

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemical sources

The following chemicals and materials were obtained from the relevant companies or individuals listed in Table 2.1.

Table 2.1: Source of chemicals

Company	Chemicals
Ajax Chemicals (Melbourne, Vic, Australia)	acetic acid, ethanol, glucose, glycine, hydrochloric acid, magnesium sulphate, methanol, perchloric acid (PCA), potassium carbonate, silver nitrate, sodium chloride, disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium hydroxide, sodium thiosulfate
Aldrich (Milwaukee, WI, USA)	α -cyano-4-hydroxycinnamate (α CHC), methylphosphonate (MeP), NEM, triethylphosphate (TEP)
ANSTO (Lucas Heights, NSW, Australia)	D ₂ O
BDH (Poole, UK)	EDTA, formaldehyde, formic acid, glycerol, metaphosphoric acid, sodium carbonate, sodium dodecylsulfate (SDS), sodium nitrite, trisodium citrate
BOC gases (Wetherill Park, NSW, Australia)	carbon monoxide, oxygen
Boehringer (Castle Hill, NSW, Australia)	GSH, Triton X-100
Calbiochem (La Jolla, CA, USA)	nicotinamide
Cambridge Isotope Laboratories (Andover, MA, USA)	[¹³ C]formate

CEA (Cedex, France)	(2,2,3,3- <i>d</i> ₄) trimethylsilyl-3-propionate (TSP or <i>d</i> ₄ -TMSP)
David Bull Laboratories (Mulgrave, Vic, Australia)	sodium heparin
Dojindo (Tabaru, Japan)	WST-1
Fluka (Buchs, Switzerland)	capsaicin, β-mercaptoethanol
Gurr's (London, UK)	bromphenol blue
ICN (Aurora, OH, USA)	DHA, lubrol
Invitrogen (Carlsbad, CA, USA)	Mark12™ unstained standards
Merck (München, Germany)	potassium chloride, tBHP
Paramjit Bansal (University of Queensland)	[<i>glycyl</i> -2- ¹³ C]GSH
Pierce (Rockford, IL, USA)	bicinchoninic acid (BCA) protein assay reagent kit
Perkin-Elmer Life Sciences Inc (Boston, MA, USA)	[<i>adenyl</i> -2,8- ³ H ₂]NAD ⁺
Roche Diagnostics (Mannheim, Germany)	lactate assay kit (kit #216)
Sigma (St Louis, MO, USA)	acrylamide/bisacrylamide 30% (w/v) solution (37.5:1), alcohol dehydrogenase, ammonium persulfate, ammonium sulphate, bovine serum albumin (BSA), CHAPS, Coomassie Brilliant Blue R250, dibutyl phthalate, 4,4'-diisothiocyanatostilbene-2,2'-sodium disulfonate (DIDS), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB), formate dehydrogenase, GSSG, Hb, HEPES, inosine, iodoacetate, iodoacetamide, lactate dehydrogenase (LDH), lysozyme, mercaptoethanol, methylene blue, molecular weight marker kit for gel filtration 29–700 kDa), NAD ⁺ , NADH, NADPH, <i>N,N,N',N'</i> -tetramethylethylenediamine (TEMED), oxalic acid, oxamic acid, pCMBS, PMS, potassium ferricyanide, pyruvate assay kit (No. 726-UV), quinacrine (atebrin), sodium cyanide, sodium phosphate, sodium pyruvate, SOD, trichloroacetic acid (TCA), Tris-HCl, Tween-80

2.1.2 Buffers and solutions

All solutions were made up in reverse osmosis water (ROW) unless otherwise stated.

Table 2.2: Common buffers and solutions used throughout experiments

Buffer / Solution	Contains
2 mM PBS	2 mM NaH ₂ PO ₄ / Na ₂ HPO ₄ , pH 7.4
	145 mM NaCl
	0.6 mM MgSO ₄
	3 mM KCl
7 mM PBS	7 mM NaH ₂ PO ₄ / Na ₂ HPO ₄ , pH 7.4
	154 mM NaCl
25 mM PBS	25 mM NaH ₂ PO ₄ / Na ₂ HPO ₄ , pH 7.4
	154 mM NaCl
50 mM PBS	50 mM NaH ₂ PO ₄ / Na ₂ HPO ₄ , pH 7.4
	154 mM NaCl
Physiological Saline	154 mM NaCl, pH 7.4
Buffer A	50 mM Tris-HCl, pH 8.0,
	50 mM NaCl
	0.1% (w/v) lubrol
Buffer B	50 mM Tris-HCl, pH 8.0
	150 mM NaCl
	0.1% (w/v) lubrol

2.2 General Methods

2.2.1 pH determination

Solution pH was determined using a digital pH meter (TPS; Brisbane, Qld, AUS) which was calibrated with pH 4, 7 and 10 standards (Merck; Kilsyth, Vic, AUS). The pH was adjusted by the addition of HCl or NaOH. Solutions were adjusted to physiological pH prior to addition to RBC suspensions.

2.2.2 Osmolarity determination

Solutions added to RBCs should have an osmolarity of ~ 290 mOsmol kg^{-1} to prevent swelling or shrinking of cells. Osmolarities were tested on a Vapro vapour pressure osmometer (model 5100C Wescor Instruments, Logan, UT, USA) calibrated with 100, 290 and 1000 mOsmol kg^{-1} standards from Wescor.

2.2.3 Filtration

All solutions were filtered to remove dust and paramagnetic species (for NMR studies). Filtration was performed using $0.45 \mu\text{m}$ cellulose nitrate membranes (MFS, Dublin, CA, USA) and a water-vacuum pump.

