable

relative intensity data with the rapidly cycled spin-echo method (Kuchel, *et al.*, 1984).



**Figure 1.8:** Aliphatic region of the  $^1\text{H}$  NMR spin-echo spectrum ( $\tau = 60$  ms) of human RBCs suspended in PBS,  $\text{Hc} = 0.7$ . Abbreviations: Ala, alanine; C; carnitine; Ch, choline; Cr, creatine; Cys, cysteine; Erg, ergothioneine; Gly; glycine; Glu; glutamate, GSH, glutathione; Lac, lactate; Pyr, pyruvate; TSP, (2,2,3,3- $\text{d}_4$ ) trimethylsilyl-3-propionate.

Ergothioneine is well established as a stable endogenous and internal reference for  $^1\text{H}$  NMR (Rae, *et al.*, 1993). It is a thiol-containing fungal metabolite, of dietary origin, that has no known role in RBC metabolism (Mitsuyama and May, 1999). The  $^1\text{H}$  NMR resonance remains at constant intensity during oxidative stress by NEM, making it a stable reference peak for studies of oxidative processes. Ergothioneine does not appear to have an antioxidant role in human RBCs, although it can scavenge some oxidants *in vitro* (Mitsuyama and May, 1999). Quantitative chemical analysis on ergothioneine suggests that it is unlikely to form disulfides, despite its higher redox potential than GSH; it exists predominately as a thione at physiological pH (Rae, *et al.*, 1993).

A number of metabolites can be readily measured in  $^1\text{H}$  NMR spin-echo spectra of RBCs (Figure 1.8). At 400 MHz the resolved resonances in spin-echo spectra such as this are those of the glycolysis products pyruvate ( $\sim 2.3$  ppm) and lactate ( $\sim 1.3$  ppm) as well as glutathione, which has four clearly distinguishable peaks; Gly  $\alpha\text{CH}_2$ ; Cys  $\beta\text{CH}_2$ ;  $\gamma\text{Glu } \gamma\text{CH}_2$  and  $\gamma\text{Glu } \beta\text{CH}_2$ . The spin-echo method provides a means of direct measurement of the conversion of GSH-to-GSSG and vice versa during the thiol exchange within the cell (Reglinski, *et al.*, 1991). The concentration of GSH cannot be measured directly by  $^1\text{H}$  spin-echo NMR. However, the ratio of GSH-to-GSSG can be determined (Reglinski, *et al.*, 1991) by comparing the signal intensities of the Gly  $\alpha\text{CH}$  glutathione resonance, which reflects the GSH content of the sample, with the  $\gamma\text{Glu } \beta\text{CH}_2$  glutathione resonance, whose intensity is virtually independent of the oxidation state of glutathione (Kuchel, *et al.*, 1984) and thus represents the total free glutathione in the sample.

## 1.4 Thesis outline

For this thesis I studied electron import and electron export systems in RBC plasma membranes. There is only limited literature data on electron import systems for any cell line; hence I furthered the investigations of Ciriolo and colleagues (1993) into the effect of extracellular glutathione on intracellular RBC metabolism and studied the possibilities of other reduced compounds acting as extracellular electron donors.

For Chapter 3 I explored the effects of extracellular glutathione on intracellular metabolism using NMR and spectrophotometric assays.

For Chapter 4 I extended the investigation to other potential extracellular electron donors, in particular, NADH and its effects on the lactate-to-pyruvate ratio.

Chapter 5 continued the work of Chapter 4. I looked at the effect of extracellular NADH on metHb reduction and clarified the mechanism of NADH-induced changes to RBC metabolism.

For Chapter 6 I investigated the use of WST-1 as an NMR probe of electron export reactions. In an attempt to elucidate the mechanism of the redox reaction, WST-1 reduction by RBCs of a number of different animal species was compared to that by human RBCs.

Chapter 7 describes the preliminary attempts to purify NADH:ferricyanide and NADH:WST-1 oxidoreductases from human RBC plasma membranes.

The results presented in this thesis are then reviewed in a General Discussion in Chapter 8.