Measurement of Glutathione Synthesis by Isotope Ratio Mass Spectrometry in Systemic Inflammation

Yukiko Kimura

DEPARTMENT OF PAEDIATRIC SURGERY

INSTITUTE OF CHILD HEALTH

UNIVERSITY COLLEGE LONDON

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Declaration

I, Yukiko Kimura, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: _____

Dated: _____

Abstract

During sepsis and critical illness, a systemic inflammatory response can significantly produce reactive oxygen and nitrogen species, which can damage host tissues. Usually, several different antioxidant defences protect host cells from these reactive species. One of the important antioxidant defences is glutathione, a tripeptide synthesised from the precursor amino acids cysteine, glutamate and glycine. The aim of this study was to measure glutathione synthesis *in vivo*, using deuterated glycine as a tracer.

Firstly, a new method for analysis of glutathione and glycine using both gas chromatography-mass spectrometry (GC-MS) and gas chromatography-isotope ratio mass spectrometry (GC-IRMS) was developed. The derivatives of both compounds were measured in a single chromatographic analysis, and the method was compatible with both GC-MS and GC-IRMS, and capable of measuring low carbon-13 or deuterium enrichment of glutathione in 50μl of erythrocytes.

Erythrocyte glutathione synthesis was measured, using GC-IRMS, in critically ill infants and children who had been infused with deuterated glycine. Glutathione fractional synthesis rate (FSR) was not statistically different between septic and non-septic patients.

In order to determine whether glutamine is able to increase GSH synthesis by acting as a glutamate precursor, erythrocyte GSH synthesis was measured using GC-IRMS in septic infants and children randomised to receive glutamine dipeptide or placebo. There was no significant difference in glutathione FSR between those given glutamine and those given placebo.

Intestinal glutathione depletion is known to occur following intestinal ischaemia-reperfusion injury. I measured glutathione synthesis in a rat model of intestinal ischaemia-reperfusion injury, and found that hypothermic rats with ischaemia-reperfusion injury had a higher intestinal glutathione synthesis than normothermic rats, providing a potential mechanism by which hypothermia may maintain intestinal glutathione levels.

In conclusion, the newly developed method of glutathione analysis using GC-IRMS was useful for compound specific isotope analysis of biological samples.

Acknowledgement

Abbreviation

2D-SDS-PAGE	Two-dimensional SDS-polyacrylamide gel electrophoresis
ABTS	2,2'-Azinobis(3-ethylbenzothiazoline 6-sulphonate)
Ala	Alanine
APE	Atom percent excess
ASR	Absolute synthesis rate
BF_3	Boron trifluoride
$CoQH_2$	Ubiquinol
CSIA	Compound specific isotope analysis
Cys	Cysteine
DAD	Diode array detector
DTNB	5,5'-dithiobis-(2-mitrobenzoic)-acid
DTT	Dithiothreitol
ECD	Electrochemical detection
ECF	Ethyl chloroformate
EDTA	Ethlendiaminetetraacetic acid
EGTA	Ethyleneglycol-bis-β-aminoethylether)-N,N,N',N'-tetraacetic acid
FSR	Fractional synthesis rate
GC-C-IRMS	GC-Combustion-IRMS
GC-MS	Gas chromatography mass spectrometry
GC-TC-IRMS	GC-Temperature Conversion-IRMS
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine

GPX	Glutathione peroxidases
GSH	Glutathione
GSSG	Oxidised glutathione
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFBTA	Heptafluorobutyric anhydride
HPLC	High-performance liquid chromatography
I/R	Ischaemia and Reperfusion
IAA	Iodoacetic acid
IL-1,-6	Interleukin-1,-6
IRMS	Isotope ratio mass spectrometry
KH_2PO_4	Potassium dihydric orthophosphate
LC-ELSD	LC-evaporative light scattering detection
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein
MALDI	Matrix Assisted Laser Desorption/Ionization
MBB	Monobromobimane
MCF	Methyl chloroformate
MDA	Malonaldehyde
MeOH	Methanol
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide

NEM	*N*-ethylmaleimide
NICU/PICU	Neonatal / Paediatric Intensive Care Unit
NO	Nitric oxide
n-PCF	*n*-Propyl chloroformate
NPP	*n*-pivaloyl & *o*-isopropylate
OPA	*ortho*-phthal aldehyde
PCA	Perchloric acid
PTV inlet	Programmed temperature vaporization inlet
RBC	Red blood cells/erythrocytes
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBD-F	Ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate
SIM	Selected ion monitoring mode
SIRS	Systemic inflammatory response syndrome
SMA	Superior mesenteric artery
SOD	Superoxide dismutase
SSA	Sulfosalicylic acid
TBA	Thiobarbituric acid
TBAH	Tetrabutylammonium hydroxide
t-BDMS	*tert*-Butyl-dimethylsilyl
TBP	Tributylphosphine
TCEP	Tris-(2-carboxylethyl)-phosphine
TFA	Trifluoroacetic acid
TMS	Trimethylsilyl

TNF-α   Necrosis factor-alpha

TPN   Total parenteral nutrition

VPDB   Vienna Peedee Belemnite

VSMOW   Vienna Standard Mean Ocean Water

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Chapter 1

Introduction

Chapter 1:   Introduction

1.1 Systemic inflammatory response syndrome and sepsis

Systemic inflammatory response syndrome (SIRS) is a leading cause of morbidity and mortality during critical illness or following severe injury (Ali et al. 1998). SIRS due to detectable infection is then termed sepsis. Sepsis is the deleterious systemic response to infection (Bone et al. 1992). One potential consequence of infection and/or the deleterious systemic response to infection is organ dysfunction. Failure of more than one organ is called multiple organ failure (MOF) or multiple organ dysfunction syndrome (MODS). MOF or MODS following SIRS remains the leading cause of death among critically ill patients in medical and surgical intensive care units (Nystrom 1998).

Sepsis is the most common cause of MODS in the paediatric population. Despite recent advances in the care of critically ill children, sepsis and MODS remain a major source of morbidity and mortality in paediatric patients (Upperman & Ford 2002). The mortality rate for paediatric patients with two or more dysfunctional organs remains much higher than for children without MODS (Upperman & Ford 2002). Most infections result from the failure of the host's intrinsic defence mechanisms to combat virulent factors from invading micro-organisms. Children with impaired immune systems are particularly affected by infection. Some authors suggest that the pattern of organ failure in paediatric patients may differ from adult patients due to developmental differences (Upperman & Ford 2002). Infections

represent a common inciting mechanism for the development of MODS, because uncontrolled infection can lead to systemic sepsis and may ultimately result in MODS. Although sepsis is the most common cause of MODS, in many cases a specific micro-organism or bacterial by-product responsible for initiating the systemic inflammatory response that leads to MODS may not be detected. This is because the identification of the micro-organism responsible for an infection still relies, for the most part, on classic microbiological selective media methods, which is not particularly sensitive. More recently, more sensitive molecular biology techniques, such as detection of 16s rRNA, have been developed which are more effective at detecting infective agents (Qiao et al. 2009). Nevertheless, many cases of SIRS may in fact be due to an infection which has not been identified, and treatment of SIRS is by empirical antibiotics until a causative micro-organism is directed to enable targeted therapy to be initiated.

In humans, SIRS consists of a cascade of events characterised by an elevated temperature, cardiovascular collapse or shock and decreased organ perfusion with associated dysfunction (Bone et al. 1992). Hence, septic patients usually present with fever, tachycardia and leukocytosis.

1.1.1 *Intestinal ischaemia-reperfusion injury and the role of hypothermic protection*

MODS and sepsis can be both a cause and a consequence of intestinal ischaemia and reperfusion (I/R) injury (Fink 1993;Schoenberg & Beger 1993). Intestinal I/R injury represents a major clinical problem in infants, children and

adults. Diseases associated with this condition include necrotizing enterocolitis, midgut volvulus, acute mesenteric arterial occlusion and haemodynamic shock (Schoenberg & Beger 1993). Intestinal I/R can also occur as a consequence of surgical procedures such as cardiopulmonary bypass, aortic aneurysm repair and intestinal transplantation (Schoenberg & Beger 1993). Reperfusion of ischaemic tissues can activate endothelial cells to produce superoxide and consequently inflammatory mediators are released. The actions of these deleterious reactive species can be prevented by antioxidants, either given exogenously, or with endogenous antioxidants such as glutathione (GSH). The intensity of the inflammatory reaction in postischaemic tissues can be also great that the injury response to reperfusion is also manifested in distant organs (Neary & Redmond 1999). Thus, intestinal ischaemia-reperfusion injury can lead to multiple organ failure (Neary & Redmond 1999). Experimental intestinal ischaemia reperfusion injury has been shown to lead to damage to the liver (Vejchapipat et al. 2001), lungs (Iglesias et al. 1998), heart (Stefanutti et al. 2004) and kidneys (LaNoue, Jr. et al. 1996) and can thus be considered a useful model of SIRS and MODS.

Some studies suggested that hypothermia may protect the gut in intestinal I/R injury by induction of heme oxygenase-1 above the levels observed at normothermia (Attuwaybi et al. 2003) and prevents an increase in nuclear factor Kappa-B (NF-kappaB) and inducible nitric oxide synthase (iNOS) expression (Hassoun et al. 2002). Hypothermia has been shown to prevent liver bioenergetic failure and mortality during experimental intestinal I/R injury (Vejchapipat et al. 2001). The protective effect of hypothermia is associated not only with a reduction in intestinal injury (Attuwaybi et al. 2003;Vejchapipat et al. 2002), but also with prevention of hepatic

bioenergetic failure (Vejchapipat et al. 2001), preservation of myocardial metabolism (Stefanutti et al. 2004), reduced lung neutrophil infiltration (Vinardi et al. 2003), and decreased oxidative stress in plasma, lungs and kidneys (Stefanutti et al. 2005). Interestingly, in a study of the effects of moderate hypothermia on oxidative stress following intestinal ischaemia-reperfusion injury, it has been shown that GSH levels were maintained by moderate hypothermia, and were even higher than control normothermic animals in control hypothermic animals (Stefanutti et al. 2005). Hence, during hypothermic intestinal ischaemia-reperfusion, GSH synthesis may even be increased above the rate observed under normal conditions, whereas decreased rates of glutathione synthesis during normothermic intestinal ischaemia-reperfusion may explain the depletion of GSH in liver (Turnage et al. 1991) and intestine (Bhaskar et al. 1995;Gibson et al. 1993) following intestinal I/R.

1.2 Inflammation, infection and free radical production

Inflammation is controlled by white blood cells, macrophages and endothelial cells, and by the cytokines they produce. Infections may activate macrophages, which causes enhanced production of pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6 and tumour necrosis factor-alpha (TNF-α). These cytokines induce the production of secondary mediators like nitric oxide, arachidonic acid metabolites, bradykinin, and histamine. Then, in turn, these may further activate macrophages, leukocytes and endothelial cells to perpetuate the process (Despond et al. 2001). During sepsis, oxygen radicals and nitric oxide (NO) are released at the same time (Goode et al. 1995);(Tanjoh et al. 1995). Release of both factors by macrophages may be a normal response to bacterial infection, but the regulation of release of oxygen radicals and NO varies and interacts among these substances (Groeneveld & Sipkema 2000). Another potential source of reactive oxygen and nitrogen species is activated neutrophils. These cells kill pathogens by producing a variety of cytotoxic chemicals, including hydrogen peroxide and the hypochlorite ion. NADPH oxidase of neutrophils and macrophages is an enzyme whose main function is generation of superoxide for bacterial killing. It catalyses the reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet -}$$

However, excessive superoxide produced in this cascade is harmful to host tissues and affects cell structures which may trigger organ dysfunction. Another important source of oxygen free radicals in the cell is mitochondria. The organelle consumes more than 90% of the inhaled oxygen. Normally only about 1 ~ 2% of total oxygen reduced in mitochondria is converted to superoxide (Thomas &

Balasubramanian 2004). Mitochondrial function is affected after sepsis (Taylor et al. 1998). Once mitochondrial dysfunction occurs, the amount of superoxide generation can increase considerably making this organelle an important cellular source of reactive oxygen species (ROS) (Thomas & Balasubramanian 2004). Reactive oxygen and nitrogen species can also react together and produce further recative species. An example of this is peroxynitrite, $OONO^{\bullet-}$, which is produced by the reaction of nitric oxide with superoxide (Beckman & Koppenol 1996). Peroxynitrite is extremely recative and can damage many proteins by nitration (MacMillan-Crow et al. 1998). Its production is greatly increased during sepsis, and this results in an increase in protein nitration (Fukumoto et al. 2004;Marcondes et al. 2001).

In general, oxidative stress, such as production of ROS and reactive nitrogen species (RNS), can cause damage to proteins, membrane lipids, carbohydrates and DNA. These, in turn, can trigger further chemical, biochemical, cell signalling and immunological reactions which ultimately result in organ failure. In order to protect against these effects and to prevent oragn damage, antioxidant defences are extremely important.

1.3   Antioxidant defences

Antioxidant defences can often be induced by exposure of organisms to ROS/RNS and to cellular signal molecules such as cytokines. Antioxidant defences can be enzymatic, where an enzyme has a specific function in reacting with reactive oxygen or nitrogen species (Mates et al. 1999). Examples of antioxidant enzymes are superoxide dismutase, catalase and glutathione peroxidase (Mates et al. 1999). Alternatively, antioxidants can be chemical anti-oxidants, where a chemical interacts directly with reactive oxygen or nitrogen species (Eaton 2006). These chemicals may require the action of enzymes to reconvert them to active forms. In addition to chemicals whose main function is to provide an antioxidant defence, such as vitamin E and vitamin C, many other chemicals within the body can act as antioxidants even though they have another main function (Roche et al. 2008;Sedlak et al. 2009). I will provide a brief overview of the main antioxidant defence systems, and will then consider the glutathione system in greater detail, as it is the main focus of this thesis.

1.3.1   *Superoxide dismutase*

One of the most important antioxidant defence enzymes is superoxide dismutase (SOD), which exists in both cytosol and mitochondria. SOD (CuZn-SOD, Mn-SOD and Fe-SOD) was discovered at the same time as the discovery of toxicity of ROS/RNS (Fridovich 1995). The metal atoms form the active site of the enzyme, and so an adequate supply of copper, zinc and manganese is necessary for adequate mammalian anti-oxidant defences (Fe-SOD is found only in plants and bacteria (Fridovich 1995)). Mn-SOD is found exclusively within mitochondria (so is not

found in erythrocytes) whereas CuZn-SOD is found in the cytosol, lysosomes, the nucleus and in the mitochondrial intermembrane space (Fridovich 1995). Another isoform of CuZn-SOD is found extracellularly. Mutations in the human gene encoding the cytosolic CuZn-SOD cause amyotrophic lateral sclerosis (a neurodegenerative disease). The biological role of SOD is to scavenge superoxide, with the catalytic activity:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

It is important to note that this reaction therefore scavenges 2 mol superoxide, but in turn produces 1 mol hydrogen peroxide, which is itself reactive and can damage enzymes and membranes (Halliwell & Gutteridge 2004d).

1.3.2 *Catalases*

Two different classes of enzymes have specific roles in detoxifying hydrogen peroxide; catalase and peroxidases. Hydrogen peroxide is not only produced by the activity of superoxide dismutases, but can also be produced by the action of several oxidases, including urate oxidase, D-amino acid oxidases and acyl-CoA oxidase. These oxidases are mostly found within the peroxisome, which is the main subcellular location for catalase. Importantly, mitochondria contain little or no catalase, so that hydrogen peroxide generated by the mitochondria Mn-SOD must be disposed of via another mechanism (Kirkman & Gaetani 2007). The reaction of catalase is as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$
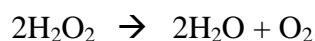
Mutations in the human gene encoding catalase cause the disease acatalasaemia, which is a relatively benign disorder leading to mouth ulcers (Goth, Rass, & Pay 2004).

1.3.3 *Glutathione peroxidase*

As mitochondria completely lack catalase, hydrogen peroxide must be detoxified via another route. The enzyme glutathione peroxidase catalyses the reaction of reduced glutathione (GSH) with hydrogen peroxide to form oxidised glutathione:

$$2GSH + H_2O_2 \rightarrow GSSG + H_2O$$
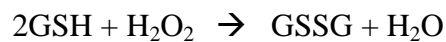
However, glutathione peroxidase (Gpx) is also active towards lipid peroxides, catalysing the general reaction to the corresponding alcohol:

$$2GSH + LOOH \rightarrow GSSG + LOH.$$
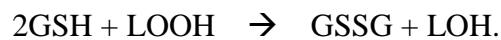
In mammalian tissues, there are four major GPx isozymes: (a) GPx1, which is found in erythrocytes, liver, lung and kidney; (b) gastrointestinal GPx (GPx2); (c) plasma GPx (GPx3), also found in kidney, lung, heart and muscle, and (d) phospholipid GPx (GPx4), which is also broadly distributed in different tissues. GPx1 is found in the cytosol, nucleus and mitochondria; GPx2 in the cytosol and nucleus; GPx3 is a secreted protein also found in the cytosol and GPx4 is present in the nucleus, cytosol, mitochondria and bound to membranes (Margis et al. 2008). These GPx isoforms all contain selenocysteine at the active site, and so an adequate dietary selenium supply is essential for adequate GPx activity. As the British diet is lower in selenium than it was, glutathione peroxidase activity is submaximal in many

individuals (Rayman 2008). Glutathione, the essential co-substrate for GPx activity, will be considered in more detail in section 1.4 below.

1.3.4 *Chemical anti-oxidants*

In addition to enzymatic methods for detoxifying reactive oxygen and nitrogen species, direct chemical antioxidants are also important within the body. A huge range of compounds, many of which are dietary constituents or can be taken as dietary supplements, has been suggested to have anti-oxidant activity (Halliwell & Gutteridge 2004a). However, whether many of these compounds have relevant *in vivo* antioxidant activity is uncertain. I will not aim to provide a comprehensive review of chemical anti-oxidants, but instead will briefly consider systems of known biological importance.

1.3.4.1 *Vitamin E*

Vitamin E, a fat soluble vitamin, is essential in the diets of animals (Clarke et al. 2008). It consists of several related tocopherols and tocotrienols, the most common of which is *dl*-α-tocopherol (Clarke et al. 2008). Its role is the protection of biological membranes, which it does by interruption of the chain of lipid peroxidation (Traber & Atkinson 2007). After small molecule reactive oxygen and nitrogen species attack lipids in membranes, they produce lipid peroxides, which react with other unsaturated lipid in membranes to produce further lipid peroxides, thus amplifying damage (Traber & Atkinson 2007). These peroxides can be scavenged by glutathione peroxidase (see above), or by tocopherols, which are within the lipid membranes themselves. Vitamin E has a hydrophobic chain, which

allows it to insert into biological membranes, and a hydrophilic chromanol ring which is responsible for reacting with lipid peroxides (Traber & Atkinson 2007) (Figure 1.1). Vitamin E cannot be synthesised by animals, but is present in many dietary sources and so human deficiency is rare.