2.2.4 Haematocrit (Hc) determination and cell counting

The Hc were determined in triplicate using Hc capillaries with an internal diameter of 1.5–1.6 mm. The capillaries were spun in a micro-Hc centrifuge (Clements, Sydney, NSW, Australia) and read with a micro-Hc reader (Hawksley, Poole, England). The RBC cell count and ratio of RBCs to white blood cells in a washed sample were determined using a Sysmex KX-21 automated haematology analyzer (Sysmex Corporation, Kobe, Japan). The RBC-to-leukocyte ratio was greater than 5000:1 in all experiments.

2.2.5 Spectrophotometric measurements

All spectrophotometric measurements were made using a dual beam Shimadzu UV-1601 UV visible spectrometer (Shimadzu Corporation, Kyoto, Japan). Microtitre plate (Greiner bio-one, Frickenhausen, Germany) measurements were made using a Labsystems Multiscan RC microtitre plate reader, with appropriate wavelength filters, and Genesis 3.03 software (Labsystems, Finland).

2.2.6 RBC preparation

Human blood cells were obtained either from banked blood (Australian Red Cross Blood Bank, Sydney, NSW, Australia) or fresh from healthy consenting volunteers by venipuncture into heparinised tubes. Animal blood was collected into heparinised tubes by venipuncture; the presence of heparin prevents clotting of the blood prior to removal of plasma and isolation of RBCs. In all cases blood was used within 48 h of collection; it was centrifuged at 3000 g for 5 min at 4 °C, and the plasma and ‘buffy coat’ of white cells and platelets were aspirated. The cell pellet was washed three times with 5 volumes of ice-cold physiological saline and suspended to the desired Hc in the appropriate buffer prior to use in experiments. In cells prepared for NMR, carbon monoxide was bubbled through the washed suspension to convert oxy/deoxy-Hb to carbon monoxy-Hb, which, due to its stable diamagnetic nature provides maximum resolution in NMR spectra (Himmelreich and Kuchel, 1997).

2.3 Preparative methods

2.3.1 Preparation of RBC methaemoglobin (metHb) and metHb reduction experiments

RBC Hb was converted to metHb by incubating cells (Hc = 0.5) with an equal volume of 150 mM sodium nitrite (in physiological saline) for 15 min at 37 °C. After five washes with physiological saline, the cells were resuspended in 7 mM PBS (Hc = 0.2) and incubated at 37 °C with 5 mM glucose, 5 mM NADH and 30 mM nicotinamide (to prevent NADH hydrolysis; Kuchel and Chapman, 1985), or both glucose and NADH. In experiments using pig blood, 5 mM inosine was used instead of glucose.

2.3.2 Preparation of A-side [nicotinamide-4R-²H] adenine dinucleotide (NADD) and [adenyl-2,8-³H₂]NADH

NADD was made from NAD⁺ using a modification of the stereospecific method described by Ottolina and colleagues (1989). In brief, 15 mM NAD⁺ was incubated at room temperature with 50 mM sodium carbonate, 50 mM deuterated formic acid

and 20 IU of formate dehydrogenase and titrated to pH 8.5. [*Adenyl*-2,8- $^3\text{H}_2$]NADH was made using the same method, with the following changes; [*adenyl*-2,8- $^3\text{H}_2$]NAD $^+$ was converted to [*adenyl*-2,8- $^3\text{H}_2$]NADH in the presence of 0.05 M formic acid.

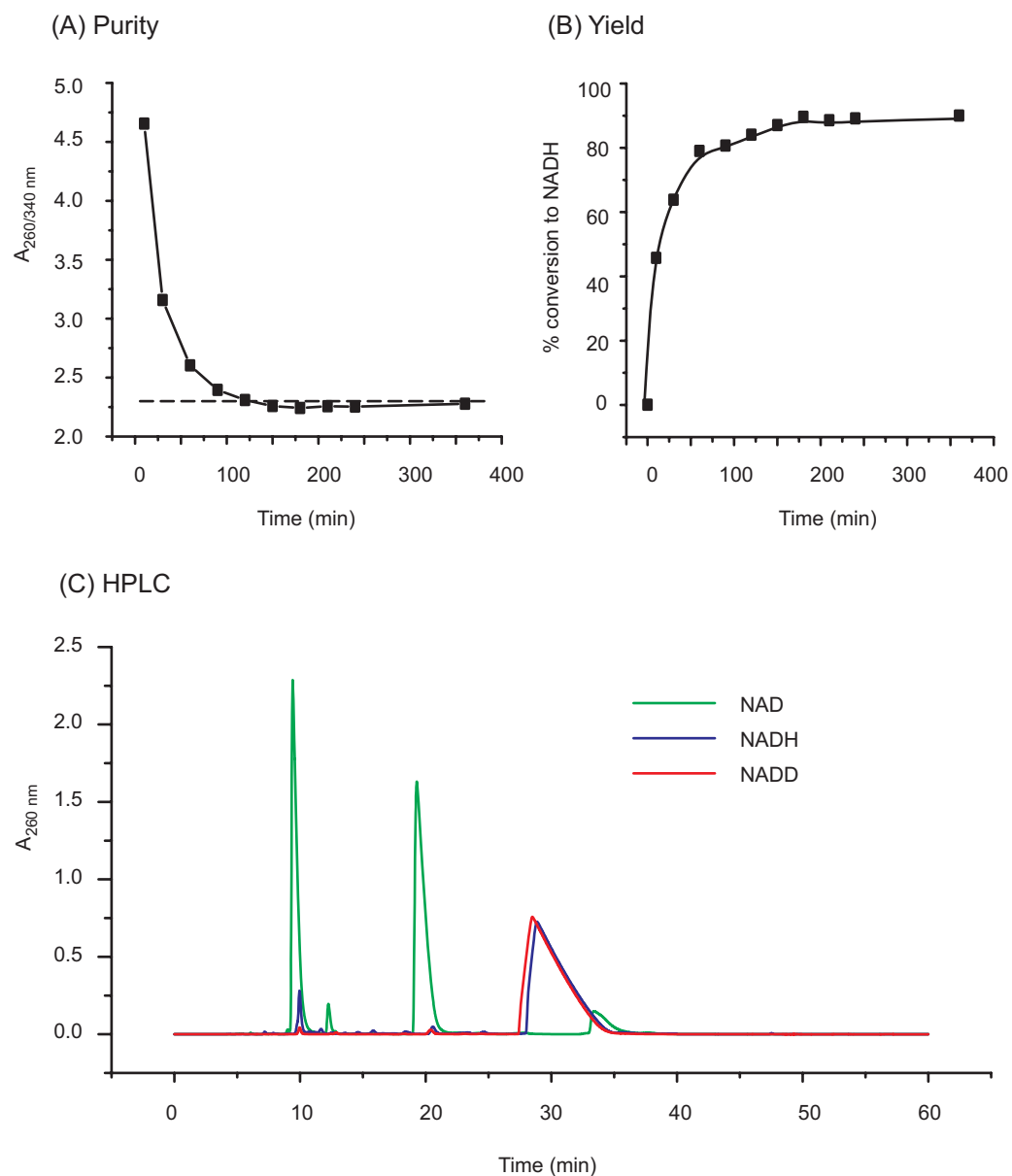


Figure 2.1: Preparation of [*adenyl*-2,8- ^3H]NADH. [*Adenyl*-2,8- ^3H]NADH was made from [*adenyl*-2,8- ^3H]NAD $^+$ as detailed in §2.3.2. (A) The purity was checked by the ratio of $A_{260/340}$, values less than 2.3 (dashed line) indicate that the majority of the nicotinamide dinucleotide is in the reduced form. (B) The yield of the reaction as a function of time and (C) the HPLC elution profiles of NAD (—), NADH (—), and NADD (—) made from [*adenyl*-2,8- ^3H]NAD $^+$ by the formate dehydrogenase reaction described in the text.

The reaction was considered complete when the $A_{260/340}$ was less than 2.3 (Figure 2.1A); the reaction yield was approximately 90% (Figure 2.1B). Formate dehydrogenase was removed by centrifugation through a 30000 MW Ultrafree[®]-MC centrifugal filter (Millipore, Bedford, USA). The purity of the preparation was checked by analytical high performance liquid chromatography (HPLC; Figure 2.1C) on a reverse phase Vydac 218TP54 C18 column (5 μ m) equilibrated with 20 mM phosphate buffer using a GBC (Dandenong, Vic, Australia) HPLC system (LC1150 HPLC pump, LC1210 UV/VIS detector, LC1445 system organiser, and Winchrom Chromatography Data System version 1.32). The HPLC profile of synthesised NADH is essentially identical to that of NADH available from commercial suppliers (Figure 2.1C).

2.3.3 Preparation of RBCs with intracellular GSSG

RBCs in 50 mM PBS were adjusted to 70% Hc and treated with 2.5 mmol (L RBC)⁻¹ tBHP in physiological saline to give approximately equimolar concentrations of tBHP and GSH.

2.4 Enzyme and substrate assays

2.4.1 Glutathione assay

Aliquots of RBC suspensions (Hc = 0.7) were centrifuged through 100 μ L dibutyl phthalate for 30 s at 12000 g (Eppendorf centrifuge 5415D, Hamburg, Germany). This allowed for the separation of cells (more dense) from supernatant (less dense). Packed RBCs (200 μ L) were haemolysed with 1.8 mL MilliQ water. Precipitating solution (3 mL; 208 mM metaphosphoric acid, 5.13 M NaCl, 6.84 mM EDTA) was added and the solution left to stand for 5 min. The sample was then centrifuged at 4500 g for 5 min and the supernatant separated from the protein pellet. Aliquots of the supernatant were used for assaying GSH concentrations.

GSH concentrations were determined according to the method of Beutler (1984) which was adapted for use on a Cobas Fara Centrifugal Analyser (Roche, Lane Cove, NSW, AUS) with GSH assay reagent (69.7 mM sodium citrate, 65 μ M DTNB). The optical density was measured at a wavelength of 412 nm but this instrument provides GSH concentration itself as the output. Each assay was performed in duplicate on the day of the experiment. Details of Cobas Fara setup are in Figure 2.2.

GENERAL		ANALYSIS	
Measurements mode	: ABS	P Sample volume	: 20.0 μ L
Reaction mode	: reag/dil	Diluent name	: H ₂ O
Calibration mode	: logit/log4	Diluent volume	: 50.0 μ L
Reagent Blank	: reag/dil	Cleaner cycle	: off
Wavelength	: 412 nm	Reagent volume	: 250 μ L
Temperature	: 25.0 °C	Time control	: no s
Decimal position	: 3	T Temp delay	: 1 s
Unit	: mmol L ⁻¹	A Readings	
CALIBRATION		First	: 0.5 s
Calibration Interval	: each run	Number	: 8
Number of standards	: 4	Interval	: 60 s
Position of standard 1	: 7	M	: no s
Std1: 0.00500		CALCULATION	
Std2: 0.02000		Sample check limit	: no Δ A
Std3: 0.04000		Reaction direction	: increase
Std4: 0.07000		Reaction check	: on
Replicate	: duplicate	Conversion factor	: 1.00000
Deviation	: 5 %	Conversion offset	: 0.00000
Correction Std	: no	Test range	
Reagent Range		Low	: off
Low	: no A	High	: off
High	: no A	Antigen excess	: no %
Blank range		Range Type	: norm range
Low	: no Δ A	Norm Range	
High	: no Δ A	Low	: no mmol L ⁻¹
CONTROL		High	: no mmol L ⁻¹
Control interval	: on request	Number of steps	: 1
Monthly report	: off	Calculation	
Cs1 position	: no	Step A	: endpoint
Cs2 position	: no	First	: 1
Cs3 position	: no	Last	: 7

Figure 2.2: Acquisition parameters for COBAS-FARA GSH assay.