Vitamin E: $dl - \alpha - $ tocopherol

Vitamin C: Ascorbate   Dehydro-ascorbate
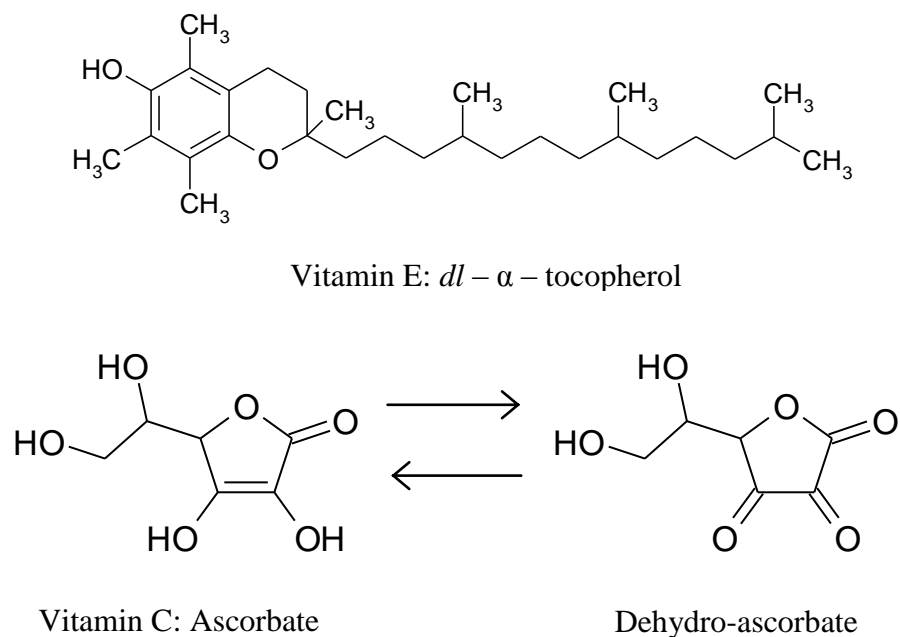
Figure 1.1 Vitamin E and Vitamin C

1.3.4.2 *Vitamin C*

Vitamin C (Ascorbic acid), the other major chemical antioxidant within the body, can be synthesised from glucose in plants and most animals but not in humans in whom the enzyme of the terminal step, gulonolactone oxidase, is not present. Therefore, humans require vitamin C to be present in the diet, and scurvy is a well known disease in humans with lack of vitamin C. Vitamin C is one of the water-soluble vitamins, and so is present in both extracellular and intracellular fluids,

whereas vitamin E is in membranes. Ascorbate, upon scavenging radicals, is converted to the ascorbyl radical and then to dehydroascorbate (Figure 1.1). Ascorbic acid will tend to reduce more-reactive species such as hydroxyl radicals, oxygen free-radicals and urate radicals and is able to scavenge a wide variety of both recative oxygen and recative nitrogen species. In addition to its activity as an anti-oxidant, ascorbate is also an essential co-factor for some enzymes, such as some hydroxylases. As ascorbate and the glutathione systems have overlapping functions, glutathione can replace some of the roles of ascorbate *in vivo* and *vice versa* (Meister 1994). In addition, glutathione appears to be also able to influence the recycling of dehydroascorbate to ascorbate (Meister 1994), although whether reduced glutathione is a substrate of a specific enzyme whose sole function is to recycle dehydroascorbate to ascorbate is unclear (Linster & Van Schaftingen 2007). Nevertheless, young rats given inhibitors of glutathione synthesis show markedly decreased levels of ascorbate and increased levels of dehydroascorbate, as well as the expected glutathione deficiency (Martensson & Meister 1991).

1.3.4.3 *Other chemical anti-oxidants*

In addition to these two well-defined anti-oxidant chemicals, several other biological compounds have anti-oxidant activity and may be relevant *in vivo*. Some of these other antioxidant compounds, such as bilirubin and hormones are shown in Figure 1.2.

Bilirubin is an end-product of haem degradation in mammals. *In vitro*, bilirubin is a powerful scavenger of peroxy radicals and singlet oxygen. Bilirubin bound to albumin can protect both albumin itself, and albumin-bound fatty acids

against free-radical damage, although the mechanism for the apparent protective effects of elevated bilirubin levels are unknown (Sedlak & Snyder 2004).

The female sex hormones oestrandiol, oestrone and oestriol can inhibit lipid peroxidation (including low-density lipoprotein (LDL) peroxidation) in vitro at micromolar concentration (Keaney, Jr. et al. 1994). They can act as chain-breaking antioxidants in a way similar to that of vitamin E (Keaney, Jr., Shwaery, Xu, Nicolosi, Loscalzo, Foxall, & Vita 1994), but the levels if these hormones *in vivo* may be too low to exert significant anti-oxidant activity (Halliwell & Gutteridge 2004e).

The hormone melatonin is mainly produced by the pineal gland at the base of the brain to help regulate circadian rhythms. Melatonin shows antioxidant activity *in vitro* (probably by donation of hydrogen by the NH group), and may show anti-oxidant effects through upregulation of synthesis of antioxidant defence enzymes rather than via any direct anti-oxidant effect *in vivo* (Anisimov et al. 2006).

Lipoic acid, thioctic acid (1,2-dithiolane-3-pentanoic acid) is an essential cofactor as its amide form (lipoamide) in the multi-enzyme complexes. The complexes catalyse the decarboxylation of α-keto acid such as pyruvate and α-ketoglutarate in the citric acid cycle. Both the oxidised and reduced forms of lipoic acid show antioxidant properties *in vitro* (Packer, Witt, & Tritschler 1995) and some authors have also suggested an *in vivo* anti-oxidant role in combination with acetyl-carnitine (Hagen et al. 2002).

Coenzyme Q (ubiquinone) plays an essential role in the mitochondrial electron-transport chain, undergoing simultaneous oxidation and reduction via a free-radical intermediate, ubisemiquinone. *In vivo*, ubiquinol ($CoQH_2$) can scavenge

organic radicals and thereby inhibit lipid peroxidation. Like vitamin E, coenzyme Q is found within biological membranes, and thus protects lipids and low-density lipoprotein (LDL) from lipid peroxidation (Bentinger et al. 2007). Ubiquinol can regenerate α-tocopherol (vitamin E) from its radical in lipoproteins and membranes, so coenzyme Q may be synergistic with vitamin E *in vivo* (Quinn et al. 1999;Stoyanovsky et al. 1995).
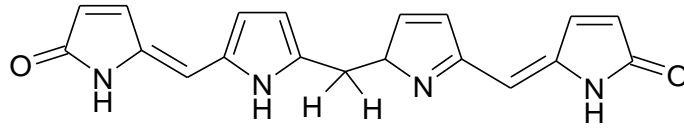
Bilirubin
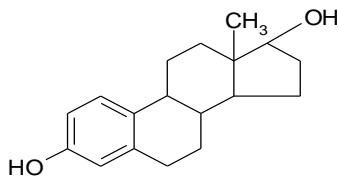
Oestradiol
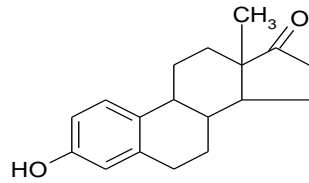
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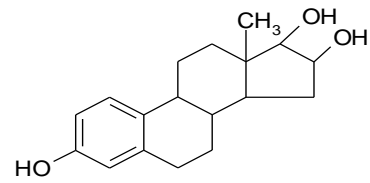
Oestriol
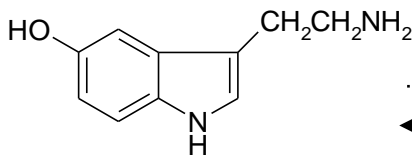
Serotonin
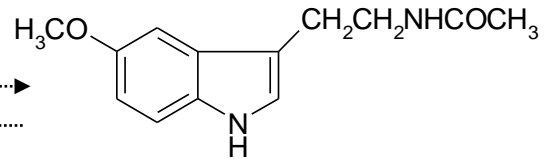
Melatonin
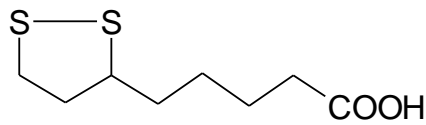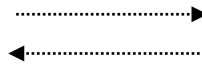
Lipoic acid
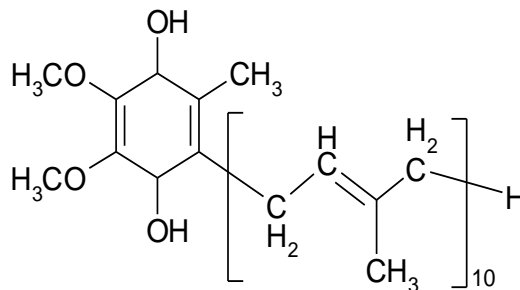
Co-enzyme Q

Figure 1.2 Chemical antioxidants

1.4   Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH), a tripeptide, is synthesized *de novo* within many cells from the precursor amino acids cysteine, glutamate and glycine, and is a major low molecular weight thiol in mammalian cells (Figure 1.3).
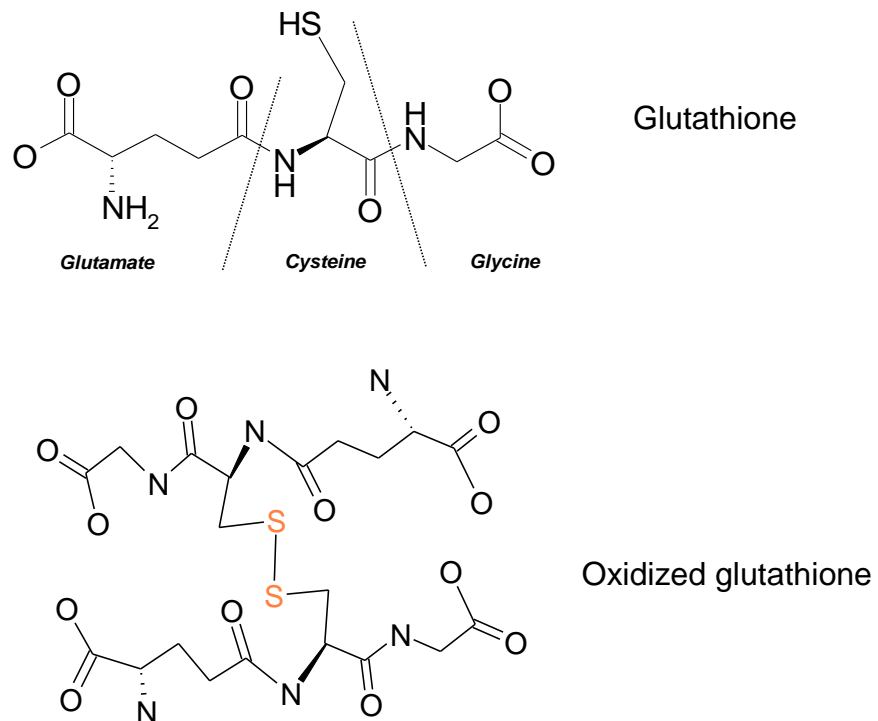
Figure 1.3 Chemical structure of Glutathione
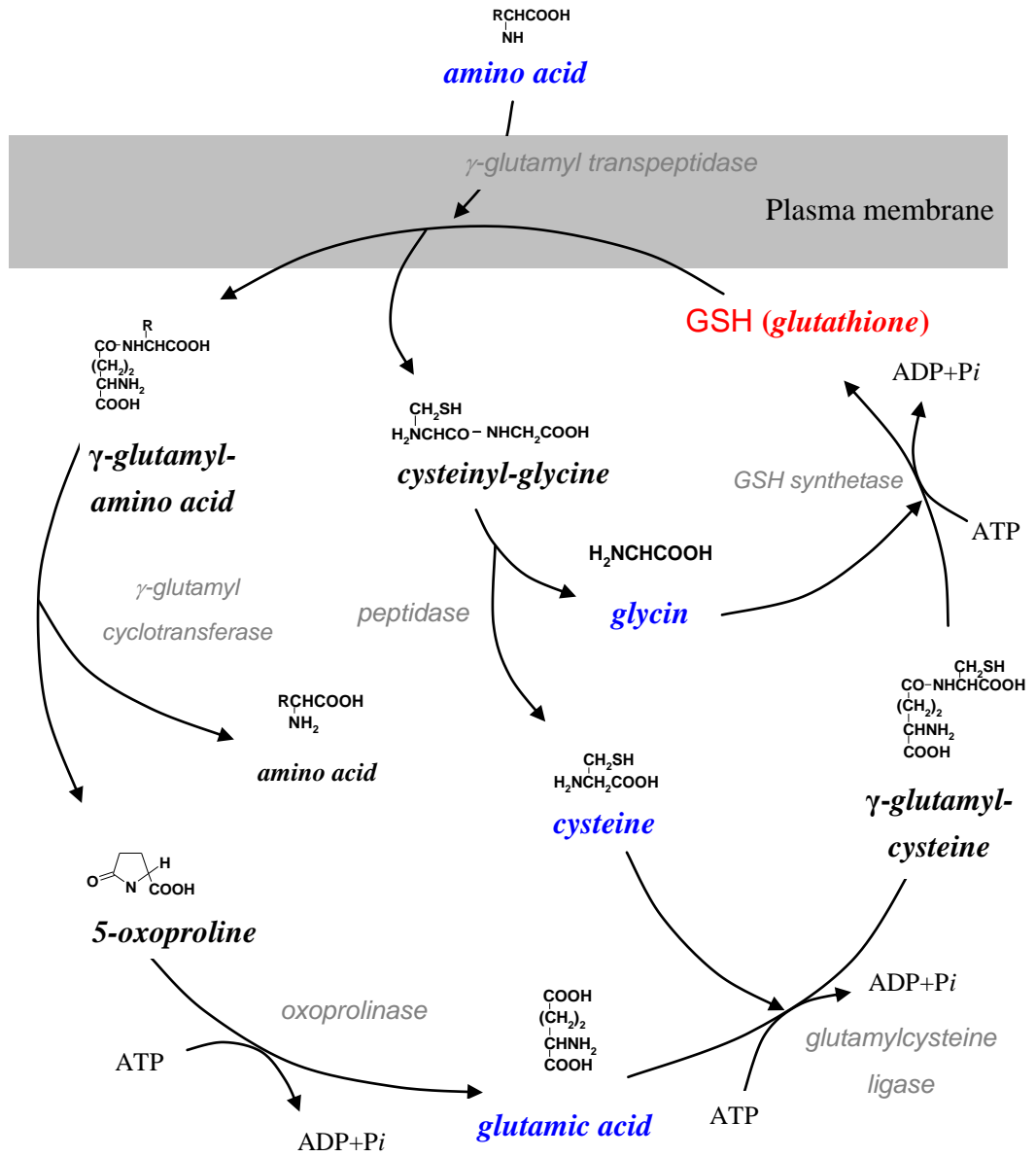
Figure 1.4 GSH synthesis and metabolism

The intracellular level of GSH in mammalian cells is in the millimolar range (0.5 − 10 mM), whereas micromolar concentrations are typically found in blood plasma (Meister & Anderson 1983). A small proportion of the total cellular GSH pool (10–15%) is found in mitochondria, where it reaches a concentration similar to that found in the cytosol (Griffith & Meister 1985). GSH plays a major role in cellular defenses against oxidative stress. It takes part in the reductive detoxification of hydrogen peroxide, although hydrogen peroxide can also be reduced by catalase, which is present only in the peroxisome. GSH in the mitchondria is particularly important because there is no catalase (Fernandez-Checa et al. 1997). The biosynthesis of GSH (Figure 1.4) is also associated with transport of extracellular amino acids intracellularly (Meister & Anderson 1983). The glutamyl group of GSH is readily transferred by γ-glutamyl transpeptidase to form the γ-glutamyl derivatives of free amino acid in the first reaction of the γ-glutamyl cycle. Since γ-glutamyl transpeptidase is usually membrane bound, it has been suggested that this reaction might serve to capture amino acids from one side of the membrane (Beutler 1989;Meister 1973). Synthesis of GSH requires two steps: firstly the formation of γ-glutamyl-cysteine from L-glutamate and L-cysteine, catalysed by γ-glutamyl-cysteine ligase, an ATP-requiring enzyme; and secondly the formation of GSH from γ-glutamyl-cysteine plus L-glycine, catalysed by GSH synthetase, also requiring ATP. The first of these enzymes is inhibited by GSH and is thought to be responsible for much of the control of GSH synthesis (Lu 1998).

1.4.1 *Glutamine as a GSH Precursor*

Although glutamate is the immediate precursor for GSH synthesis, glutamine has a role, particularly important in critical illness, as a GSH precursor. For most cell types, extracellular glutamine is a better source of intracellular glutamate than extracellular glutamate, so that synthesis of GSH is supported by extracellular glutamine during increased oxidative stress such as that which occurs during critical illness (Babu et al. 2001). Glutamine is a better precursor than glutamate for GSH synthesis because: (i) the extracellular concentration of glutamine is much higher than that of glutamate; (ii) some cells, such as erythrocytes, do not have a specific glutamate transporter and so glutamate uptake is extremely low (Ellory et al. 1983), so that extracellular glutamine, taken up by the Na^+-dependent system N, is a better source of intracellular glutamate. Various groups have shown depletion of muscle GSH during critical illness and/or surgical trauma (Brealey et al. 2002;Hammarqvist et al. 1997;Luo et al. 1996), and this depletion can be counteracted by provision of glutamine (Flaring et al. 2003). Hepatic GSH can also be replenished by provision of glutamine after depletion in experimental animals (Hong et al. 1992), and plasma GSH depletion can be prevented by glutamine, both experimentally (Denno et al. 1996) and clinically (Luo et al. 2008), but the metabolic and clinical significance of plasma GSH is uncertain.

1.4.2. *Antioxidant function of GSH*

As described in section 1.3.3 above, the major anti-oxidant role of GSH is as a substrate of GSH peroxidases to enable detoxification of hydrogen peroxide and lipid hydroperoxides. In this reaction (Figure 1.5), GSH is oxidised to GSH disulfide (GSSG). GSSG is then reduced intracellularly to GSH by GSH reductase at the expense of NADPH (Beutler 1989;Meister & Anderson 1983). The GSH reduction/oxidation (redox) state reflects the balance between the levels of oxidation and reduction in the GSH buffer system. At normal levels of oxidative stress, intracellular GSSG levels are extremely low, and GSH makes up over 99% of total GSH levels (Akerboom et al. 1982;Griffith 1999;Meister & Anderson 1983). However, GSSG can be lost from cells rapidly, leading to a decrease in cellular total GSH during oxidative stress, when GSSG levels are increased (Rahman et al. 1995;Srivastava & Beutler 1969). Additionally, GSH may become depleted during oxidative stress by reaction with hypochlorous acid produced as part of the bacterial killing process (Pullar et al. 2001).
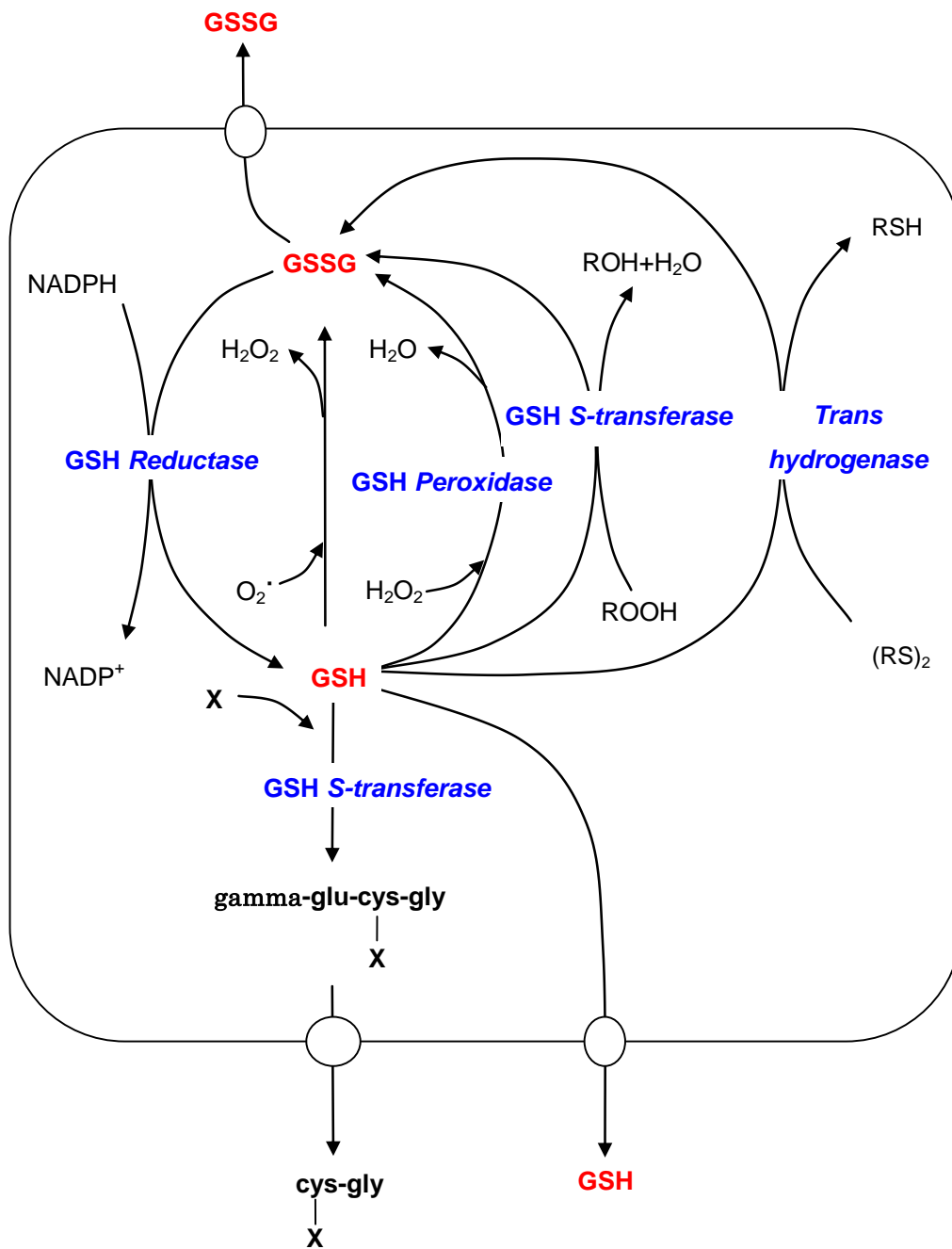
Figure 1.5   Antioxidant function of GSH

As well as its role in the GSH peroxidase reaction, GSH can also act in other important ways to protect against oxidative stress. GSH *S*-transferases act as detoxification enzymes defending cells against peroxides induced by toxic chemicals. Hence, for example 4-hydroxynonenal can be conjugated to GSH by GSH *S*-transferases and excreted in bile (Alin et al. 1985;Warnke et al. 2008) . Many lipid peroxides can therefore by detoxified either by conjugation to GSH by GSH *S*-transferase or reduced by GSH peroxidase (Chandan 1997;Shelly 1999). GSH is also extremely important for maintaining intracellular thiol groups (for example free thiol groups of proteins) in the reduced state. Cysteine thiol groups of proteins are vulnerable to oxidative damage during inflammation and oxidative stress. Glutathionylation of these cysteine residues provides a reversible protection mechanism, as well as a redox-sensing mechanism by means of which transcription factors can be triggered by oxidative stress (Gianazza et al. 2009;le-Donne et al. 2009). The metabolism of intracellular thiols is complex, with thioredoxin and glutaredoxin both playing crucial roles in addition to GSH (Johansson et al. 2004;Jung & Thomas 1996).