2.4.2 Lactate assay

Lactate was assayed using a lactate assay kit (kit #216, Roche Diagnostics). In brief, 200 μ L RBCs were extracted 1:1 with 10% (w/v) TCA, and the extract centrifuged at 1500 g for 10 min prior to the addition of 100 μ L of physiological saline. The supernatant was then analysed for lactate concentration using a Hitachi 912

analytical analyser (Hitachi, Tokyo, Japan) with the working reagents provided in the kit. The Roche/Hitachi system provides the lactate concentration as output.

2.4.3 LDH assay

LDH was assayed according to the method of Beutler (1984). In brief, the assay was performed in a 1 mL cuvette containing 100 μ L of 1 M Tris buffer, 5 mM EDTA pH 8.0, 100 μ L of 2 mM NADH and a 50 μ L aliquot of sample in a total volume of 900 μ L. Whole cell samples were prepared by making a 1 in 200 haemolysate in β -mercaptoethanol stabilizing solution (0.005% (v/v) β -mercaptoethanol, 0.1% (w/v) EDTA); supernatant samples were prepared by separating the cell and supernatant fractions by centrifuging the cell suspension through 100 μ L dibutyl phthalate. Blank assays were also performed. The cuvette was incubated for 10 min at 37 °C prior to addition of 100 μ L of 10 mM sodium pyruvate (final concentration 1 mM). The change in absorbance at 340 nm was measured against the blank at 37 °C. LDH activity was calculated using the following equation:

$$\text{LDH activity [mmol (L supernatant)}^{-1} \text{ min}^{-1}] = \frac{(\Delta A_{\text{sample}} - \Delta A_{\text{blank}})}{6.22 \text{ mM}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}^{-1}} \times 20$$

Where ΔA_{sample} is the change in absorbance after the addition of pyruvate to the reaction, ΔA_{blank} is the change in absorbance of the blank reaction, $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ is the extinction coefficient for NADH at 340 nm, and 20 is the dilution of the sample in the assay.

2.4.4 Measurement of NADH uptake by RBCs

To determine whether NADH was transported into RBCs, cells were prepared as in §2.2.6, suspended in 7 mM PBS to 20% Hc, and incubated with 5 mM NADH/[*adenyl*-2,8- $^3\text{H}_2$]NADH over 8 h. To measure the uptake of radioactive NADH, samples of the cell suspension were separated by centrifugation and the packed cell fraction washed three times with 7 mM PBS to remove radioactive NADH in the supernatant. The cellular and supernatant fractions were then mixed with 0.5% (v/v) Triton X-100 to lyse cells, and the protein precipitated with 0.5 mL of 33% (w/v) TCA. Radioactivity in the deproteinated cellular and supernatant

fractions was measured using a 200CA Tri-Carb[®] liquid scintillation counter (United Technologies, Packard, IL, USA).

2.4.5 MetHb assay

The metHb concentration was measured by the method of Hegesh and co-workers (1970). Briefly, 0.2 mL of a 20% RBC suspension was incubated with 0.55 mL ROW for 3 min to haemolyse the cells. Then 0.25 mL phosphate buffer (0.5 M, pH 7.2) was added and the sample centrifuged at 4500 g for 10 min. The absorbance of the supernatant (0.6 mL) was measured at 632 nm (A_1). In a second cuvette 0.05 mL of supernatant was mixed with 0.55 mL ferricyanide-phosphate mixture (4 mM potassium ferricyanide, 125 mM phosphate buffer, pH 7.2), to oxidise any remaining Hb, and the absorbance measured at 632 nm (A_2). Neutralised cyanide solution (20 μ L; 1.02 M sodium cyanide, 0.94 M acetic acid) was added to cuvettes 1 and 2, whose contents were mixed gently and left to stand for 1 min prior to measuring the absorbance at 632 nm (A_3) and (A_4). The concentration of metHb was calculated using the following equation:

$$\%MetHb = \frac{A_1 - A_3}{(A_2 - A_4)} \times 100$$

2.4.6 Pyruvate assay

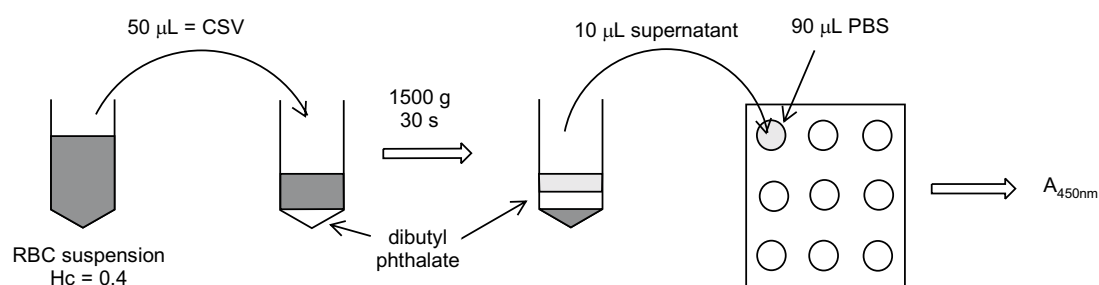
Pyruvate was assayed using a pyruvate assay kit (No. 726-UV, Sigma Diagnostics). In brief, RBCs were diluted 1:2 with ice cold 8% (v/v) PCA and kept on ice for 5 min prior to neutralisation with 1 M K_2CO_3 . The neutralised extract was centrifuged at 1500 g for 10 min prior to assay. In a cuvette, 1 mL of PCA extract was mixed with 0.25 mL TRIZMA[®] Base solution (1.5 M Tris(hydroxymethyl)aminomethane, 0.05% (w/v) sodium azide) prior to the addition of 0.25 mL NADH (3 mM in TRIZMA[®] Base solution). The absorbance at 340 nm was read against ROW (initial A). Then 0.025 mL of LDH (1000 IU/mL) was added with mixing and the absorbance read at 340 nm until an absorbance minimum was reached (final A). The concentration of pyruvate was calculated using the following equation:

$$\text{pyruvate [mmol (L RBC}^{-1}\text{)]} = \frac{\Delta A_{340} \times 1.5 \text{ mL}}{6.22 \text{ mM}^{-1} \text{ cm}^{-1} \times 0.333 \text{ mL RBC} \times 1 \text{ cm}}$$

where: ΔA_{340} = (initial A–final A), 1.5 mL is the reaction volume, 6.22 $\text{mM}^{-1} \text{ cm}^{-1}$ is the extension coefficient for NADH at 340 nm and 0.333 mL is the volume of RBC in the 1 mL PCA extract placed in the cuvette.