In addition to protecting against peroxides and hypochlorous acid, GSH can act to decrease high levels of nitric oxide and thereby protect against potent nitrating chemicals, such as peroxynitrite, generated when nitric oxide levels are high, as in sepsis and other inflammatory conditions. Reaction of GSH with nitric oxide generates *S*-nitrosoglutathione (Foster et al. 2009;Zhang & Hogg 2005). *S*-nitrosation of proteins can alter function, and GSH can be nitrosated to *S*-nitrosoglutathione by nitrosated proteins (Foster et al. 2009;Zhang & Hogg 2005). *S*-nitrosoglutathione can subsequently be de-nitrosated by nitrosoglutathione

reductase (a recently discovered function of formaldehyde dehydrogenase); thus, GSH appears to have an important role as an intermediate in de-nitrosation pathways (Haqqani et al. 2003;Liu et al. 2004;Steffen et al. 2001). It can also act as a nitric oxide carrier between tissues, so has nitrosating properties too. The function of GSH in modulation of cellular *S*-nitrosation is an area of great current interest. Peroxynitrite is produced during sepsis, partially by the reaction of superoxide with nitric oxide, and is potentially very damaging to host tissues as it is highly reactive towards tyrosines, tryptophans and other residues in proteins. Whether GSH reacts directly with peroxynitrite or decreases peroxynitrite damage by scavenging nitric oxide is uncertain, as the reaction of GSH with peroxynitrite *in vitro* is rather slow (Schrammel et al. 2003).

These functions of GSH are very important as defence factors of critically ill patients. Adequate GSH levels prevent excessive oxidative stress induced by infections or an inflammatory response, to prevent further organ dysfunction. It is also strongly associated with the immune system to protect neutrophils and macrophages against the bactericidal compounds that they produce. Cells of the immune system, when stimulated, produce huge quantities of reactive species for bacterial killing, and GSH is essential to protect these cells from the deleterious effects of these reactive species on host cells, for example by reaction with hypochlorous acid (Pullar et al. 2001). The redox state of GSH (i.e. the ratio of cytosolic GSH to GSSG) is a key component of the signalling system influencing the translocation of the transcription factor NFκB which regulates the synthesis of cytokines and adhesion molecules. Thus GSH can be regarded as anti-inflammatory (Peristeris et al. 1992;Saccani et al. 2000;Sato et al. 1996) but is

necessary for cytotoxic T-cell activity and neutrophil function (Droge & Breitkreutz 2000;Staal et al. 1992;Villa et al. 2002) hence regulation of GSH levels within cells of the immune system is an important part of host defence during infection and sepsis.

The deficiencies of GSH and GSH related enzymes can be lethal if the disorder occurs from either congenital or acquired diseases. Inherited deficiencies of γ-glutamylcysteine synthetase (very rare), GSH synthetase (less rare), GSH reductase, γ-glutamyltranspeptidase, and 5-oxoprolinase have been reported (Kamerbeek et al. 2007;Ristoff & Larsson 2007). In addition, congenital GSH peroxidase deficiency can be caused by lack of dietary selenium during pregnancy (Perona et al. 1979), as selenocysteine is essential for the active site of the enzyme, and GSH peroxidase activity can be affected both by selenium status and gene polymorphisms (Hesketh 2008). As erythrocytes are very dependent on the pentose phosphate pathway to supply NADPH to recycle GSSG to GSH, invididuals with glucose-6-phosphate dehydrogenase deficiency (common in Medinterranean populations) can also suffer from lack of reduced GSH and a functional decrease in erythrocyte GSH peroxidase activity (Kurata et al. 2000)

Newborn mice with GSH reductase deficiency show early cataracts (Meister 1995). Glutathionemia (excess GSH in the blood) occurs with aberrant expression of γ-glutamyltranspeptidase. Transgenic mice with lack of γ-glutamyltranspeptidase showed elevated plasma GSH but decreased tissue levels, suffered growth retardation and developed cataracts, and most died between 10 and 18 weeks of age (Lieberman et al. 1996). γ-Glutamyltranspeptidase deficiency can lead to an imbalance in glutamic acid homeostasis, so the disease is sometimes

classed as an inherited disorder of dicarboxylic acid catabolism. (Townsend et al. 2003) A severe GSH synthetase deficiency (5-oxoprolinuria) results in an increase in γ-glutamylcysteine synthetase activity through a feedback mechanism, leading to an accumulation and excretion of 5-oxoproline (Meister & Larsson 1989). In humans, γ-glutamylcysteine synthetase activity is diminished in patients with haemolytic anaemia (Beutler et al. 1999).

1.4.3 *GSH metabolism in infants and children.*

There is limited knowledge on GSH metabolism specific to infants and children. Birth itself is marked by a period of relative hypoxia, followed by oxygenation, and thus can be considered analogous to ischaemia-reperfusion. In term infants, anti-oxidant defences, including GSH, are adequate to counteract this challenge, because of the increases in anti-oxidant defences in late gestation (Friel et al. 2004). However, anti-oxidant defences on preterm infants may be less effective because of immaturity of anti-oxidant enzymes and decreased concentration of anti-oxidants and their precursors (Thibeault 2000).

   Although preterm infants show diminished availability of other components of the antioxidant defense systems, the GSH concentrations in cord blood of preterm infants at birth exceed those of term infants (Ahola et al. 2004;Jain et al. 1995;Jean-Baptiste & Rudolph 2003). GSH concentrations, however, fall rapidly after birth in preterm infants (Ahola et al. 2004;Jain et al. 1995;Jean-Baptiste & Rudolph 2003), despite the presence of significant hepatic gamma-glutamyl cysteine synthetase activity from the second trimester of pregnancy (Levonen et al.

2000). This appears to be due to lack of early amino acid supply, as earlier intervention with amino acids increases GSH concentration in preterm infants (Braake et al. 2008). Paradoxically, although it would be expected that amino acid administration, especially cysteine, would increase GSH fractional synthesis rate, the effect of amino acid mixtures on GSH concentration appears to be mediated via a decrease in GSH consumption rather than an effect on GSH synthesis (Braake et al. 2009;Rook et al. 2010;te Braake et al. 2008). After the neonatal period, there is little available literature on alterations in GSH metabolism in later infancy and in childhood. The little available data suggests that there may be a lower GSH concentration in plasma in infants than in adults (Ono et al. 2001), although other authors have found no evidence for any age-related differences (Pastore et al. 1998). In septic infants and children, GSH concentration and synthesis rates are decreased compared with controls (Lyons et al. 2001), and this did not appear to be due to decreased cysteine availability. In contrast, in children with protein energy-malnutrition, the decreased rates of GSH synthesis *do* appear to be related to lack of availability of cysteine (Badaloo et al. 2002;Reid et al. 2000).

1.5   Methods to study antioxidant defences

In order to understand how anti-oxidant defences are useful and oxidative stress is important, a very wide variety of methods have been established. As free radical themselves are very reactive and short lived, they are rarely measured in body fluids or tissues, although they can be measured *in vivo* using specialised equipment and techniques. Most studies on anti-oxidants and oxidative stress therefore measure either end products ("footprints") of oxidative damage, or measure changes in concentration of anti-oxidants. I do not aim to provide a comprehensive review of these methods, but a very brief introduction to areas of methodology relevant to this thesis.

The thiobarbituric acid reactive substances (TBARS) test is one of the most frequently used measured of lipid peroxidation. It measures mainly (but not exclusively) malondialdehyde (MDA; malonaldehyde). In fact, MDA is formed only in small amounts during the peroxidation of most lipids, although larger amounts are produced during the peroxidation of liver microsomes in the presence of iron salts (Esterbauer et al. 1991). Other aldehydic lipids (e.g. 4-hydroxynonenal) are also products of lipid peroxidation which can be measured. Another major group of lipid peroxidation products is the isoprostanes. Isoprostanes are formed by peroxidation of phospholipids containing arachidonic acid. During conditions of oxidative stress, levels are increased. F_2-isoprostanes are useful markers in human blood plasma and urine, and 8-epi-$PGF_{2\alpha}$ which is a powerful renal vasoconstrictor is also measured (Roberts & Morrow 1997). These can be measured with highly

specific assays by HPLC, GC-MS and antibody-based determination (Halliwell & Gutteridge 2004b).

During oxidative stress *in vivo*, one or more antioxidants, such as ascorbate and GSH, can become depleted. Loss of individual antioxidants and generation of oxidation products from them can be measured as an index of oxidative stress (Halliwell & Gutteridge 2004c). In addition, various assays have been developed as estimates of "total antioxidant activity" of body fluids (Miller et al. 1993;Wayner et al. 1987). An example of this is the ABTS (2,2'-Azinobis(3-ethylbenzothiazoline 6-sulphonate)) assay, which has been used in plasma. The contribution of various plasma components to this "total antioxidant capacity" can also be calculated (Table 1.1.; adapted from Miller et al. 1993).

Table 1.1 Antioxidant hierarchy in the plasma water.

	Concn. (µmol/l)		Antioxidant activity (% of total plasma activity)
	Range	Mid-point	
Albumin	535 - 760	640	43
Urate	180 - 420	300	33
Ascorbate	34 - 111	73	9
α-Tocopherol	14 - 44	29	3
Bilirubin	< 20	10	2
Unmeasured antioxidants			10

The remaining 10% of the total plasma antioxidant activity is accounted for by substances such as cysteine, GSH, β-carotene, dihydrolipoate and ubiqinone (Miller et al. 1993).

1.6　Analysis of Glutathione

Many different methods have been used for assessing GSH metabolism. The choice of method depends on what is required to be studied. As GSH exists in both oxidised and reduced forms, it is also importamt to decide whether it is necessary to know the concentration or metabolism of both oxidised and reduced forms, or whether measurement of the total GSH pool is adequate.

Intracellular GSH and GSSG constitute the free GSH forms. Proteins can become glutathionylated, and proteomics methods such 2D-SDS-PAGE followed by MALDI-TOF analysis (Cotgreave & Gerdes 1998),(Fratelli et al. 2002) have been used to determine which proteins GSH is bound to. As GSH and GSSG have a low molecular weight, they are soluble in strong mineral acids, and the first step of GSH analysis is usually de-proteinization (protein precipitation) by acidification (e.g. trichloroacetic acid, perchloric acid or sulfosalicylic acid). However, care must be taken at this stage if it is necessary to preserve the GSH/GSSG redox state in a physiological condition, as GSH can be oxidised to GSSG in strong acid, especially in the absence of metal chelators (Rossi et al. 2002). Deproteinization and centrifugation usually yields a clear, protein-free supernatant containing GSH and GSSG. As further manipulations in sample preparation lead to further thiol oxidation, at this stage the samples are usually subjected to either:

(i) where it is necessary to measure GSH and GSSG independently, the free thiol group of GSH is blocked by various agents (e.g. *N*-ethylmaleimide, iodoacetic acid, iodoacetamide or 2-vinyl pyridine), and then GSH (with blocked thiol) and GSSG are measured by the analytical method of choice

(ii) where it is more of interest to measure the total GSH pool, the sample is reduced by an excess of reducing agent, which can be performed with a variety of reducing compounds, such as sodium borohydride (Ivanov et al. 2001;Ivanov et al. 2000a;Ivanov et al. 2000b;Senft et al. 2000), dithiothreitol (DTT) (Cereser et al. 2001;Chung-Shi et al. 1995;Luo et al. 1995;Parmentier et al. 1998), 2-mercaptoethanol and trialkylphosphines: tributylphosphine (TBP) (Tang et al. 2000) and tris-(2-carboxyethyl)-phosphine (TCEP) (Abukhalaf et al. 2002;Anderson et al. 1999;Han & Han 1994), and the total GSH pool measured as GSH. More rarely, the entire GSH pool is oxidised, and measured as GSSG (Schierbeek et al. 2007).

The spectrophotometric assay for GSH, based on an enzymatic recycling reaction was first detected by Owens and Belcher (Owens & Belcher 1965) and then developed by Tietze (Tietze 1969). This method uses Ellman's reagent (5,5´-dithiobis-(2-nitrobenzoic)-acid, DTNB), yeast GSH reductase and NADPH and to measure the absorbance at 412nm. The mixture results in an excess colour yield, and GSH is detectable at concentrations as low as 10ng/ml (Chung et al. 2005),(Pastore et al. 2003). As the assay recycles oxidised to reduced GSH, which then reacts with DTNB, it measures total GSH status. Where it is necessary to measure GSH and GSSG independently using this method, GSH is first blocked, e.g. with 2-vinyl-pyridine. Then, only GSSG is able to react with DTNB, after conversion to GSH, thus obtaining the concentration of GSSG. Another assay is then carried out in the absence of 2-vinyl-pyridine, to obtain total GSH, and GSH calculated as the difference between total GSH and GSSG.

High-performance liquid chromatography (HPLC) for many years has been the major method of measuring GSH and related thiols in biological samples.

HPLC methods in determination of GSH and related compounds use several different detection methods.

HPLC with electrochemical detection (ECD) has been used for the analysis of redox-reactive compounds, such as thiols and disulfides (Richie, Jr. & Lang 1987). In this preparation, samples after protein precipitation with metaphosphoric acid are diluted with mobile phase. Thiol elution from the column is detected by dual electrochemical detector of two gold-mercury electrodes. This method has the advantage of speed and simplicity, and has also been used for analysis of related thiol compunds, such as gamma-glutamyl-cysteine (Gegg et al. 2002). One potential disadvantage is that as GSSG is not electrochemically active, GSH and GSSG cannot both be analysed in a single run.

HPLC with photometric detection, such as UV-Visible absorbance detectors, or diode array detectors (DAD), is simple and applicable to determination of GSH and GSSG. GSH and GSSG are both lacking in chromophores so derivatization with a suitable chromophore, e.g. DTNB or Sanger's reagent (2,4-dinitrofluorobenzene), is necessary. GSH and GSSG can be analysed in a single run together with other related thiols (Reed et al. 1980), but these methods generally have low sensitivity and specificity in comparison with ECD, fluorometric and MS determination (Camera & Picardo 2002).

HPLC with fluorometric detection of GSH was first described by Imai et al., using ammonium-7-fluoro-2,1,3-benzoxadiazole-4-slufonate (SBD-F) for determination of GSH and other thiols (Imai et al. 1983). This method has been improved and modified by Toyo'oka and Oe et al. (Oe et al. 1998;Toyo'oka & Imai 1983). GSH and other low-molecular-mass thiols are derivatised with SBD-F and

quantified after elution from a reverse-phase column. The methods based on SBD-F are sensitive and specific, and there are no particular interfering peaks in the chromatogram. However, it requires high temperature (60°C) and a long reaction time (Pastore et al. 2003). Monobromobimane (MBB) rapidly reacts with thiols at room temperature to produce a highly fluorescent thio-ether (Fahey et al. 1981). The method requires pH adjustment following derivatization, and MBB derivatives are usually separated from other thiols by gradient elution. MBB has been used for determining reduced, oxidised and protein-bound GSH in whole blood (Svardal et al. 1990),(Pastore et al. 2001). $NaBH_4$ is also used as a reduction reagent and total recovery of oxidised GSH can be obtained. *ortho*-phthalaldehyde (OPA) derivatization pre-column is another method of HPLC with fluorescence detection. OPA reacts widely with GSH and related amino thiols. However, derivatization of GSH with OPA is pH-dependent (Parmentier et al. 1998) and GSSG cannot react with OPA to form a fluorescent derivatives due to alkaline hydrolysis at pH 12 to produce GSH (Yan & Huxtable 1995). Improvement of chromatographic separation in several amino thiols derivatives is based on aqueous-polar organic mixtures with ion pairing reagents, such as tetrabutylammonium hydroxide (TBAH), trifluoroacetic acid (TFA) and sodium *n*-decysulfate (Chung-Shi et al. 1995;Ivanov et al. 2000b;Parmentier et al. 1998;Tang et al. 2000).

HPLC has been combined with mass spectrometry (MS), either as LC-MS or LC-MS-MS and (tandem mass spectrometry), and these methods have expanded the potential for high-throughput analysis of GSH and other low-molecular-mass thiols in biological samples. The advantages of LC-MS-MS methods are (i) derivatization is not usually necessary and (ii) the analysis time is very short

because chromatographic resolution of different components from each other is not important. Norris et al. described the assay of GSH and GSSG in mouse liver samples by using tandem MS (Norris et al. 2001). Petritis et al. tried the analysis of underivatised small peptides by using ion-pair LC-ELSD (LC coupled with evaporative light scattering detection) and tandem MS (Petritis et al. 2002). Guan et al. used LC-MS for analysis of biological samples in rats, and quantified GSH, GSSG, cysteine, homocysteine and homocystine (Guan et al. 2003).

Gas chromatography mass spectrometry (GC-MS) methods for analysis of GSH and other thiols have also been reported. GC-MS methods can analyse very low amount of samples for identification and quantification. GC-MS detection system requires volatile condition of compounds consequent to evaporation in a high temperature inlet port. Then, the eluants from the column reach the MS detector where they are ionized and detected (Eiceman et al. 2000). Derivatization in GC-MS is very important and is needed to lower the boiling point of the analyte. Only a few gas chromatographic methods for GSH have been described, as GSH itself is non-volatile, and derivatization yields bulky derivatives which are difficult to elute from GC columns. Kataoka and co-workers first used chloroformates as derivatizing agents for GSH, although they used flame photometric detection (Kataoka et al. 1995;Takagi et al. 1996). These derivatives have been subsequently exploited for GC-MS analysis of GSH (Capitan et al. 1999;Humbert et al. 2001), as described in more detail in Chapter 2. Other groups have used alternative derivatization methods such as derivatization to a complex bicycloglutarimide (Lyons et al. 2000), or methyl ester trifluoroacetyl derivatives (Faber et al. 2002). Although GC-MS analysis of GSH has been used to measure concentration, it is

more frequently used to measure isotopic enrichment. Another alternative approach applicable to measurement of GSH enrichment, has been to isolate GSH by HPLC, then to hydrolyse the collected peak and measure enrichment in glycine by GC-MS (Jahoor et al. 1999).

The methods for analysis of GSH are summarised on Table 1.2 below.

Table 1.2 Glutathione analysis

Method of analysis	*Analyte*	*Comments*	*References*
Proteomics (MALDI-TOF)	glutathionylated proteins	Includes 2D-SDS-PAGE	Cotgreave *et al*. 1998 Fratelli *et al*. 2002
Spectrophotometric assay (412nm absorbance)	GSH/GSSG	Enzymatic recycling Ellman's reagent (5,5´-dithiobis-(2-nitrobenzoic)-acid, DTNB)	Owens & Belcher 1965, Tietze 1969, Chung *et al*. 2005, Pastore *et al.* 2003
HPLC (electrochemical detection)	either GSH or GSSG	GSH and GSSG cannot both be analysed in a single run	Richie, Jr. *et al*. 1987, Gegg, Clark, & Heales 2002
HPLC (photometric detection)	GSH/GSSG	DTNB or Sanger's reagent (2,4-dinitrofluorobenzene) analysis in a single run	Reed *et al*. 1980,
HPLC (fluorometric detection)	GSH/GSSG and protein bound GSH	Ammonium-7-fluoro-2,1,3-benzoxadiazole-4-slufonate (SBD-F) Derivatives: Monobromobimane (MBB) *Ortho*-Phthalaldehyde (OPA) Tetrabutylammonium hydroxide (TBAH) Trifluoroacetic acid (TFA) Sodium *n*-decysulfate	Imai *et al.*1983 Oe *et al*. 1998 Fahey *et al*. 1981 Parmentier *et al*. 1998 Chung-Shi *et al*. 1995, Ivanov,

			Nazimov, & Baratova 2000, Parmentier *et al.* 1998, Tang, Wen, & Santschi 2000
Tandem Mass Spectrometry (LC-MS-MS)	GSH/GSSG	Derivatisation is not usually necessary	Norris *et al.* 2001
LC-ELSD (LC coupled with evaporative light scattering detection)	Small peptides	Underivatisation	Petritis *et al.* 2002
LC-MS	GSH/GSSG, CysSH, HCysSH and homocystine	Ellman's reagent	Guan *et al.* 2003
GC-MS	GSH (total)	Chloroformates *N*-pivaloyl & *O*-isopropylates (NPP) Bicycloglutarimide Methyl ester +Trifluoroacetylate	Kataoka *et al.*1995, Husek 1998, Eiceman *et al.* 2000 Metges *et al.*1996 Lyons *et al.* 2000 Faber *et al.* 2002
GC-IRMS	GSH	Chloroformates ^{13}C-/^{2}H-GSH ^{13}C-GSH	Kimura *et al.* (*SIMSUG*), 2007 Tea *et al.* 2007
LC-IRMS	GSH/GSSG	^{13}C-GSH	Schierbeek *et al.* 2007

1.7 Isotope ratio mass spectrometry in metabolic research

Stable isotope tracer methodologies are well characterised for measurement of metabolism *in vivo*. However, these methods mostly use GC-MS for measurement of tracer or product enrichment; some other methods use liquid-chromatography-mass spectrometry (LC-MS) or nuclear magnetic resonance (NMR). However, these techniques are only sensitive to fairly large changes in enrichment, for example down to 0.5% atom percent excess (Meier-Augenstein 1999a). Gas isotope ratio mass spectrometry (IRMS), based on magnetic sector instruments, has been a standard tool in archaeology, geochemistry and environmental science for analysis of isotopic enrichment of gases, water, or bulk samples (via an elemental analyser). However, the IRMS instruments have been used with GC via a combustion interface, for compound-specific isotope analysis of $^{13}C/^{12}C$, since 1990. These instruments are now commercially available. More recently, compound-specific analysis of $^2H/^1H$, $^{18}O/^{16}O$ and $^{15}N/^{14}N$ has also become possible using appropriate high-temperature reactions.

Since then, GC-C-IRMS has been applied to other areas of analytical chemistry in where GC-MS and LC-MS are commonly used for analysis in complex matrix or variety concentrations of substances. GC-C-IRMS is coupled a dual collector mass spectrometer to a GC via combustion interface for recording of $(m + 1)/m$ isotope ratio by detecting two successive masses simultaneously (Meier-Augenstein 1999a;Wolfe 1992). GC-C-IRMS can measure isotope composition at very low enrichment and natural abundance level. It means that minute variations in small amount of heavier isotope are detected in comparison with large amount of

lighter isotope (Meier-Augenstein 2004). Isotopic differences which cannot be detected by GC-MS can be detected by GC-IRMS. GC-C-IRMS enables the more precise analysis of compound-specific isotope especially at natural isotopic abundance level. Compound-specific isotope analysis at natural abundance level can provide information on the biogenic relation and origin of a given organic compound. Minute differences in the isotopic abundance of ^2H, ^{13}C, ^{15}N or ^{18}O can be useful in testing adulteration of foodstuff, or doping of sport players or race hoses in comparison with reference data. Compound-specific isotope analysis in tracer studies is of increasing interest. This approach is concerned with quantitative studies of biochemical processes. This isotope tracer studies is for assimilation or incorporation of nutrients, turnover rates of biologically important molecules, and quantification of protein synthesis (Meier-Augenstein 1999a).

IRMS instruments achieve highly precise measurement of isotopic abundance at the expense of the flexibility of scanning MS. For isotope ratio measurement, the analyte must be converted into a simple gas which is isotopically representative of the original sample. Isotope ratio measurements of $^2H/^1H$, $^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ use continuous flow of gases of H_2, N_2, CO_2, CO and SO_2, respectively. IRMS determines the difference in isotope ratio with great precision and accuracy rather than the absolute isotope ratio. It provides the information of isotopic abundance of the analyte gas relative to the measured isotope ratio of a standard or reference gas (Meier-Augenstein 1999a).

In measurement of $^{15}N/^{14}N$ as N_2 gas, Metges, C. et al. measured ^{15}N isotopic abundances of *N*-acetyl-n-propyl (NAP)- and *N*-pivaloyl-i-propyl (NPP)-amino acid esters in physiological samples by using GC-C-IRMS (Metges, Petzke,

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& Hennig 1996). This was a pilot study of amino acid synthesis in minipigs. The same group had analyzed individual plasma free amino acids of human adults at natural abundance to measurement of $^{15}N/^{14}N$ isotopic composition (Metges & Petzke 1997).

In measurement of $^{13}C/^{12}C$ as CO_2 gas, the data comprise three different isotope combinations of $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$ with their corresponding masses at m/z 44, 45 and 46, respectively. Utility of GC-C-IRMS has been expanded in compound specific isotope analysis (CSIA) by using ^{13}C-labelled compound. Infusion of ^{13}C-labelled glutamine tracers used for measurement of the rates of production, utilization and oxidation of glutamine's carbon skeleton *in vivo*. GC-C-IRMS can allow to measure more accuracy low ^{13}C enrichment in plasma free glutamine (Menand et al. 1997). Metges, C. et al. who had analyzed $^{15}N/^{14}N$ abundances of amino acids by former pilot study in GC-C-IRMS had used ^{13}C-labelled casein as a ^{13}C-labelled tracer and analysed NPP-ester amino acids in tissues and plasma samples (Metges & Daenzer 2000). Montigon et al. measured ^{13}C- and ^{15}N-enrichment of glutamine in rat plasma and human plasma, respectively (Montigon et al. 2001).

Measuring the $^2H/^1H$ (D/H) ratio of materials by using GC-Thermal Conversion-IRMS is more complicated. The first commercial GC-IRMS instrument to measure $^2H/^1H$ was produced in 1998 (Sessions 2006). Hydrogen in samples is converted to H_2 via pyrolysis or coupled oxidation/reduction and flows into an IRMS. In all cases, mass-2 (H_2) and mass-3 (HD) ion beams are monitored continuously, and D/H ratios are based on integration of the mass-2 and mass-3 ion current signals. Basically data-processing procedures are similar to those carbon

isotopic analyses (Sessions et al. 2001a). The abundance of ^{2}H (D) is low relative to that of ^{1}H (H). It means, at natural abundance, most organic materials contain less than 100 ppm D. To avoid confounding contributions from other, such as ^{13}C in CH_4, molecular H_2 as the sample gas needs to get mass spectrometric determination of H_2 and HD abundances for precise measurements of hydrogen isotopic ratios. The reaction ($H_2^+ + H_2 \rightarrow H_3^+ + H^-$) in the ion source of the mass spectrometer ensues from using H_2 for these measurements. In materials containing a natural abundance of D, H_3^+ can account for as much as 5-30% of the m/z 3 signal. Therefore, the correction for H_3^+ is significant (Sessions et al. 2001b). The H_3^+ factor is determined as the value of K which is described the formula: $i_{HD} = i_3 - K(i_2)^2$, where i_2 and i_3 are the raw mass-2 and mass-3 ion currents, i_{HD} is the corrected ion current. The value of K minimizes the mean absolute error of the 9 - 10 peaks of varying size and determined using this method are generally within 5% of those determined conventionally. Raw mass-3 ion currents are corrected for contributions from H_3^+ on a point-by-point basis (Sessions et al. 1999). When the H_3^+ factor has been corrected, isotope ratios are determined using the same techniques as a carbon measurement in GC-C-IRMS. Thus, frequent determination of the H_3^+ factor, and stability of the H_3^+ factor within a run and between runs, is a key requisite for accurate compound-specific analysis of ^{2}H/^{1}H.

The ability to measure low deuterium enrichments is very attractive, as many metabolic processes incorporate deuterium from water and so can in theory be measured *in vivo* by administering D_2O (Dufner & Previs 2003). Deuterated water as a heavier isotope tracer has been used for measurement of *de novo* triacylglycerol synthesis in humans (Scrimgeour et al. 1999). Scrimgeour et al.

used GC-IRMS techniques for the measurement of deuterium enrichment. Cogo et al. also measured surfactant palmitate disaturated-phosphatidylchline (DSPC) synthesis in human infants by using deuterated water. They used GC-IRMS for deuterium labelled lipid synthesis and measured deuterium enrichment of urine and tracheal aspirates. In this study, there were no side effects from the administration of D_2O (Cogo et al. 2005). Deuterated water (D_2O) does not cause any adverse physiological effects unless used in very high concentrations. As GC-C-IRMS is more sensitive than GC-MS, even in selected ion monitoring (SIM) mode, lower D_2O infusion rates can be used. It is because GC-C-IRMS can detect minute variations of very small amount of the heavier isotope in the presence of large amounts of the lighter isotope.

1.8   Aim of this thesis

The aims of the work described in this thesis were:

(i) to establish a method for analysis of GSH enrichment and synthetic rate using isotope-ratio mass spectrometry, which would be suitable for use with deuterated and ^{13}C- labelled amino acid precursors

(ii) to use this method to measure GSH synthesis in critically-ill infants and children

(iii) to use this method to measure GSH synthesis in septic infants and children given parenteral glutamine as a potential GSH precursor

(iv) to use this method to measure GSH synthesis in rats undergoing intestinal ischaemia-reperfusion injury as a model of systemic inflammation.

Chapter 2

Development of methods to measure
enrichment of Glutathione and Glycine
by Gas Chromatography-
Isotope Ratio Mass Spectrometry

Chapter 2: _____ _____

2.1 Introduction

The use of GC-IRMS in medical biochemical research is very recent, as it has been mainly previously used in archaeology, geology and environmental chemistry. Although the IRMS is able to detect very small (ppm) differences in isotopic enrichment, simple gases, such as CO_2, H_2, N_2 and CO must be analysed, so that organic biochemical compounds have to be converted by a high temperature interface.

GC-Combustion/Thermal Conversion-IRMS is based on a GC and IRMS attached to GC via a thermal conversion interface (Figure 2.1). A combustion interface is used for analysis of carbon isotopic enrichment in organic compounds and high temperature conversion ("pyrolysis") interface used for hydrogen isotopic analysis in organic compounds.
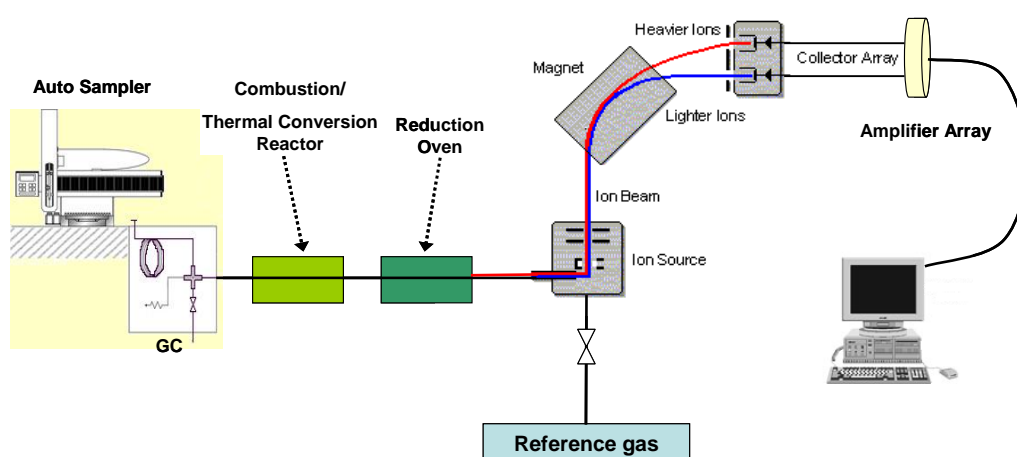
Figure 2.1 Set-up of an isotope ratio mass spectrometer to a gas chromatograph via a combustion interface to measure $^{13}C/^{12}C$ (carbon mode) or high temperature conversion interface to measure $^{2}H(D)/^{1}H$ (hydrogen mode).

Gas chromatography-mass spectrometry is often used for measurement of stable isotope tracer enrichments in biochemistry and biomedicine but it is not an ideal method. When GC-MS is used for tracer studies, either high tracer enrichments and/or selective ion monitoring (SIM) mode have to be used. As GC-IRMS has been developed for specifically for analysis of isotopic ratios, it should be a much more sensitive detector than GC-MS even in SIM mode, allowing lower tracer infusion rate or more complex analysis in which the infusion tracer is several biochemical steps away from the measured compound.

Methods of sample derivatization using GC-MS have been developed and studied intensively. Silylation agents (e.g. trimethylsilyl (TMS) and *tert*-butyl-dimethylsilyl (*t*BDMS)) and fluorinating agents (e.g. trifluoroacetates (TFA) and boron trifluoride (BF_3)) have enjoyed great popularity. However, many of these methods are not compatible with GC-C/TC-IRMS. Using either TMS or *t*BDMS derivatization causes a high load of carbon and which, in the case of carbon isotope ratio measurement, causes dilution of $\delta^{13}C$-value of the measured compound. In addition, the life of the combustion reactor may be shortened by silylation. Fluorinated derivatives have also a huge risk of damage to combustion reactor. As fluorine forms extremely stable fluorides with cupper (Cu) and nickel (Ni), if CuO/Pt is used as combustion catalyst, this effect will show up immediately (Meier-Augenstein 2004). In addition, fluorine-containing ions produced in the source can be very corrosive to be the mass- spectrometer.

For analysis of amino acids using GC-MS, some groups have used chloroformates (e.g. methyl chloroformate (MCF) and ethyl chloroformate (ECF)) (Capitan, Malmezat, Breuille, & Obled 1999;Husek 1998), and Metges et al. used

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N-pivaloyl & *O*-isopropylates (NPP) (Metges & Petzke 1997;Metges, Petzke, & Hennig 1996). The derivatization has the advantages that (i) it does not involve damaging silylation or fluorinating agents, (ii) the carbon and/or hydrogen load on the target compound are relatively low, and (iii) the chloroformate reaction takes place readily in aqueous solution. GSH is not an amino acid but it is a tripeptide. This derivatization method, therefore, could be used for analysis of GSH using GC-C/TC-IRMS.

I aimed to establish a new methodology to analyse GSH and its precursor amino acids simultaneously using GC-C/TC-IRMS. The new methodology is also compatible with GC-MS.

2.2 Aim

My aims were (i) to establish a method for measurement of $^{13}C/^{12}C$ ratios in GSH and glycine using GC-MS and GC-C-IRMS and (ii) to establish validate a method for measurement of $^{2}H/^{1}H$ ratio in GSH and glycine using GC-TC-IRMS.

2.3 Methods

2.3.1 Chemicals

All chemicals came from the sources describe in Appendix 2 (Chemicals and Procedures).

2.3.2 GC-MS

GC-MS measurements were carried out with an Agilent 6890 Series Gas Chromatograph coupled to an Agilent 5973 Series Mass Spectrometer, in electron impact (70 eV) ionisation mode.

2.3.3 GC-IRMS

Isotope ratios of either $^{13}C/^{12}C$ or $^{2}H/^{1}H$ was measured by GC-IRMS, which was Thermo Finnigan[TM] GC Combustion III and Thermo Finnigan[TM] DELTA[Plus] XP with ISODAT software. Combustion mode ($^{13}C/^{12}C$) was carried out using a ceramic tube with a copper oxide lining, operated at $950^{\circ}C$. The copper oxide was periodically re-oxidised by backflushing with O_2 gas. High temperature conversion ($^{2}H/^{1}H$) was carried out in a ceramic tube at $1425^{\circ}C$ in the absence of oxygen. The inner surface of the tube was coated with elemental carbon, which acts as a catalyst and scavenges residual oxygen. The tube was periodically re-carbonised (every ~200 injections, or when peak heights were noticeably diminished) by backflushing with methane gas (0.8-1.0ml/min for 10 minutes).

2.3.4 Gas chromatography (GC-MS and GC-IRMS)

Sample injections were made in splitless mode, the injector temperature was at 250°C and helium flow rat was 1.2 ml/min. The injection volume was 2 μl. Column was 10m of Phenomenex® ZB-50 (ID 0.25 mm × film thickness 0.25 μm). Two oven temperature gradients were programmed. One was a temperature programme from 70°C to 180°C at a rate of 5°C/min, then from 180°C to 280°C at a rate of 30°C/min and from 280°C to 320°C at a rate of 5°C/min, holding at 320°C for 1 min. Another was programmed temperature from 70°C to 180°C at a rate of 14°C/min, then from 180°C to 280°C at a rate of 30°C/min and from 280°C to 310°C at a rate of 3°C/min, holding at 310°C for 3 min.

2.3.5 Samples

Standard glycine (Gly) and GSH (L-γ-glutamyl-L-cysteinylglycine) were used in this study. Chemical structures are shown in Figure 2.2.

Glycine   GSH
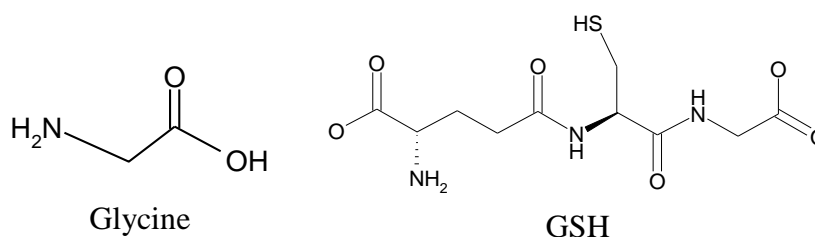
Figure 2.2 Chemical structure of glycine and GSH

$^{13}C_2$-Glycine and Gly$^{13}C(C_2)$-^{15}N-GSH, as stable isotope standard samples, were used. Chemical structures are shown in Figure 2.3.
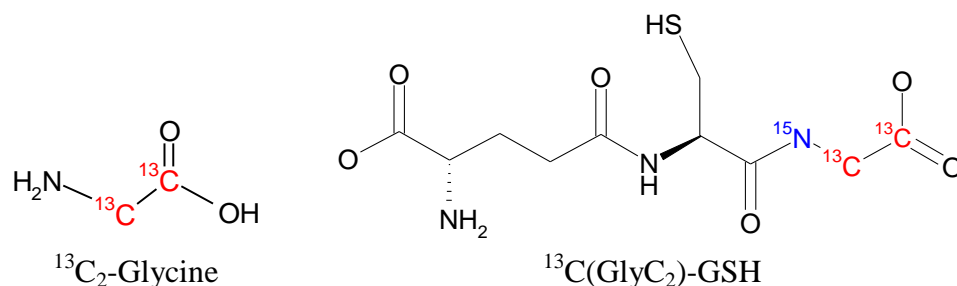
Figure 2.3 Chemical structure of ^{13}C labelled glycine and GSH

2.3.6 Generation of d$_2$-Glutathione

As d$_2$(GlyC$_2$D$_2$)-GSH is not commercially available, I generated deuterated GSH by incubating human RBC with D$_2$-glycine *in vitro*.