2.4.7 WST-1 assay

WST-1 reductase activity was measured using a microtitre plate method adapted from that reported by Berridge and Tan (1998). RBCs were incubated in 2 mM PBS containing 5 mM glucose at 37 °C with no additions, addition of 5 mM WST-1, or addition of 5 mM WST-1 and 5 μM PMS. The following were tested for their inhibitory effect on WST-1 reduction: 200 μM capsaicin, 1 mM atebirin, 1 mM iodoacetate, 1 mM pCMBS, and 20 $\mu\text{g/mL}$ SOD. At various times after addition of WST-1, PMS and the inhibitors, 50 μL aliquots of the RBC suspension were removed and centrifuged through 50 μL dibutyl phthalate for 30 s at 12000 g to separate the supernatant from the cells. A 10 μL aliquot of supernatant was then placed in a 96-well microtitre plate well (300 μL capacity) and diluted to 100 μL with 2 mM PBS. At the conclusion of the timecourse the absorbance was read at 450 nm using a microtitre plate reader (§2.2.5).



The absorbances were converted into concentration of WST-1 using the following equation:

$$\text{Formazan [mmol (L RBC)}^{-1}\text{)]} = \frac{A_{450}}{\varepsilon_{\text{eff}}} \times df \times \frac{1}{\text{CSV} \times \text{Hc}}$$

where A_{450} is the measured absorbance at 450 nm, ε_{eff} is the extension coefficient for WST-1 formazan adjusted for the microtitre plate format (see Figure 6.4), df is the

dilution factor used and equals the volume of supernatant in the original RBC aliquot divided by the volume of supernatant placed in the microtitre plate well, CSV is the cell suspension volume, and Hc is the haematocrit of the suspension.

2.5 NMR experiments

NMR spectra were acquired on a Bruker DRX-400 spectrometer, operating at frequencies of 400.13 MHz for ^1H , 100.62 MHz for ^{13}C and 161.98 MHz for ^{31}P , and on a Bruker DRX-600 spectrometer with operating frequencies of 600.13 MHz for ^1H and 150.92 MHz for ^{13}C . Samples were not spun during signal acquisition to avoid sedimentation and cell packing around the sides of the tube, ensuring sample homogeneity. ^1H and ^{13}C NMR experiments on RBCs were performed in Wilmad 507-PP NMR tubes (Wilmad, NJ, USA), whilst ^1H and ^{13}C NMR experiments on aqueous solutions were performed in Wilmad 528-PP NMR tubes, and ^{31}P NMR experiments were performed in Wilmad 513-7PP NMR tubes.

2.5.1 ^1H spin-echo NMR experiment theory

^1H spin-echo NMR spectroscopy has been used frequently to study cellular metabolism (Brindle, *et al.*, 1979; Simpson, *et al.*, 1981; Brindle, *et al.*, 1982; York, *et al.*, 1984; Reglinski, *et al.*, 1988b; Mendz, *et al.*, 1989; Rae, *et al.*, 1991). The technique takes advantage of the fact that large molecules (e.g. membrane components and proteins) have smaller relaxation times than small molecules (e.g. metabolites and substrates). The spin-echo pulse sequence is represented in schematic form in Figure 2.3. On application of a 90° pulse the magnetisation (originally on the z axis) is flipped into the xy plane. During the delay time τ the magnetisation decays due to relaxation, thus magnetisation is lost more quickly from large molecules. The application of the 180° pulse and the second delay τ refocuses the magnetisation. That magnetisation remaining in the xy plane after the second delay τ is observed in the acquired free induction decay (FID). By varying τ , the extent of contributions from the small and large molecules to the total magnetisation, and observed signal, can be altered. Spin-echo spectra are often phase modulated due to J -coupling effects.