Red blood cells were separated from whole human blood of a healthy adult volunteer by taking blood (20ml) into a Li-Heparin tube, and centrifugation at 3000rpm for 15min at 4°C. 1ml aliquots of the pellet were stored at -80°C until analysis.

10 mM d$_2$-Gly (distilled water solution) was added to an aliquot of RBC to a final concentration of 1 mM. The RBC sample was incubated for 18h (overnight) at 37ºC. The reaction was stopped by addition of EDTA (ethylendiaminetetraacetic acid) to a final concentration of 5 mM. The deuterated-(Gly)-GSH of RBC sample was kept at -80ºC until analysis. Chemical structures are shown in Figure 2.4.
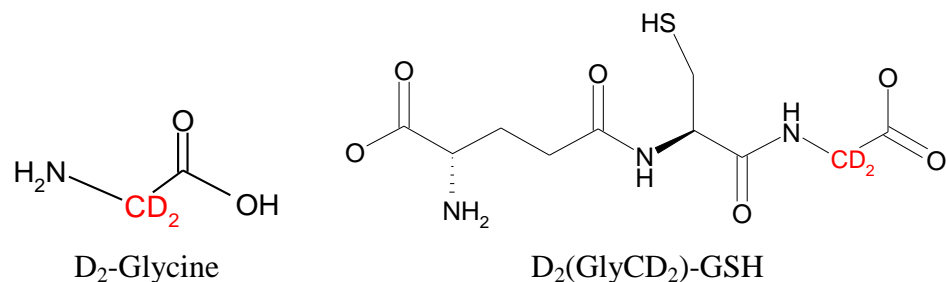
Figure 2.4 Chemical structure of deuterium labelled glycine and GSH

This method relies on the activity of the enzyme GSH synthetase, with catalyses the reaction: γ-glutamyl-cysteine + glycine ↔ GSH, and requires Mg^{2+} ions for its activity and is, therefore, inhibited by excess of chelating agents such as EDTA (Oppenheimer et al. 1979).

2.3.7 Preparation of samples and standards for analysis

Reduction of GSH and protein precipitation of erythrocyte samples

50 ~ 200 µl of RBC sample was taken into a microcentrifuge tube and 200 µl of TCEP (tris-(2-carboxyethyl)-phosphine) buffer (7.5 mM TCEP, 0.2 M KH_2PO_4, 5 mM EDTA, pH 7.5) added. The sample was mixed for 15 min at room temperature to reduce all oxidised GSH to GSH. 200 µl of 10% SSA (sulfosalicylic acid) was added into the sample and mixed vigorously. The sample was centrifuged at 1200*g* for 12 min at 4°C and the supernatant immediately derivatised as below.

Derivatization

After protein precipitation, 200 µl of the supernatant (or the standard solution) was transferred into a 10 ml screw capped glass tube. The sample was

made pH > 10 with 2 M NaOH. 50 μl of PCF (*n*-propyl chloroformate) was added to the sample and mixed for 5 min at room temperature. 1 M HCl was added into the sample to pH < 2 and then 0.5 g of NaCl added. Then, 3 ml of diethyl ether (peroxide free) was added for extraction of the organic phase. The extracted organic phase was transferred to a new tube and the aqueous phase was re-extracted. The organic phase was dried down under nitrogen air at 60°C. 200 μl of 1 M HCl in MeOH (340 μl of concentrated HCl (ca. 6 M) put into volumetric flask to make up 10 ml with MeOH (HPLC grade)) was added to the dry sample. The sample was incubated for 10 min at 80°C and then dried down under nitrogen at 60°C. The sample was re-suspended with 100 μl of ethyl acetate and transferred to an autosampler vial for GC-MS or GC-IRMS analysis, as described in section 2.3.4.

2.3.8 Statistics

Data were compared by t-tests (two samples), one-way ANOVA (more than two samples) or by linear regression analysis. $p < 0.05$ was regarded as significant.

2.4 Results

2.4.1 Method development

2.4.1.1 *N,S-ethoxycarbonyl methyl esters*

The initial attempts to develop a method for GC-IRMS-compatible analysis of GSH were based on the method of Capitan et al. (Capitan, Malmezat, Breuille, & Obled 1999), which were based on derivatization with ethyl chloroformate followed by methanolic-HCl to yield the *N,S*-ethoxycarbonyl methyl ester of GSH. Using the reaction conditions described: standards were dissolved in 0.8 ml of 0.2 M phosphate buffer, 5 mM EDTA, 80 mM DTT, pH 7.5. After addition of 100 μl ethyl chloroformate, the mixture was shaken on a tube rotator for 15 min at room temperature. The pH of the mixture was then adjusted to 1.5 with 2 M HCl. The mixture was saturated with NaCl (~0.3 g per ml) and extracted three times with 3 ml of peroxide-free diethyl ether. The ether extracts were evaporated to dryness at 80°C, then 200 μl of 1 M HCl in methanol was added, the vial capped and incubated at 80°C for 10 min in a heating block. The solvent was then evaporated to dryness at 80°C under a stream of air and the residue dissolved in 50–100 μl of ethyl acetate. These samples were analysed by GC-MS using the following conditions: the temperature of the GC injector was 270°C. The column used was a 10m x 0.25 mm I.D.×0.25 μm film thickness HP5MS. Injections (1–2 μl) were made in the split mode with a 20:1 split, with a column helium flow rate of 1ml/min.

The GC oven temperatures were: 200°C for 0.5 min, then increased to 280°C at a rate of 30°C/min, 280°C for 0.1 min, increased to 295°C at a rate of

5°C/min and 295°C for 2 min. Although this method gave the expected peaks for cysteine, cys-gly and gly-cys, no clear peaks were obtained for GSH (expected m/z 479 with fragments at 363 and 216). Despite variation in reaction pH, different methods for producing dry methanolic HCl, and ensuring samples were completely dry before esterification with methanolic-HCl, clear GSH peaks could not be obtained. In addition, other stages of the sample derivatization were also altered, such as use of ethyl- acetate to extract the *N*,*S*-ethoxycarbonyl ester, and use of methanolic acetic acid for the final esterification, all without success.

The next attempt was to use the above reaction method, but with methyl-chloroformate followed by methanolic-HCl, with the expectation of producing the *N*,*S*-methoxycarbonyl methyl ester of GSH. This would have had a lower molecular weight than the *N*,*S*-ethoxycarbonyl methyl ester, which could have circumvented any problems due to GSH-derivatives not being eluted from the HP5MS column.

2.4.1.2 *Single stage chloroformate derivatisation*

The problems regarding use of the above described method were considered to be due to the second esterification step, that of methanolic-HCl, in which the samples must be water-free. It was therefore decided to attempt to derivatize GSH in a single step procedure, in aqueous solution, using the methods of Husek (Husek 1991;Husek 1998). Initially, to a 200μl aqueous standard were added 164μl methanol, 34μl pyridine, then 2 aliquots of 20μl of methyl chloroformate, with vortexing after each addition. Then, products were extracted with 400μl chloroform, with addition of 400μl 50mM $NaHCO_3$. The chloroform phase was dried over

anhydrous sodium sulphate and injected to the GC-MS, using a temperature profile from 60°C, then 15°C/min to 275°C, then 30°C/min to 300°C. Clear single peaks were obtained for gly, cys and cys-gly. Although a derivative was obtained for GSH, multiple chromatographic peaks were obtained.

It was then attempted to optimise this method using ethyl chloroformate, n-propyl chloroformate, iso-propyl chloroformate and butyl chloroformate. In addition, several different solvents such as acetonitrile, piperidine, triethylamine, 1% NaOH previously described as variants on the Husek method were attempted. However, none of these variants gave a single clear peak of GSH.

2.4.1.3 *N-pivalolyl-2-propanol derivatization*

The next method attempted was to use the N-pivalolyl-2-propanol derivatization described by Metges et al. (Metges & Daenzer 2000;Metges & Petzke 1997;Metges, Petzke, & Hennig 1996). Although this yielded single clear peaks for alanine, glycine and other amino acids, GSH was either not derivatized or not eluted from the GC column.

2.4.1.4 *Use of a commercial amino acid analysis kit*

It was then decided to use a commercially available kit for GC-MS based amino acid analysis, EZ-Faast (Phenomenex, UK). This method also used single step chloroformate based derivatization (I e. similar to Husek), but the manufacturers did not disclose the alkyl chloroformate or the solvent used in their kit, nor was the GC column disclosed. Although the EZ-Faast kit did not derivatize GSH, it was considered to be very promising as dipeptides were amenable to

derivatization and analysis. From the mass spectra of the amino acids, the derivatives appeared to be *n*-propyl derivatives. It was therefore decided to attempt to use *n*-propyl chloroformate derivatization, followed by solvent extraction and methylation. This method, as described above in section 2.3.7, proved to be successful in analysis of GSH and glycine, with GC analysis on the 10m EZ-Faast column. Examination of the characteristics of the EZ-Faast column led to the conclusion that the ZB-50 column (Phenomenex, UK) was very similar to the EZ-Faast column, so a 10m ZB-50 column was used, as described in 2.3.4. for all future work.

2.4.1.5 *Internal standardisation*

In order to develop a method for measuring concentration, as well as enrichment, of GSH, it was necessary to try to find a suitable internal standard for GC-IRMS analysis. Usually, for GC-MS analysis of concentration, a deuterated or ^{13}C- labelled internal standard would have been used, but this was not appropriate as it would have interfered with the isotopic analysis necessary. It was therefore necessary to attempt to find an internal standard with the characteristics (i) chemically similar to GSH so losses of GSH are reflected in losses of internal standard; (ii) not found endogenously so no interference from biological samples and (iii) must appear completely separate chromatographically from all endogenous compounds. The last was especially difficult to achieve, as it must be remembered that in GC-IRMS, detection is detection of $^{2}H/^{1}H$ or $^{13}C/^{12}C$, so that any organic compound would interfere.

Two different compounds assessed as internal standards are shown on figures 2.5. and 2.6 respectively:
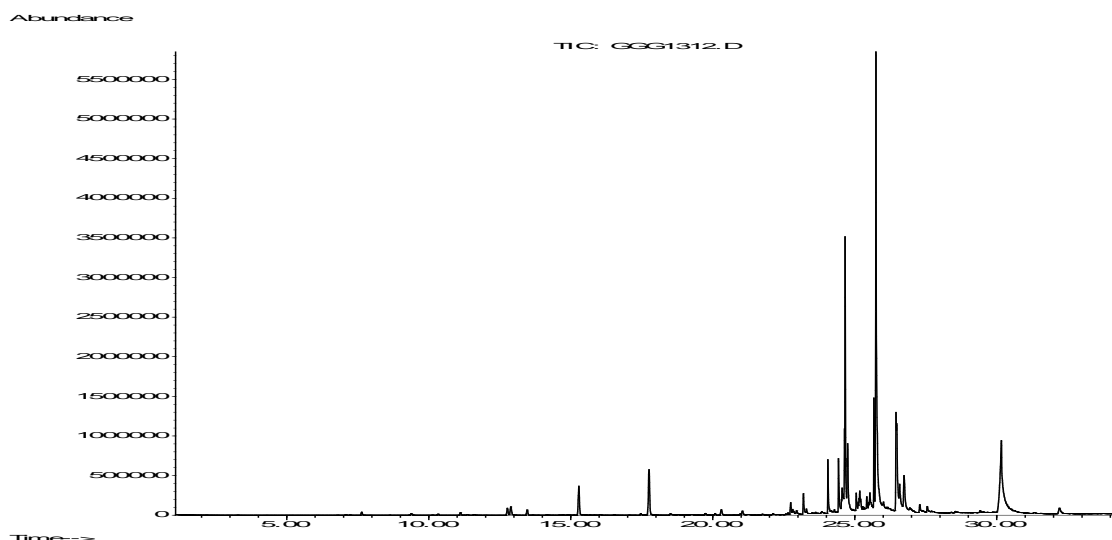
Figure 2.5 Use of gamma-glutamyl-glutamate as internal standard. GSH peak is at 30 minutes.

Gamma-glutamyl-glutamate unfortunately yielded a peak which co-chromatographed with other biological material in red blood cell samples, appearing at several split peaks between 24 and 27 minutes (Figure 2.5).

Homoglutathione (gamma-*L*-glutamyl-*L*-cysteinyl-beta-alanine) initially showed promising results, as suggested by Humbert et al. (Humbert, Nguyen, Obled, Bobin, Vaslin, Sweeten, & Darmaun 2001) and as shown in Figure 2.6, where a clear peak separate from that of GSH was observed. However, there is significant band broadening in the high-temperature conversion/ combustion ceramic tube, so that on GC-IRMS, the homoglutathione peak was not chromatographically separate from that of GSH, rendering it not suitable for use as an internal standard for concentration measurement by GC-IRMS.
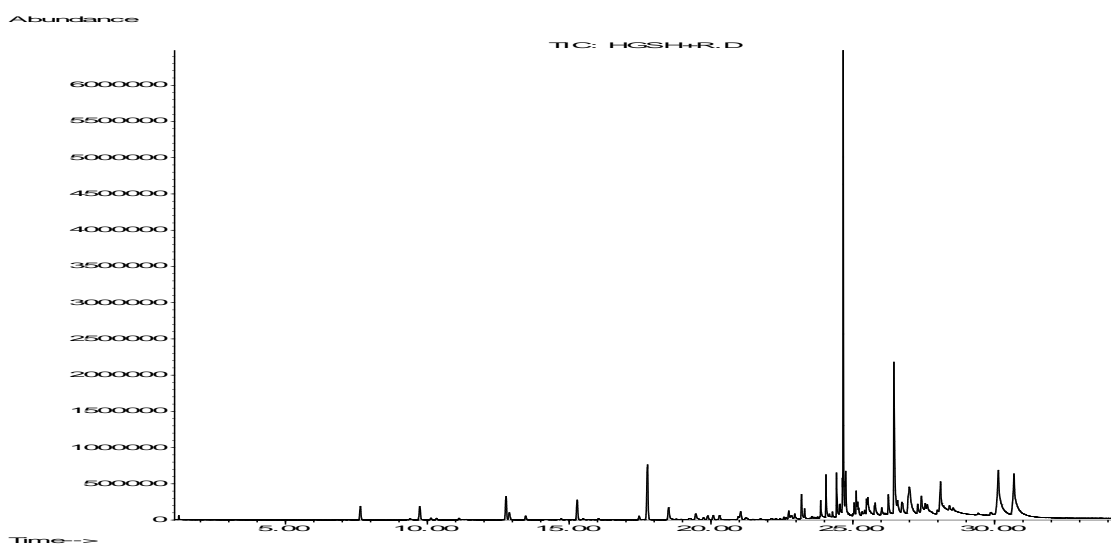
Figure 2.6 Use of homoglutathione as internal standard. GSH peak is at 30 minutes, homoglutathione at 31minutes.

2.4.2 Glycine and GSH in GC-MS

GC-MS was used for identification of glycine and GSH derivatives, as structured information can not be obtained by GC-IRMS. All structured information is lost after conversion to CO_2, H_2, or N_2 in GC-IRMS analysis. Glycine and GSH were identified by these retention time and mass spectrum patterns at GC-MS.

Glycine derivative gave the fragments; $m/z = $ 116, 88, 56, 59, 74, 90, 102, 134. ($m/z = $ 116 was the base peak ion.) Virtually no molecular ion was detected. Retention time was around 8 min using the temperature programme: from 70°C to 180°C at 5°C/min, from 180°C to 280°C at 30°C/min and from 280°C to 320°C at 5°C/min, holding at 320°C for 1 min. The structure of glycine *n*-propoxycarbonyl

methyl ester is shown at Figure 2.7, the chromatogram in Figure 2.8 and the mass

spectrum is in Figure 2.9.

M = 175
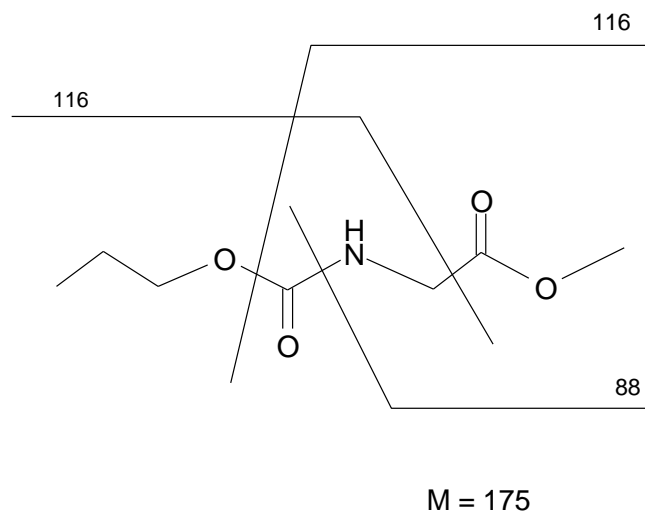
Figure 2.7 Glycine *n*-propoxycarbonyl methyl ester
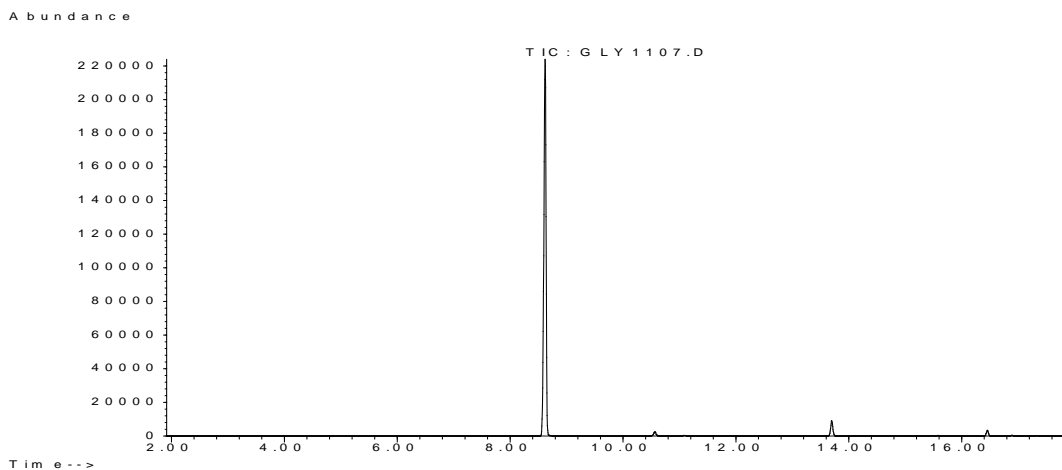
Figure 2.8 Chromatogram of Glycine *n*-propoxycarbonyl methyl ester
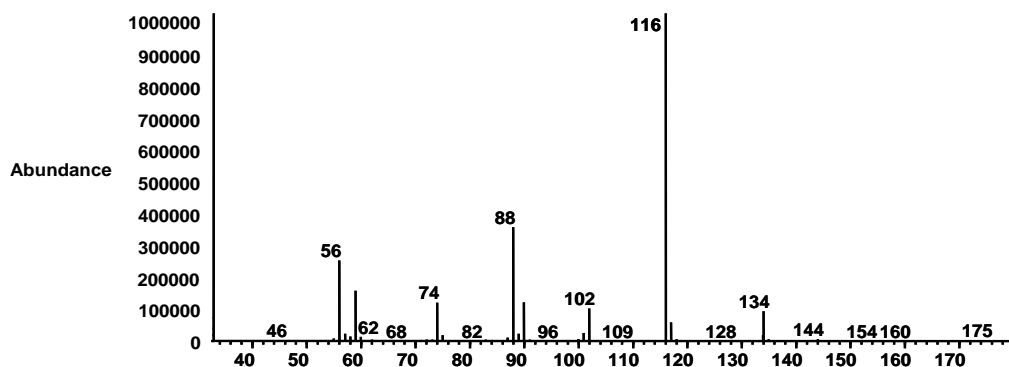
Figure 2.9 Mass Spectrum of Glycine *n*-propoxycarbonyl methyl ester

The GSH derivative gave the fragments; *m/z* = 144, 84, 162, 142, 391, 230, 56, 331, 88, 82. Again, virtually no molecular ion was detected. Retention time was around 30.4min in temperature programme: from 70°C to 180°C at 5°C/min, from 180°C to 280°C at 30°C/min and from 280°C to 320°C at 5°C/min, holding at 320°C for 1 min.

The structure of the GSH *N,S-n*-propoxycarbonyl methyl ester is shown in Figure 2.10, the chromatogram in Figure 2.11, and the mass spectrum in Figure 2.12.

The peak shape for the GSH derivatives was relatively poor, as the compound has limited volatility. A high temperature was necessary to elute GSH from the column, and a column longer than 10m could not be used as GSH did not elute at all.
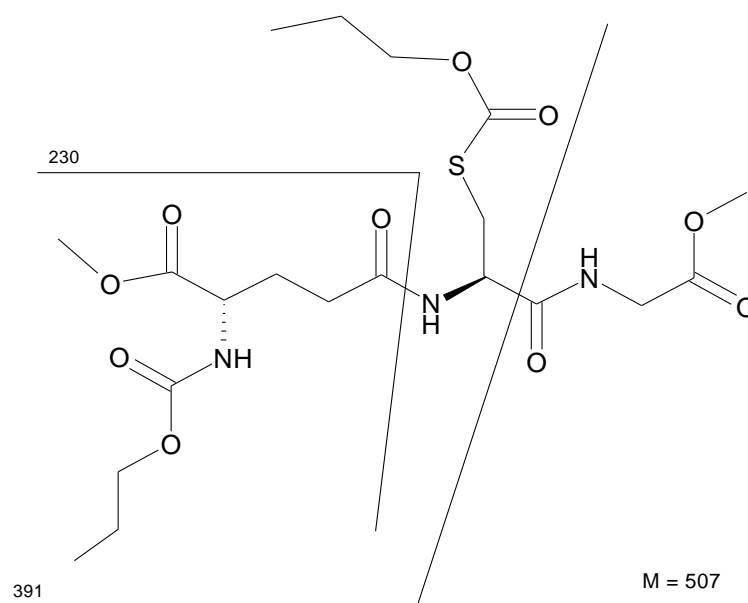
Figure 2.10 Glutathione *N,S-n*-propoxycarbonyl methyl ester
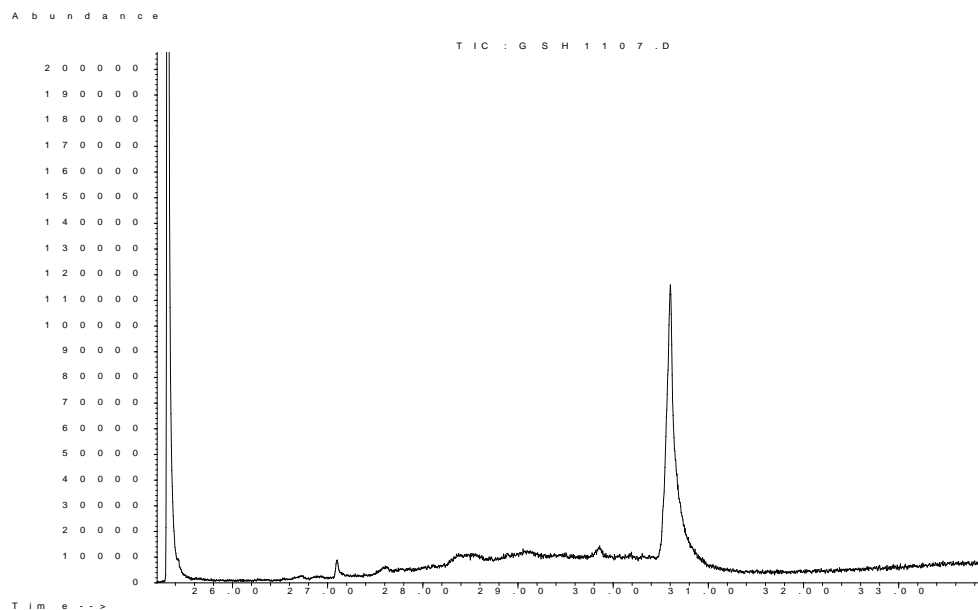
Figure 2.11 Chromatogram of Glutathione *N,S-n*-propoxycarbonyl methyl ester
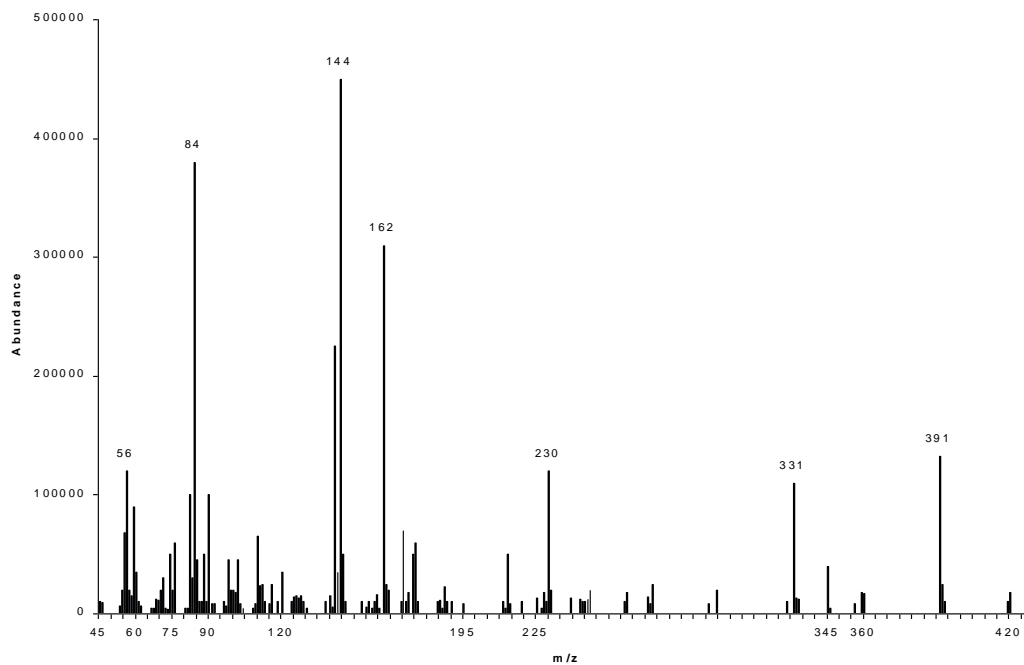
Figure 2.12 Mass Spectrum of Glutathione *N,S-n*-propoxycarbonyl methyl ester

2.4.3 Methods in GC-C-IRMS

When GC-C-IRMS had been set up at using CO_2 reference gas for analysis of $^{13}C/^{12}C$, the column was the same as in using GC-MS. The temperature gradient was also the same method in GC-MS: temperature programme from 70°C to 180°C at 5°C/min, from 180°C to 280°C at 30°C/min and from 280°C to 320°C at 5°C/min, holding at 320°C for 1 min. Injection volume was 2 μl in splitless mode.

Each eluted peak from the column was transferred on-line to a 950°C ceramic combustion tube containing copper wires (Figure 2.13). The resulting $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$ were shown as their corresponding masses at m/z 44, 45 and 46 respectively, by an on-line magnetic sector isotope ratio mass spectrometer (Thermo Finnigan^{TM} DELTA^{Plus} XP with ISODAT software). The $\delta^{13}C$ is reported against Vienna Peedee Belemnite (VPDB) (VPDB is presented by International Atomic Energy Agency, Vienna, Austria). The copper of the combustion tube was re-oxidised to Cu_2O every 200 - 300 injections by back-flashing with O_2 over night.
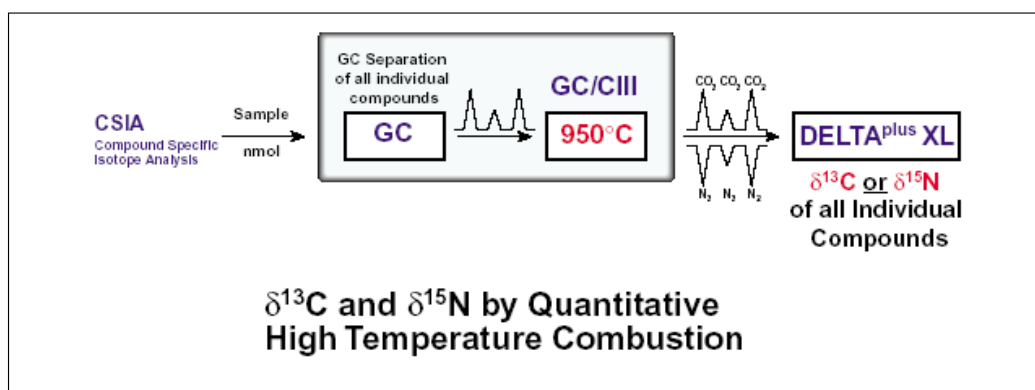
Figure 2.13 Analysis of $^{13}C/^{12}C$ in GC-C-IRMS; Each peak of $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$ is eluted and the $\delta^{13}C$ is calculated

2.4.4 Analysis of ^{13}C-Glycine and ^{13}C-GSH

Although the ^{13}C-glycine stock solution was made at 10 mM in analytical grade water (HPLC grade), the ^{13}C-GSH stock solution was made 10 mM in methanol because stable isotope labelled GSH was more stable in methanol than in water. Each standard and stable isotope labelled sample was analysed by carbon mode of GC-C-IRMS. RBC and RBC with ^{13}C-glycine were also analysed. The chromatograms are shown in Figure 2.14, Figure 2.15 and Figure 2.16.

Analysis of both standards and blood samples, was successful, showing that the derivative could be combusted online to CO_2 and detected by IRMS.
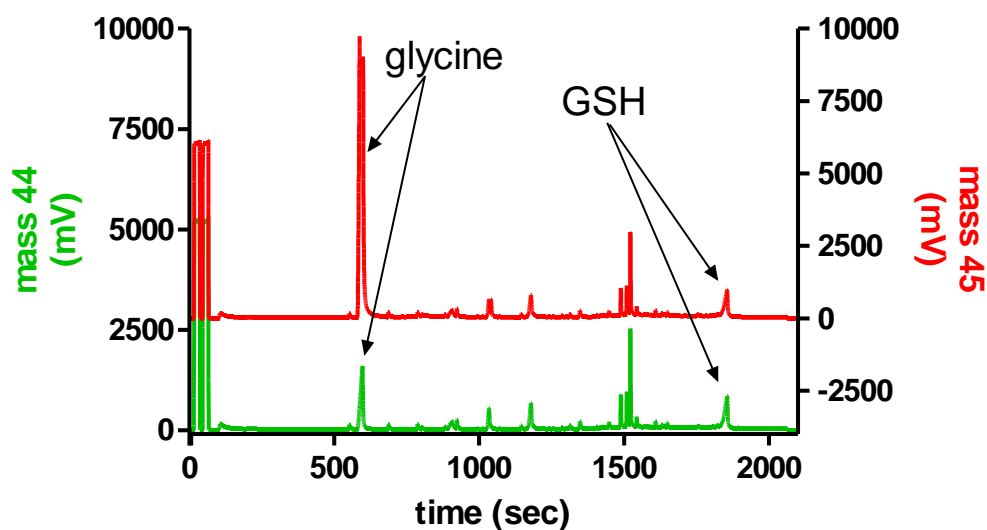
Figure 2.14 Chromatogram of ^{13}C-glycine-spiked RBC sample, GSH peak was from endogenous GSH in RBC.
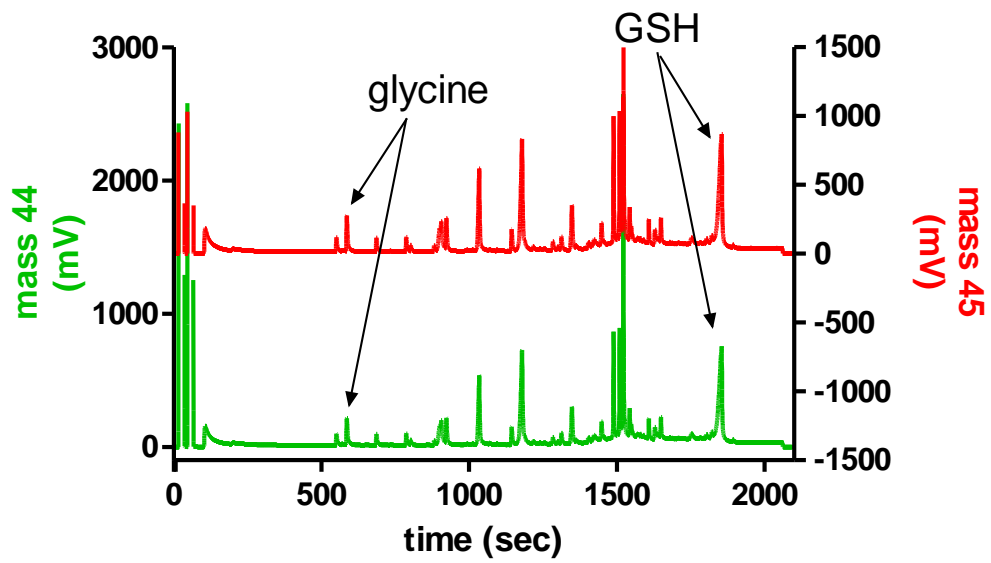
Figure 2.15 Chromatogram of unspiked RBC sample
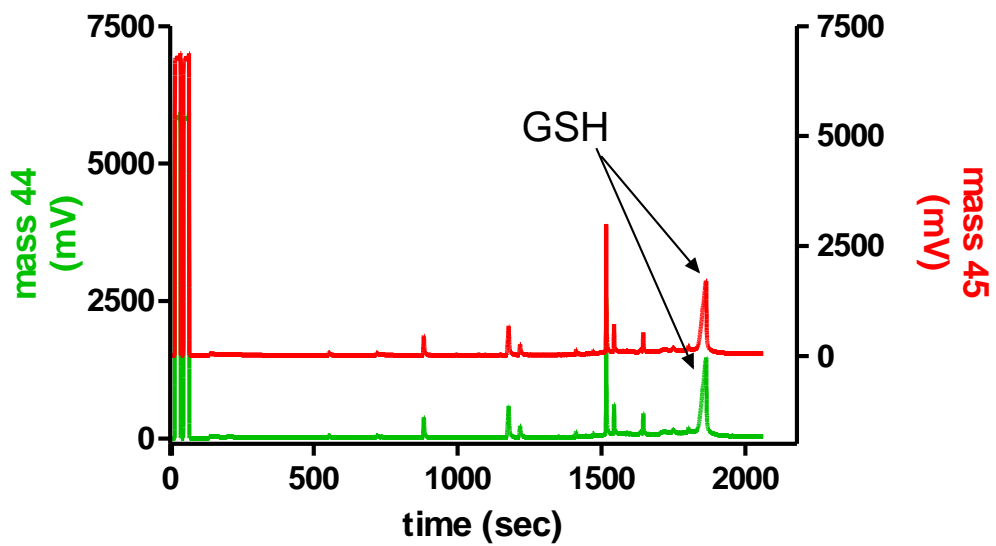
Figure 2.16 Chromatogram of GSH standard

2.4.5 ^{13}C-GSH calibration curve

10mM ^{13}C,^{15}N-GSH was mixed into GSH standard solution (10mM) to make an isotopic calibration curve from 0% to 5% of ^{13}C-GSH (the ^{15}N-label is not relevant to these studies, and was present because ^{13}C,^{15}N-GSH was available, whereas ^{13}C-GSH was not). The mixed 200μl of GSH samples were analysed. Each sample was analysed in triplicate and the result of ^{13}C/^{12}C shown per mille (i.e. parts per thousand, ‰) against VPDB. For this analysis of ^{13}C/^{12}C-GSH, a different oven temperature gradient was used: One was started from 70°C to 180°C at 5°C/min, from 180°C to 280°C at 30°C/min and from 280°C to 320°C at 5°C/min, holding at 320°C for 1 min. Another was started from 70°C to 180°C at 14°C/min, from 180°C to 280°C at 30°C/min and from 280°C to 310°C at 3°C/min, holding at 310°C for 3 min.

The other programmed temperature gradient was initial temperature of 110°C, and increased at a rate of 30°C/min from 110°C to 280°C, holding at 280°C for 0.1min, then at 5°C/min from 280°C to 320°C, holding at 320°C for 2min. It was only focused on analysis of ^{13}C/^{12}C-GSH, not including glycine.

Linear regression of ^{13}C/^{12}C vs. ^{13}C-GSH (%) gave a good linear fit with r^2 of 0.9981, and a Y intercept of -28.02 ± 0.58. Calibration curve of 0-5% of ^{13}C-GSH in GSH standard is shown in Figure 2.17 and chromatogram of 2.5% of ^{13}C-GSH in GSH standard is shown in Figure 2.18.
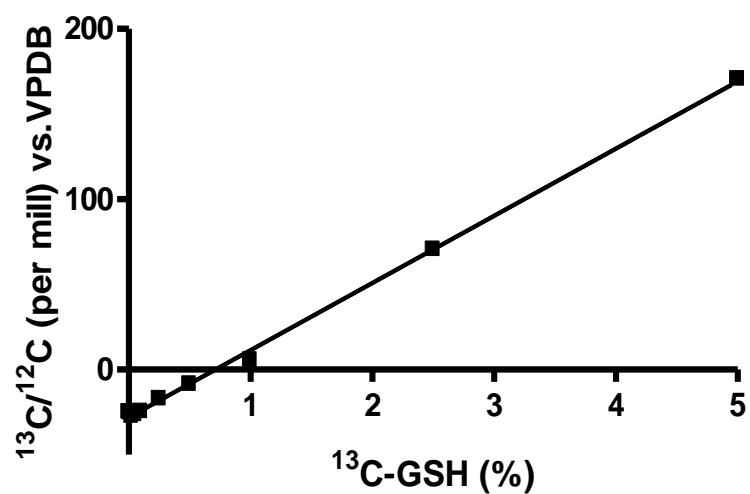
Figure 2.17 Calibration curve of 0% to 5% of ^{13}C-GSH in GSH standard
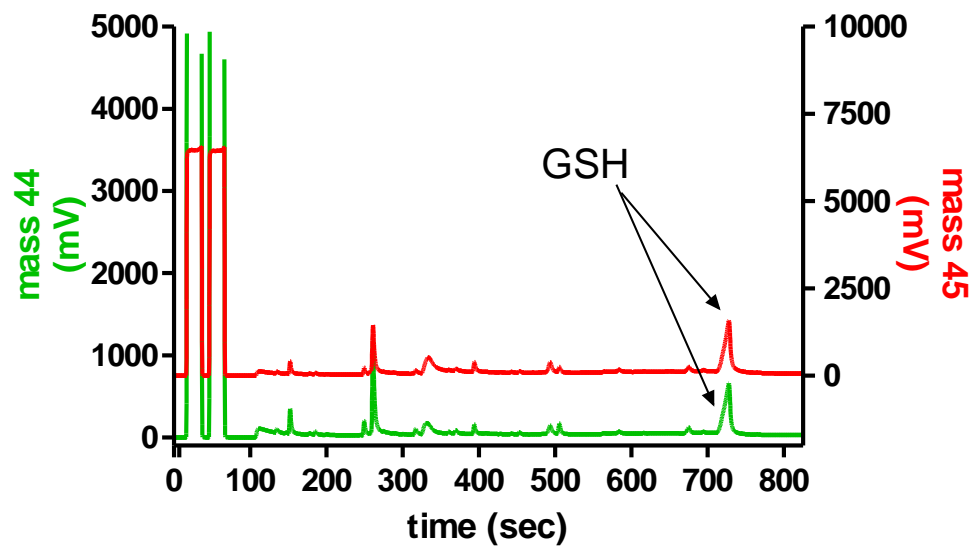
Figure 2.18 Chromatogram of 2.5% of ^{13}C-GSH in GSH standard; ^{13}C/^{12}C-GSH peak was at 728 sec of the chromatogram.