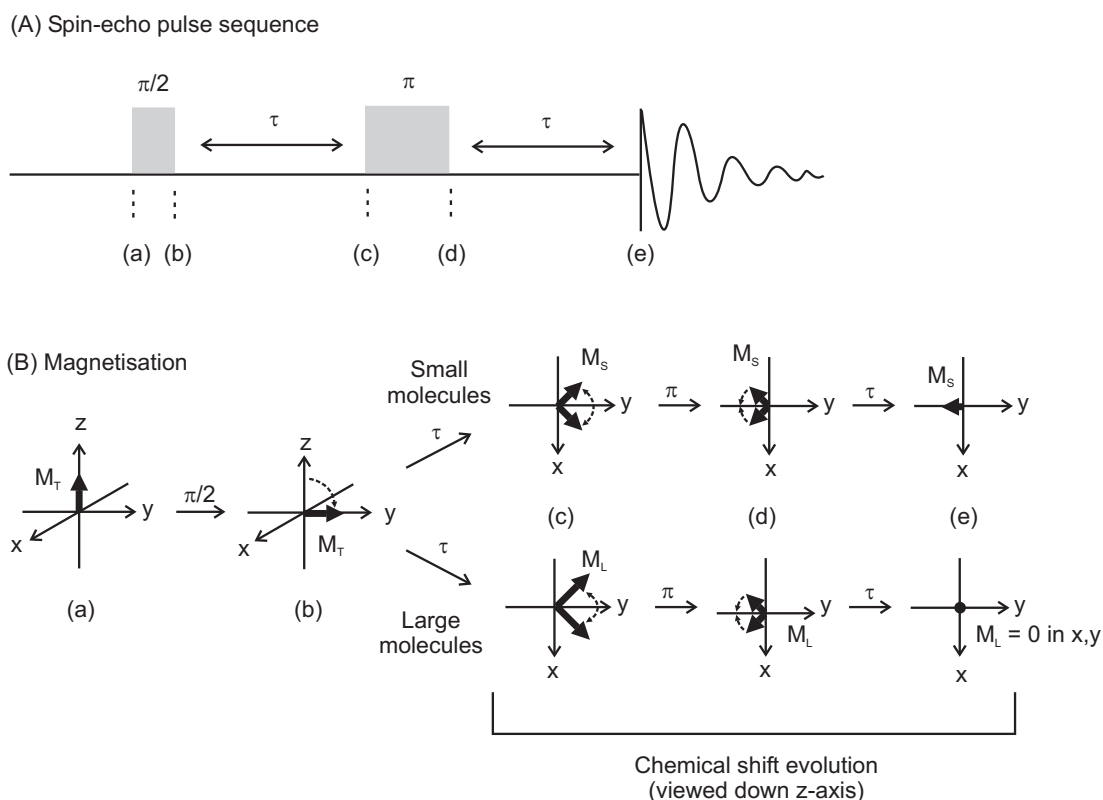


Figure 2.3: Schematic representation of the Hahn spin-echo pulse sequence (A) and its effect on the behaviour of the total magnetisation (M_T) and that for small (M_S) and large (M_L) molecules (B). Adapted from Reglinski and co-workers (1988a). The small letters in brackets in (B) indicate the magnetisation at the corresponding point in the pulse sequence (A).

2.5.2 2D NMR analysis of [glycyl-2- ^{13}C]GSH

[Glycyl-2- ^{13}C]GSH was synthesised using Fmoc methodology by Dr Paramjit Bansal, University of Queensland. The purified product was subjected to 2D NMR analysis to identify any contaminants and to confirm the location of the ^{13}C label. Spectra were acquired at 25 °C using a 5 mm triple resonance XYZ-gradient probehead on a Bruker DRX-600 NMR spectrometer. Chemical shifts are expressed relative to [^{13}C]formate $\delta(^1\text{H})$ 8.447, $\delta(^{13}\text{C})$ 171.8 and generally represent the midpoints of cross-peaks in correlation spectroscopy (COSY) spectra for protons, the midpoints of cross-peaks in heteronuclear single quantum coherence spectroscopy (HSQC) spectra for protonated carbons, and the midpoints of cross-peaks in heteronuclear multiple bond correlations spectroscopy (HMBC) experiments for

nonprotonated carbons. Standard Bruker (XWIN-NMR, version 3.5) pulse programs were used without modification.

2.5.3 Acquisition parameters for ^1H and ^{13}C NMR experiments using [glycyl-2- ^{13}C]GSH

NMR spectra were acquired at 37 °C using a 5 mm triple resonance XYZ-gradient probehead, on a Bruker DRX-600 spectrometer. ^1H and ^{13}C NMR spectral acquisitions on RBCs in 2 mM PBS containing 10% (v/v) D_2O were interleaved over 2 h time courses. ^1H NMR spectra were acquired using a spin-echo pulse sequence with an echo delay time (τ) of 60 ms, and 90° pulse duration of 13.5 μs . Low-power saturation of the water resonance during the relaxation delay (2 s) was used to reduce the intensity of the water peak. Each accumulated FID consisted of 64×1 s transients; the spectral width was 5000 Hz. All ^{13}C NMR spectra were acquired with continuous proton decoupling (WALTZ-16; Shaka, *et al.*, 1983) and a 30° tip angle for the ^{13}C pulse. The spectral width was 34722 Hz and each accumulated FID consisted of 512×1 s transients. Line broadening of 3 Hz was applied before Fourier transformation of both ^1H and ^{13}C NMR data. ^1H NMR spectra of [glycyl-2- ^{13}C]GSH/GSSG were obtained using a ‘pulse and acquire’ sequence with water pre-saturation (3 s) and a pulse duration of 3 μs . Each accumulated FID consisted of 8×4 s transients; the spectral width was 5000 Hz. ^1H and ^{13}C spectra were calibrated to the secondary reference [^{13}C]formate, $\delta(^1\text{H})$ 8.447, $\delta(^{13}\text{C})$ 171.8.

2.5.4 Acquisition parameters for ^1H and ^{31}P NMR measurements of NADH oxidation by RBCs

^1H NMR spectra were acquired at 37 °C using a 5 mm triple resonance XYZ-gradient probehead, on a Bruker DRX-400 spectrometer. ^1H spin-echo NMR spectra of RBCs in 50 mM PBS in H_2O incubated with 5 mM NADH, were acquired over 1.5–3 h with a spin-echo delay time of 60 ms and 90° pulse duration of 14.5 μs . Low power saturation of the water resonance during the relaxation delay (3 s) was used to reduce the intensity of the water peak. Each accumulated FID consisted of 64×1 s transients, and line broadening of 1 Hz was applied before Fourier transformation.

^1H spin-echo NMR spectra of RBCs incubated with 5 mM NADH and 5 mM pyruvate were acquired over 30 min with a spin-echo delay time of 68 ms and 90° pulse duration of 15 μs . Low power saturation of the water resonance during the relaxation delay (1 s) was used to reduce the intensity of the water peak. Each accumulated FID consisted of 50×2 s transients, and line broadening of 3 Hz was applied before Fourier transformation. The spectral width for all ^1H spin-echo NMR spectra was 5000 Hz. A coaxial capillary containing 1 mM TSP in D_2O was used as a chemical shift reference and for the field frequency lock in all experiments.