Samples of lower enrichment (0-1%) also showed a good linear regression with r^2 of 0.9779 and a Y intercept of -26.57 ± 0.4290. This calibration curve is shown in Figure 2.19. Although the r^2-value was good, examination of the calibration curve shows a departure from linearity below 0.25% ^{13}C-GSH. A sample chromatogram at 0.025% ^{13}C-GSH is shown in Figure 2.20.
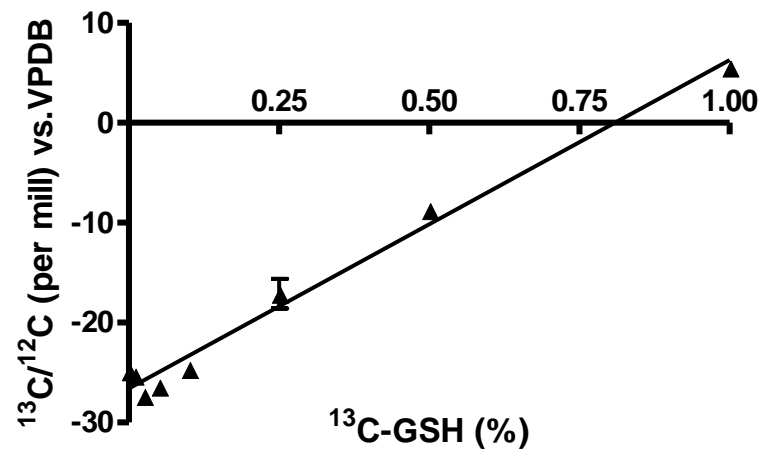
Figure 2.19 Calibration curve of 0% to 1% of ^{13}C-GSH in GSH standard
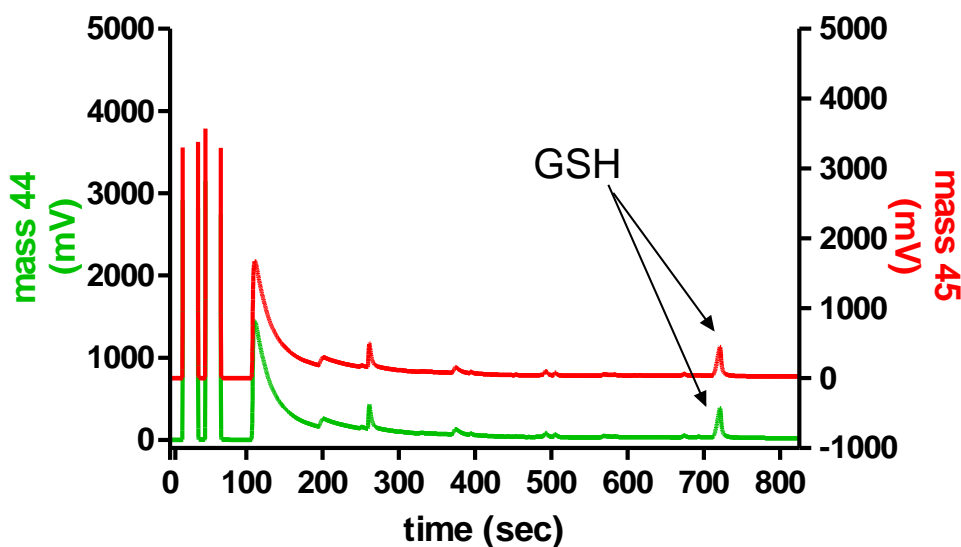
Figure 2.20 Chromatogram of 0.025% of ^{13}C-GSH in GSH standard

2.4.6 ^{2}H/^{1}H analysis of glycine and GSH in GC-TC-IRMS

As deuterium analysis by IRMS is less sensitive than ^{13}C/^{12}C, it was necessary to inject larger volumes. In order to do this, injections of 11.25 µl were made via a programmed temperature vaporization inlet (PTV inlet) in solvent vent mode. 11.25 µl of sample was injected at 1 µl/sec into a Siltek® treated liner (volume 125µl) containing Siltek®-treated glass wool to increase evaporation area. The liner was held at 37ºC and vented with helium at 100 ml/min to remove solvent. Analytes remained in the liner until, after 0.8min, the temperature of the liner was increased to 300°C at a rate of 150°C/min, and held at 300°C for 5 minutes. This rapid heating transfers analytes onto the head of the analytical column. The liner was purged with helium at 50 ml/min for the last 2.5 minutes of

the 300°C isothermal period. At the end of the run, the inlet was rapidly cooled to 37°C with liquid N_2. The column was 10m of Phenomenex® ZB-50 (ID 0.25 mm × film thickness 0.25 μm), as for GC-MS analysis. The GC oven temperature was programmed from 70°C to 180°C at a rate of 14°C/min, from 180°C to 280°C at a rate of 30°C/min and from 280°C to 310°C at a rate of 3°C/min, holding at 310°C for 3 min.

The eluted peaks were passed through a high temperature conversion oven operated at 1425°C, in the absence of oxygen, containing an Al_2O_3 ceramic tube (0.5mm i.d., 320mm long). This results in the quantitative conversion of organic compounds of H_2. The resulting H_2 and DH were shown as their corresponding masses at m/z 2 and 3, respectively (see Figure 2.21).
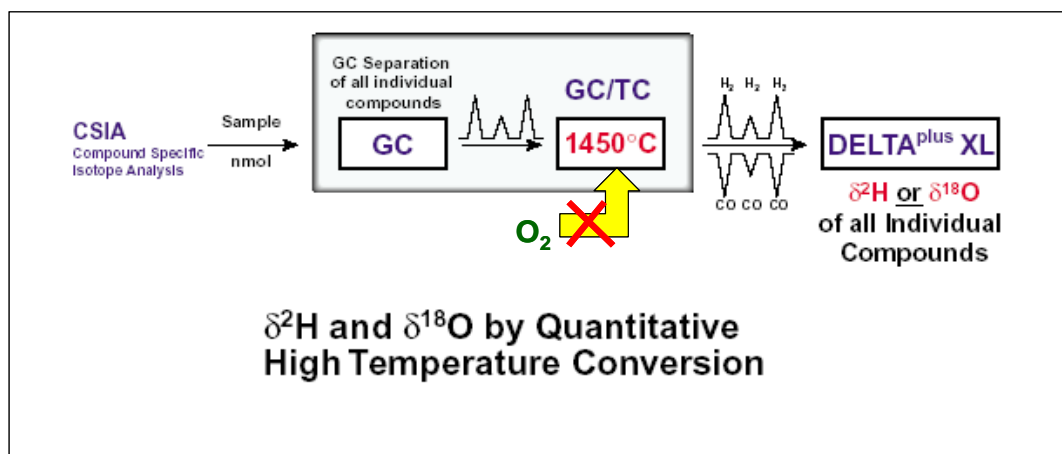
Figure 2.21 Analysis of ^{2}H/^{1}H in GC-TC-IRMS; each peak of ^{2}H^{1}H and ^{1}H$_2$ is eluted and the δ^{2}H is shown from these H$_2$ result.

2.4.7 Analysis of D_2-Glycine and D_2(Gly)-GSH

Standards of glycine (5 mM; Figure 2.22), GSH (10 mM; Figure 2.23), d_2(Gly)-GSH (generated from RBC with d_2-glycine *in vitro* as described above; Figure 2.24) and RBC (Figure 2.25) were analysed by hydrogen mode of GC-TC-IRMS.
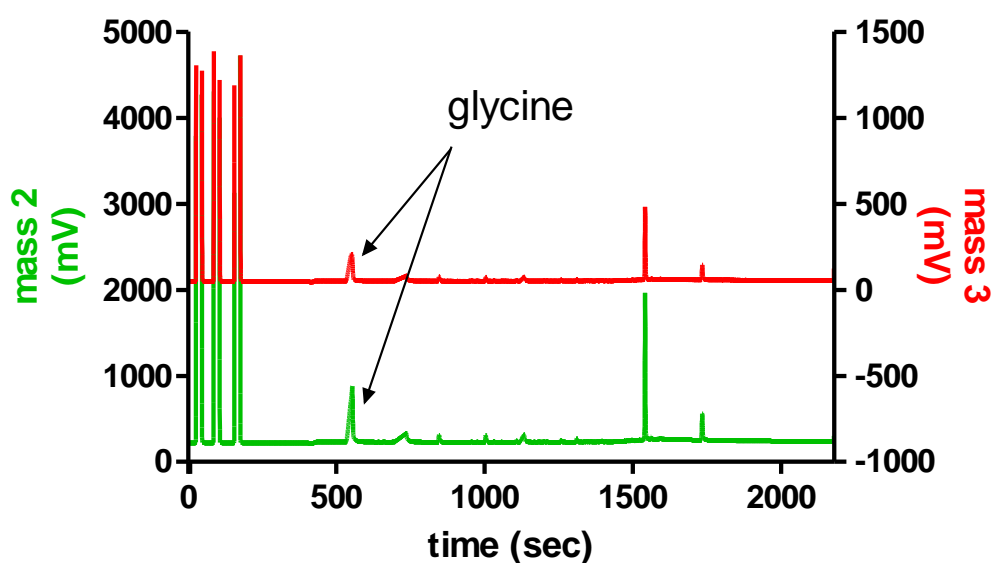
Figure 2.22 Chromatogram of glycine standard
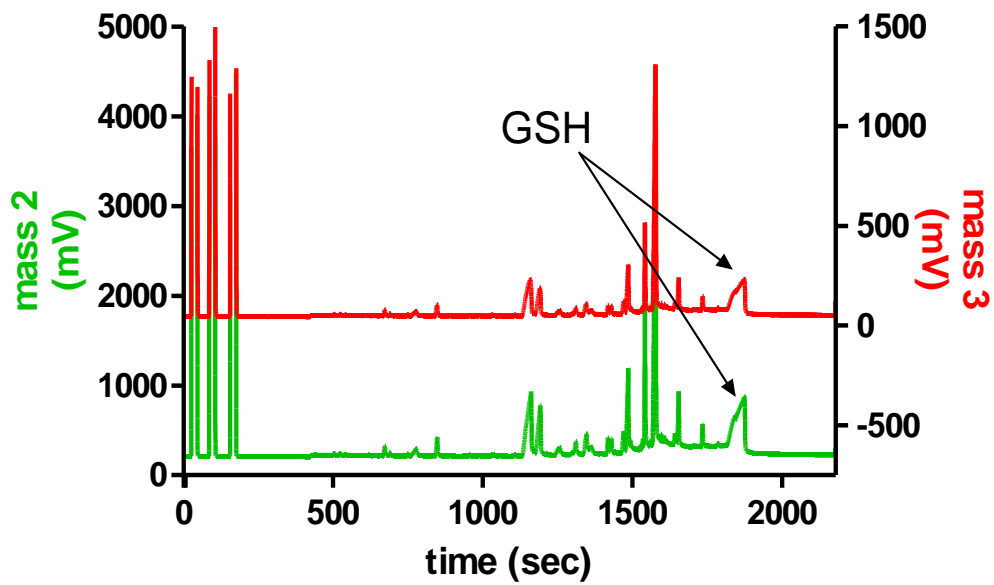
Figure 2.23 Chromatogram of GSH standard
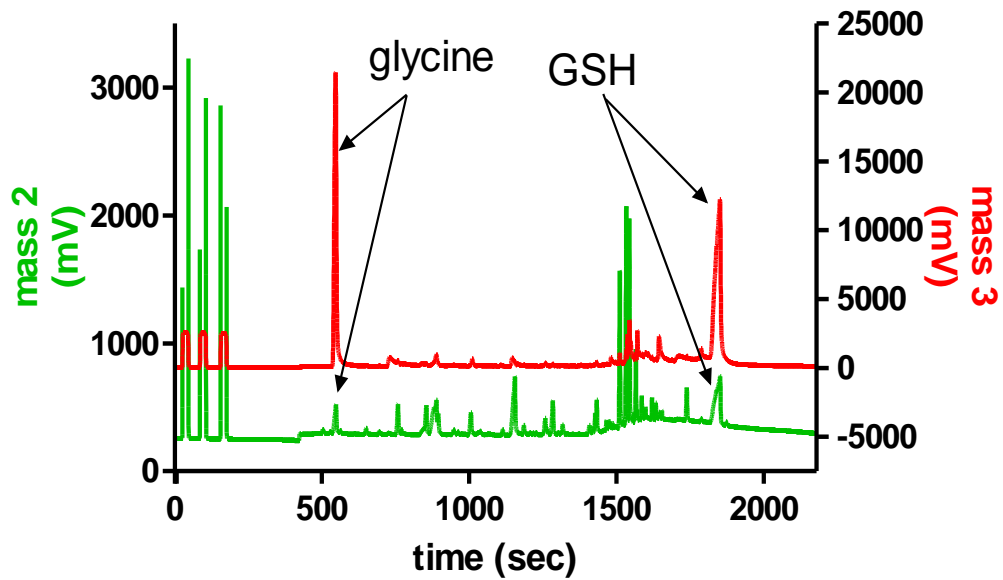
Figure 2.24 Chromatogram of Deuterated RBC sample
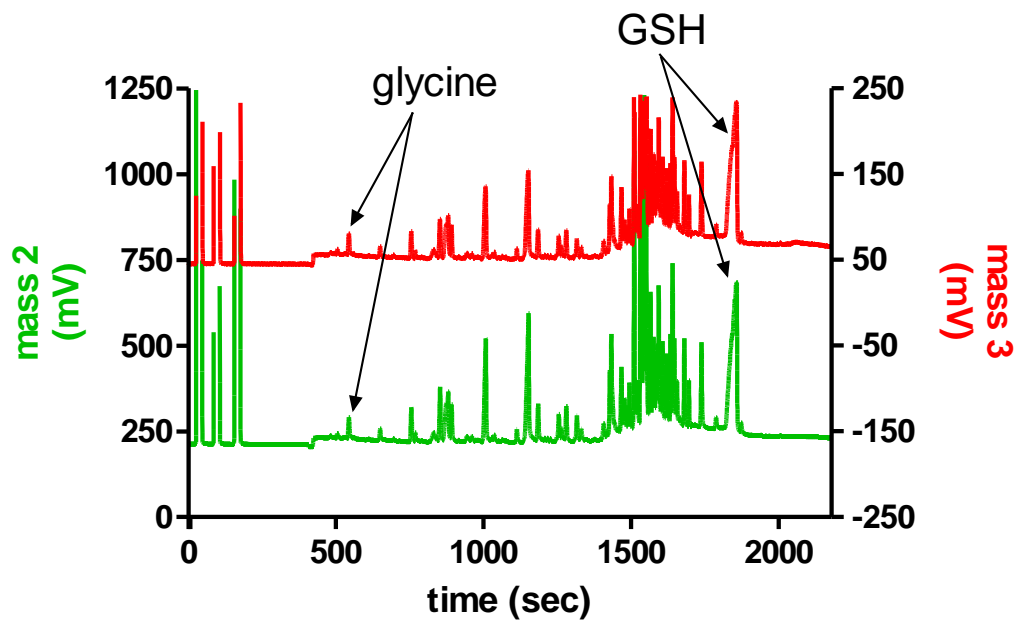
Figure 2.25 Chromatogram of RBC sample

2.4.8 Deuterated RBC

When d_2(Gly)-GSH was generated from GSH by erythrocytes (as described in section 2.3.6), 28.8% of endogenous GSH in RBC was converted to d_2(Gly)-GSH. The investigation of the yield of converted GSH was performed by analysis of both control RBC and RBC with d_2-glycine in GC-TC-IRMS. The yield of converted GSH was calculated from the results of ^{2}H/H-GSH in GC-TC-IRMS by following formula:

Converted ^{2}H-GSH (%) =

(^{2}H/H$_{(\%)}$ of GSH in RBC$_{\textbf{d2-Gly}}$ − ^{2}H/H$_{(\%)}$ of GSH in RBC$_{\textbf{control}}$) / (2/33)

(as 2 of the 33 hydrogens of GSH could be replaced by deuterium.

The GSH derivative of control RBC and the d_2(Gly)-GSH derivative of RBC with d_2-glycine was analysed. The results from GC-TC-IRMS were ^{2}H/H of GSH in RBC$_{\textbf{d2-Gly}}$ = 1.77%, and ^{2}H/H of GSH in RBC$_{\textbf{control}}$ = 0.02%. These values put into the formula: 1.77- 0.02 / (2/33) = 28.8 (%)

Although excess d_2-glycine was added into RBC in this GSH generated process, d_2(Gly)-GSH and d_2-glycine in the RBC were analysed by GC-TC-IRMS simultaneously. It was not necessary to purify the d_2(Gly)-GSH from the RBC with d_2-glycine for this study as a purpose of the study was to measure both GSH and glycine in a sample simultaneously by GC-TC-IRMS.

2.4.9   D$_2$-GSH calibration curve

A calibration curve of deuterated GSH was made by mixing RBC with d$_2$-labelled RBC in proportions from 0% to 100%. 200µl of these mixed samples were analysed in triplicate by ^{2}H/H-GSH. The results of ^{2}H/H were shown as parts per mill against reference gas. This calibration curve gave a linear regression with r^2 of 0.9892 and a Y intercept of 1669 ± 1042. The calibration curve is shown at Figure 2.26, and the chromatogram of 100% deuterated RBC is shown in Figure 2.24 above.
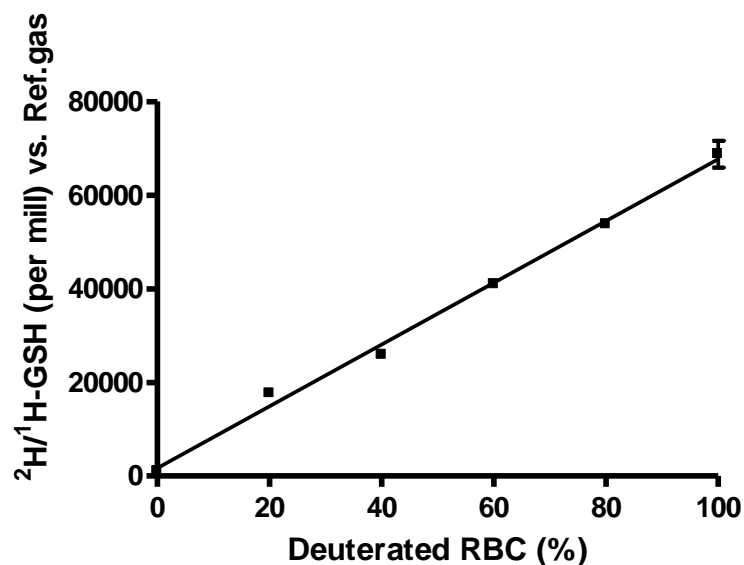
Figure 2.26 Calibration curve of d$_2$-GSH in deuterated RBC

Hence, a good linear regression was obtained. I tried to make a calibration curve of lower amounts of deuterated RBC in the mixture, from 0-20%, again measuring in triplicate. For the lowest amount, 2.5% of deuterated RBC, this

corresponds to an enrichment on the two labelled hydrogen atoms of 28.8% x 0.025 = 0.72%. An acceptable linear regression was obtained with an r^2 value of 0.965 and a Y intercept of 885.5 ± 235.5 (Figure 2.25). The chromatogram of 2.5% of deuterium RBC is shown at Figure 2.28.
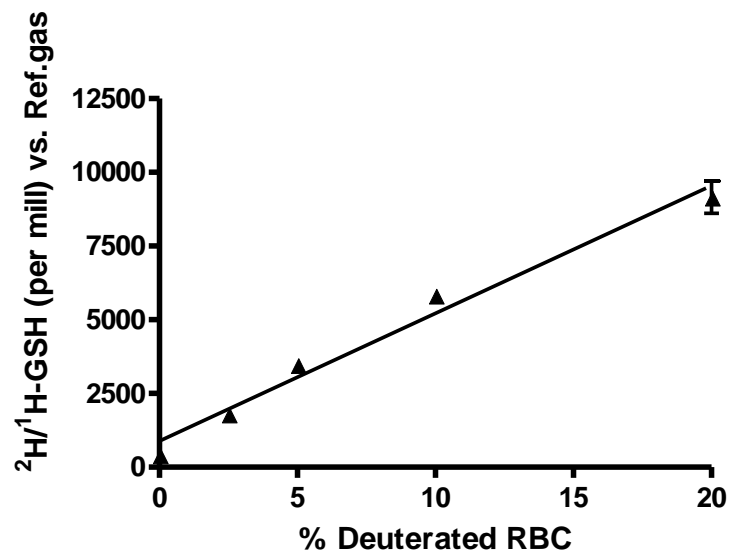
Figure 2.27 Calibration curve of 0% to 20% of d$_2$-GSH in deuterated RBC
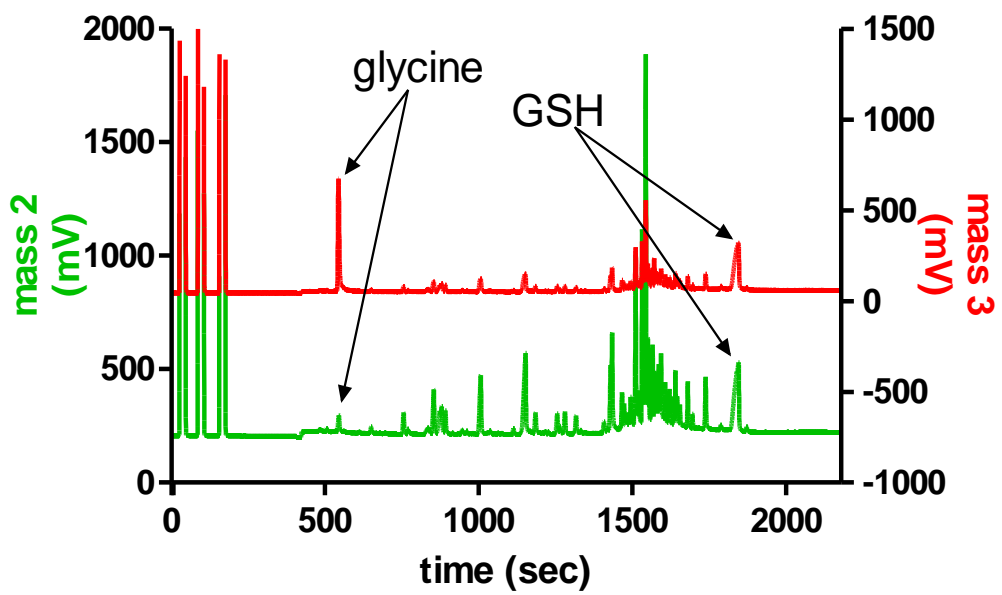
Figure 2.28 Chromatogram of 2.5% of D$_2$-RBC in control RBC

2.4.10 Stability test

The stability of the derivatives was tested as follows. Standards of glycine and GSH, and control and deuterated RBC were derivatised at the same time, and split into three aliquots. One was analysed immediately, another kept at room temperature for 10 days, and the other kept at 4°C for 10 days. The results were compared between storage these three injections.