^{31}P NMR spectra were acquired at 37°C using a 10 mm multinucleus or broadband probehead, on a Bruker DRX-400 spectrometer, with continuous proton decoupling (WALTZ-16; Shaka, *et al.*, 1983). Each accumulated FID consisted of 16×1 s transients, and a 30 s relaxation delay was used. The spectral width was 10000 Hz. Line broadening of 1 Hz was applied prior to Fourier transformation. A coaxial capillary containing D_2O was used for the frequency lock. Chemical shifts are expressed relative to TEP $\delta(^{31}\text{P})$ 0.44 (Raftos, *et al.*, 1986; Kirk and Kuchel, 1988a, 1988b).

2.5.5 2D NMR analysis of WST-1

WST-1 was characterised by a combination of 2D NMR techniques. NMR spectra were acquired at 25°C using a 5 mm triple resonance XYZ-gradient probehead, on a Bruker DRX-400 spectrometer. Chemical shifts are expressed relative to TSP $\delta(^1\text{H})$ 0.0, $\delta(^{13}\text{C})$ 0.0, and generally represent the midpoints of cross-peaks in COSY, HSQC, and HMBC spectra. Standard Bruker (XWIN-NMR, version 3.5) pulse programs were used without modification.

2.5.6 Acquisition parameters for ^1H NMR measurements of WST-1 reduction by RBCs

^1H NMR spectra were acquired at 37°C using a 5 mm triple resonance XYZ-gradient probehead, on a Bruker DRX-400 spectrometer. Three types of ^1H NMR experiment were performed on RBC suspensions in 50 mM PBS in D_2O , in the

presence and absence of 5 mM WST-1 and 5 μ M PMS: (a) Hahn spin-echo pulse sequence with echo delay time (τ) of 60 ms, and 90° pulse duration of 13.5 μ s; (b) Hahn spin-echo pulse sequence with echo delay time (τ) of 20 ms, and 90° pulse duration of 13.5 μ s; (c) Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with delay time (τ) of 20 \times 1 ms, and 90° pulse duration of 13.5 μ s. Low-power saturation of the water resonance during the relaxation delay (2 s) was used to reduce the intensity of the water peak in all Hahn spin-echo experiments. Each accumulated FID consisted of 64 \times 1 s transients. Low-power saturation of the water resonance during the relaxation delay (3 s) was used for CPMG experiments and each accumulated FID consisted of 64 \times 1 s transients. The spectral width was 5000 Hz.

2.6 Statistical analysis

Differences between controls and investigative experiments were tested for statistical significance using the nonparametric Mann Whitney U test (Wilcoxon Rank-Sum Test). The test is used for comparing means of independent groups which have a small sample size and a non-Gaussian distribution of data, the null hypothesis being that mean ranks are equal for two groups. The null hypothesis was rejected if the difference between the means of the groups was found to be statistically significant at the 95% confidence interval, $p < 0.05$ (Dawson-Saunders and Trapp, 1994).

2.7 Purification of PMOR activity from RBCs

2.7.1 Preparation of RBC membranes (ghosts)

RBC ghosts were prepared by the procedure of Price and colleagues (1989) from three packs (~1.2 L) of buffy-coat depleted blood. Washed and packed RBCs (§2.2.6) were haemolysed with 5 volumes of haemolysis buffer (15 mM Tris-HCl, 1 mM EDTA, pH 7.7) and the membranes separated from the cytosol by filtration through a Plasmaflo™ Hi-05 Asahi plasma separator hollow-fibre capillary system (Asahi Medical Co., Tokyo, Japan). The ghosts were washed with washing buffer (10 mM HEPES, pH 7.7), to remove traces of Hb. The washed fluffy white ghosts were centrifuged at 4 °C for 45 min in an SS34 rotor at 40000 g using a Sorvall®

centrifuge (DuPont Instruments) and the supernatant removed. The pelleted ghosts were then centrifuged at 4 °C for 1 h in a 70.1Ti rotor at 330000 g using a Beckman L8-70 Ultracentrifuge (Beckman, USA) to ensure a high starting protein concentration for solubilization and fractionation procedures. The packed ghosts were stored at -20 °C.

2.7.2 Membrane solubilization

RBC ghosts were solubilized by gentle mixing overnight at 4 °C in 2% (w/v) lubrol, in 50 mM Tris-HCl, pH 8.0 containing 50 mM NaCl. The solubilized ghosts were centrifuged at 330000 g for 1 h at 4 °C, the unsolubilized pellet discarded and the supernatant retained for further fractionation. Solubilized membrane proteins could be stored at 4 °C or -20 °C for several weeks with no appreciable decrease in PMOR activity. The PMOR activity was tolerant to salt concentrations to at least 0.5 M and was retained through concentration steps in a stirred cell concentrator using a YM10 diaflo ultrafiltration membrane (Amicon Inc., Beverly, MA, USA).

2.7.3 DEAE-sepharose anion-exchange chromatography

Lubrol solubilized ghosts were loaded on a 10 mL Toyopearl DEAE-650 M (Tokyo, Japan) column (hand poured within a 60 mL disposable syringe) equilibrated with buffer A. The column was washed with 5 column volumes of buffer A, and the wash fraction collected. Proteins were eluted with a 20 column volume linear gradient of 50 mM–2 M NaCl in buffer A. The column flow rate was 2 mL min⁻¹ and the 6 mL fractions were assayed for protein concentration (§2.7.6) and redox activity (§2.7.7).

2.7.4 Hydroxyapatite adsorption

The active fractions from the DEAE-sepharose column were pooled and diluted to 50 mM salt prior to loading on a hand poured (within a 60 mL disposable syringe) 10 mL Hydroxyapatite Bio-Gel[®] HTP gel (BioRad Labs, Hercules, CA, USA) column equilibrated with buffer A. The column was washed with four column volumes of buffer A prior to elution with a 10 column volume linear gradient of 0–0.5 M

NH₄SO₄ in buffer A. The column flow rate was 1 mL min⁻¹ and the 3 mL fractions collected were assayed for protein concentration (§2.7.6) and redox activity (§2.7.7).

2.7.5 Size exclusion chromatography

Gel filtration was performed using a 25 mL Sephadex-200 HR 10/30 column (Amersham Pharmacia) coupled to the BioRad Laboratories fast performance liquid chromatography (FPLC) system BioLogic v.1.2. All solutions and samples were passed through 0.22 µm filters prior to application. The column was washed with MilliQ water prior to equilibration with buffer B. Samples were eluted with buffer B at a constant flow rate of 0.5 mL/min. The column was initially calibrated with molecular weight markers; blue dextran (2000 kDa; void volume estimate), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (66 kDa), and ribonuclease A (14 kDa).

2.7.6 Determination of protein concentrations

Protein concentrations were determined by using the BCA protein assay (Smith, *et al.*, 1985) adapted for use in the microtitre plate format. In brief, 25 µL of each standard (0–2 mg/mL) and unknown sample were aliquoted into separate wells on a 96-well microtitre plate. The working assay reagent was made by mixing 50 parts of Reagent A with 1 part of Reagent B (BCA Protein Assay Reagent Kit, Pierce, IL, USA) forming a clear green solution. 200 µL of working reagent was added to each microtitre plate well and the plate incubated for 30 min at 37 °C. The absorbance was read at 562 nm using a microtitre plate reader.

2.7.7 PMOR activity assays

2.7.7.1 WST-1 assay

WST-1 reduction by RBC ghosts and partially purified membrane preparations was measured using a microtitre plate format assay. In a total volume of 200 µL, 40 µL of sample was placed in a microtitre-plate well with 20 mM Tris-HCl, pH 8.0 and 1 mM WST-1. NADH (final concentration 1 mM) was added to start the reaction and

the change in absorbance at 450 nm measured over a 30 min incubation at room temperature. The rate of change of formazan production per min was calculated using the following equation:

$$\text{Rate of formazan formation [mM min}^{-1}\text{]} = \frac{(\Delta A_{\text{sample}} - \Delta A_{\text{background}})}{\text{calibration factor}} \times 5$$

Where ΔA_{sample} is the change in $A_{450\text{nm}}$ in the presence of sample and $\Delta A_{\text{background}}$ is the change in $A_{450\text{nm}}$ of the assay components without the sample. The calibration factor is the relationship of the concentration of WST-1 formazan to its absorbance in the microtitre-plate well and 5 is the dilution of the sample in the assay.

2.7.7.2 Ferricyanide assay

Ferricyanide reduction by RBC ghosts and partially purified membrane preparations was also measured using a microtitre plate format assay. In a total volume of 200 μL , 40 μL of sample was placed in a microtitre-plate well with 20 mM Tris-HCl, pH 8.0 and 1 mM ferricyanide. NADH (final concentration 1 mM), was added to start the reaction and the change in absorbance at 405 nm was measured over 30 min at room temperature. The rate of change of ferricyanide reduction per min was calculated using the following equation:

$$\text{Rate of ferricyanide reduction [mM min}^{-1}\text{]} = \frac{(\Delta A_{\text{sample}} - \Delta A_{\text{background}})}{\text{calibration factor}} \times 5$$

Where ΔA_{sample} is the change in $A_{405\text{nm}}$ in the presence of sample and $\Delta A_{\text{background}}$ is the change in $A_{405\text{nm}}$ of the assay components without the sample. The calibration factor is the relationship of the concentration of ferricyanide to its absorbance in the microtitre-plate well and 5 is the dilution of the sample in the assay.

2.7.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on the combination of 10% resolving gels (9.73% (w/v) acrylamide/0.27% (v/v) bisacrylamide, 380 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.07% (v/v) TEMED), and 4% stacking gels (3.9% (w/v) acrylamide/0.1% (v/v) bisacrylamide, 124 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.07% (v/v) TEMED). The SDS running buffer consisted of 25 mM Tris, pH 8.3, 0.192 mM glycine and 0.1% (w/v)

SDS. Samples were prepared by one-to-one dilution in SDS loading buffer (250 mM Tris, 10% (w/v) SDS, 50% (v/v) glycerol, 25% (v/v) β -mercaptoethanol, 0.5% (w/v) bromophenol blue) and heated at 90 °C for 2 min. Mark12™ unstained standards (Invitrogen, USA) were used as approximate markers of molecular weight on all gels. Approximately 0.1–15 μ g of protein was added per lane. Proteins were electrophoresed at 100 V for 1.5 h.

2.7.9 Coomassie Brilliant Blue stain

SDS-PAGE gels were heated in Coomassie Brilliant Blue stain (0.12% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol, 10% (v/v) acetic acid) in a microwave oven (until the solution started to boil, usually ~30 s on high setting, 1400 W). Gels were then transferred to destain solution (30% (v/v) methanol, 10% (v/v) acetic acid) and soaked, with agitation, until bands were clearly visible.

2.7.10 Silver stain

Gels were silver stained using a modification of the protocol described by (Shevchenko, *et al.*, 1996). Gels were heated twice in fixative (10% (v/v) acetic acid, 30% (v/v) ethanol) in a microwave oven for 1 min at high power (1400 W). The gels were then heated twice in 20% ethanol for 1 min in a microwave oven at high power (1400 W), followed by rinsing in milliQ water for 5 min. Gels were soaked in sensitizer solution (0.02% (w/v) sodium thiosulfate) for 1 min and then rinsed three times in MilliQ water (20 s each). Gels were then exposed to 0.2% (w/v) silver nitrate for 1 min in a microwave oven at full power (1400 W) then rinsed in MilliQ water for 5–10 s. Gels were then soaked in developer solution (0.3% (w/v) sodium carbonate, 0.025% (v/v) formaldehyde and 0.001% sodium thiosulfate) until bands were sufficiently clear. Staining was stopped by exposure to 5% (w/v) Tris base and 2.5% (v/v) acetic acid.