2.4.10.1 *Glycine stability*

Using 150 µl of 10 mM glycine standard, after derivatization step the glycine sample was re-suspended with 300µl of ethyl acetate and 100µl aliquot into 3 vials. The result of glycine stability showed no significant difference between the sample of day 0 and day 10, even between in room temperature and 4°C (see Figure 2.29).
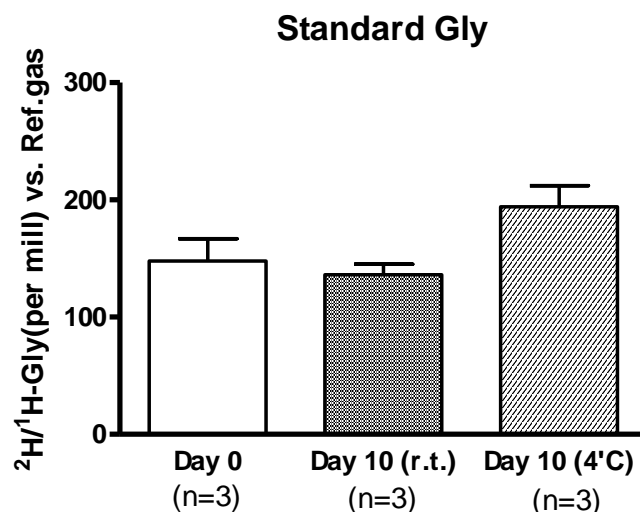
Figure 2.29 Standard glycine stability

2.4.10.2 *GSH stability*

Using 200 μl of 10 mM GSH standard, after derivatization step the GSH sample was re-suspended with 300 μl of ethyl acetate and 100 μl aliquot into 3 vials. The result of GSH stability showed also no significant difference between the sample of day 0 and day 10, even between in room temperature and 4°C (see Figure 2.30).
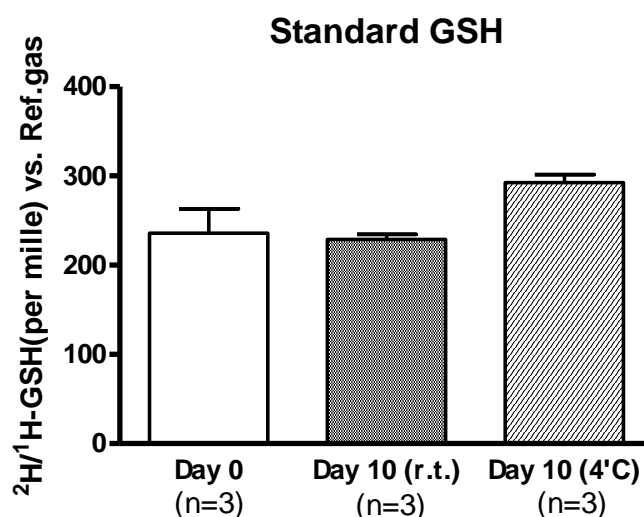
Figure 2.30 Standard GSH stability

2.4.10.3 *RBC stability*

Using 200 μl of control RBC, after protein precipitation and derivatization the RBC sample was re-suspended with 300μl of ethyl acetate and 100 μl aliquot to 3 vials. Glycine in RBC showed stability over the three samples (see Figure 2.31). However, there was a slight alteration in GSH after being kept at 4°C for 10 days.
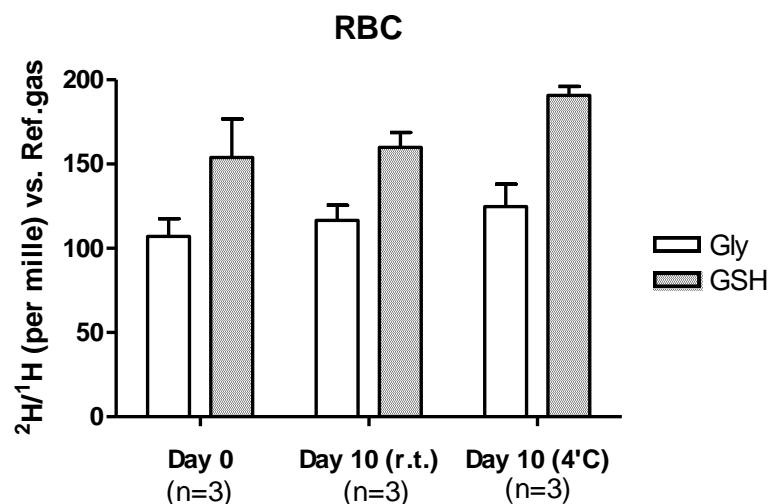
Figure 2.31 RBC stability. Samples were analysed in duplicate at each time point, error bars are SEM

2.4.10.4 *Deuterated RBC stability*

Using 150μl of deuterated RBC, after protein precipitation and derivatization, the deuterated RBC sample was re-suspended in 300 μl of ethyl acetate and 100 μl aliquot into 3 vials. Glycine in deuterated RBC showed stability over the three samples (see Figure 2.32). However, GSH in the deuterated RBC showed a change in enrichment, both when kept at room temperature for 10 days, and when stored at 4°C, but the change in enrichment at 4°C was less than that at room temperature (see Figure 2.33).
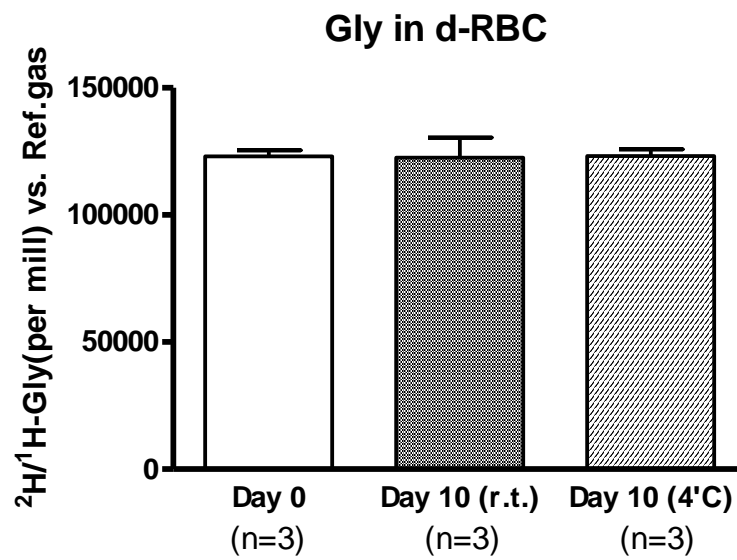
Figure 2.32 Glycine stability in deuterated RBC
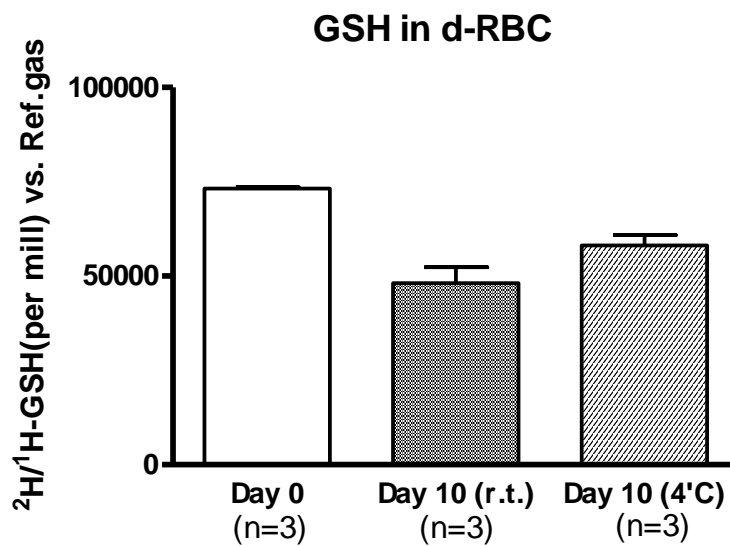
Figure 2.33 GSH stability in deuterated RBC

2.5 Discussion

GC-C/TC-IRMS is in some ways similar to GC-MS but is up to a hundred times more sensitive to isotopic differences than GC-MS (Meier-Augenstein 1999b), although it has a lower analytical sensitivity. However, GC-IRMS cannot be used for identification of unknown compounds because only CO_2 or H_2 are measured and all structural information is lost. Therefore, a combination of GC-MS and GC-IRMS can be important when it is necessary to analyse unknown compounds. In that case, it is necessary to use the same column in GC-IRMS as GC-MS. The step of sample derivatization is also important to use both machines. GC-IRMS can be suitable for compound specific isotope analysis (CSIA), but the silylated and fluorinated derivatives which are often used in GC-MS may cause damage to the IRMS.

A new derivatization method using *n*-propyl chloroformate and methanolic hydrochloric acid has been developed in this study. This method was able to be used successfully for both GSH and glycine. GSH is a tripeptide synthesized from the precursor amino acids, cysteine, glutamate and glycine. It might be more difficult to make GSH derivative than to make derivatives of single amino acid such as glycine and glutamate. The derivative of GSH should be compatible for using both GC-MS and GC-IRMS. Moreover, it is desirable that derivatization is simple and straightforward, and yields stable derivatives. The new derivatization method which I developed is based on chloroformates. For analysis of amino acids by chloroformates, several derivatization methods had been established; there are mainly four methods, acetyl/methylation, ethylchloroformates

(ECF), *n*-pivaloyl and *o*-isopropylates (NPP), and *n*-acetyl and *o*-propylates (Capitan et al. 1999;Husek 1998;Metges & Petzke 1997;Metges et al. 1996;Wolfe 1992). Some groups had also analysed GSH as well as other amino acids for using GC-MS (Capitan et al. 1999;Kataoka et al. 1995). I was especially interesting in using chloroformate as the derivative of GSH.

The use of alkyl chloroformates (Husek 1998) as derivatization agents is attractive for GSH and related peptides as the amino and sulphydryl functions are simultaneously derivatised in aqueous solution, allowing subsequent alkylation of the carboxylic acid groups (Capitan et al. 1999;Kataoka et al. 1995;Takagi et al. 1996). Capitan et al. used ethyl chloroformate (ECF) and methanolic hydrochloric acid, in two steps. Firstly, sulfhydryl groups (-SH) and amine groups (-NH$_2$) of GSH or an amino acid were esterified with ECF, and then methanolic hydrochloric acid reacted with the carboxylic acid group. I had difficulty obtaining reliable derivatization using this method and therefore explored the derivatization of glycine and GSH using a range of different alkyl chloroformates and alcohols. However, as GSH is a tripeptide, many of the derivatives generated were difficult to elute from a GC column. Use of *n*-propyl chloroformate and methanolic hydrochloric acid to generate the *N,S-n*-propoxycarbonyl methyl ester of GSH allowed chromatographic separation on a 10m column, with the added advantage that glycine, which I intended to use as a stable isotopic label for measurement of GSH synthesis, could be derivatised and analysed in the same sample. The derivatives from chloroformate are more polar than other derivatives such as trimethylsilylates (TMS). Polar GC columns tend not to be used at high temperature because the stationary phase decomposes. However, a moderately

polar column can be used at high temperature. I used a ZB-50 column, which is the highest polarity phenyl phase available (50% Phenyl 50% Dimethylpolysiloxane) and is reported by the manufacturers to be stable to 320ºC (isothermal) or 340ºC for short periods or grandients. It was necessary to operate the column to 320ºC to elute the GSH derivative. The column was reasonable stable under these conditions, but even so required changing every few months.

Moreover, it was necessary to analyse stable isotope labelled GSH and glycine as one of precursor amino acids of GSH at once, if possible. An advantage of using *n*-propylchroloformate and methanolic hydrochloric acid is that it allowed analysis of glycine and GSH in a single chromatogram. This is especially important in hydrogen mode GC-TC-IRMS, where it was necessary to inject in excess of 10μl of derivatised sample in order to be able to detect GSH. Thus, detection of both glycine and GSH in a single injection enabled samples to be analysed by duplicate injections. This derivatization method can potentially be used for many other amino acids and dipeptides as well as single amino acids. The column can be used for both GC-MS and GC-IRMS and also the derivatised sample can be analysed by both machines.

In order to able to calculate the dilution of the labels within the glycine and GSH derivatives, it was important to characterise the structure of each derivative by GC-MS. The GSH derivative showed characteristic fragments of *m/z* 230 and 391, and glycine derivatives also showed *m/z* 116. Although the molecular ion could be detected for the glycine derivative ($m/z = 175$), the GSH derivative was fragmented very easily and its molecular ion ($m/z = 507$) could not be detected. Glycine and GSH derivative were identified by the retention time and characteristic

fragmentation in GC-MS. My initial aim was to identify a suitable internal standard that could be used in GC-IRMS, which would have allowed calculation of the amount of GSH as well as enrichment. I tested several other di- and tri-peptides for potential use as internal standard. In particular, γ-glutamyl glutamate and homoglutathione (γ-Glu-Cys-β-Ala) were tested. However, none of the tested compounds were clearly separated from other interfering peaks in GC-IRMS analysis, so I was unable to identify a suitable internal standard.

For analysis of biological samples, it is necessary to reduce oxidised GSH (GSSG) to GSH. I chose tris-(2-carboxyethyl)-phosphine (TCEP) for reduction of disulfide, although dithiotreitol (DTT) is commonly used for reduction of thiol compounds. When I compared DTT with TCEP to blood samples in this derivatization method, TCEP treated samples gave fewer interfering peaks and noise than DTT (Anderson et al. 1999). It may because of the structure of TCEP chemical, which has a carboxyethyl group. The carboxylation of GSH by propylchroloformate was less affected from TCEP than DTT. TCEP can be a good reductant over a relatively wide range of pH (2 ~ 11), and thus will continue to reduce thiol groups during the subsequent steps, in which the sample is first acidified and then made alkaline, and then re-acidified. Thiol group of DTT changes disulfide-bridge into circled chemical structure *per se* when DTT reduces other thiol compounds. In comparison, TCEP does not contain thiol group and also non-volatile.

After establishing the temperature gradient and the derivatization method, I started to analyse ^{13}C-glycine, (glycine-^{13}C,^{15}N)-GSH (because glycine-^{13}C-GSH

is not commercially available), and $^{13}C/^{12}C$-glycine and GSH in RBC by GC-Combustion-IRMS. Calibration curves for ^{13}C-GSH were generated and were linear. Before I continued to analysis of $^2H/^1H$ in samples, I needed to analyse ^2H-glycine-GSH. As this is not commercially available, I generated deuterated GSH from human blood *in vitro*. I found that when glycine, cysteine or glutamate were added to RBC collected in a Li-Heparin tube, the peak area of the GSH derivative increased, whereas when the RBC was collected in an EDTA tube, there was no change in peak area. I concluded that the enzymes of GSH synthesis are active in heparinised blood, but their activity can be stopped by EDTA. Thus, I used the method of heparinised blood incubated with ^2H-glycine, followed by stopping the reaction with 5mM EDTA, for generation of ^2H-GSH *in vitro.* Stopping the reaction with EDTA is important otherwise the isotopic enrichment of GSH would have changed over time as the enzymes would still have been active. Analysis of *in vitro* generated ^2H-GSH showed that approximately 29% of the glycine moieties in GSH had been replaced by $^2H_2$-glycine, and thus the *in vitro* generated ^2H-GSH could be used to create a calibration curve of deuterated GSH in RBC. As $^2H/^1H$ compound specific isotopic analysis is inherently less sensitive than $^{13}C/^{12}C$ analysis (Sessions 2006), in order to increase the amount of analyte on column, it was necessary to use large volume injection via a programmable temperature vaporization (PTV) inlet. Using this method, it was possible to make duplicate injections of 11.25μl from the same sample, which allowed measurement of $^2H/^1H$ enrichment of glycine and GSH in a single chromatogram.

Calibration curves were made by mixing deuterated with non-deuterated RBC in various proportions. The calibration curve was acceptable, with $r^2=0.965$,

p<0.001. The relatively poor correlation coefficient may be due to the fact that programmable temperature vaporization with solvent venting is less reproducible than other GC injection techniques. Incomplete transfer of GSH during heating of the inlet from 37 to 300°C could introduce a variable fractionation. In addition, the chromatographic peak height for GSH sometimes fell below 1000mV, thus leading to inaccurate quantification of ^{2}H/^{1}H of GSH (the amplifiers of the IRMS are most linear in response between 1000mV and 10,000mV). Thus the method is acceptable for studies of enriched samples, where the fractional synthesis rate is calculated from the slope of the enrichment over time, but is not yet suitable for studies close to, or at, natural abundance of ^{2}H/^{1}H, or where an absolute measurement of enrichment relative to VSMOW (Vienna Standard Mean Ocean Water) is required.

In terms of stability of the new developed derivatization, chloroformate-mediated derivatives are relatively stable (Husek 1998;Montigon et al. 2001), but the derivative samples would be normally analysed as soon as possible, or at least within 24h on an autosampler sequence. However, IRMS machines are frequently temperamental, so that after preparing a batch of samples for analysis, it is not always possible to analyse them immediately until the machine problems were solved. In order to determine whether samples that were stored could be reliably analysed later, I carried out a preliminary assessment of sample stability. The results suggested that standards of both glycine and GSH, and RBC sample were reasonably stable, although RBC samples appeared to be less stable than standards, especially if kept at room temperature. Therefore, if there was a delay between derivatization and analysis, I stored samples at 4°C and tried to analyse as soon as

possible, although the stability results suggested that the derivatives could be stable for a week, even at room temperature.

Since I carried out the practical work appearing in this thesis, two further papers measuring GSH synthesis using labelled glycine have been published. Tea et al. described a determination of ^{13}C isotopic enrichment of GSH and glycine using GC-C-IRMS (Tea et al. 2007) They also used GC-MS for analysis of two compounds. They used ethyl chloroformate (ECF)/methanolic HCl to derivatise both GSH and glycine, and an apolar column (HP-5MS, 5% phenyl polysilphenylene-siloxane) which Capitan group had used (Capitan et al. 1999), In my preliminary experiments with ECF-methanolic HCl and an HP-5MS column, although glycine gave a clear peak when analysed by GC/MS, GSH did not give a clear peak or consistent results. The paper by Tea et al. also shows a poorly-defined GSH peak (see Figure 2.34).

Figure 2.34 GSH peak from GC-C-IRMS using HP-5MS column (Tea et al. 2007)
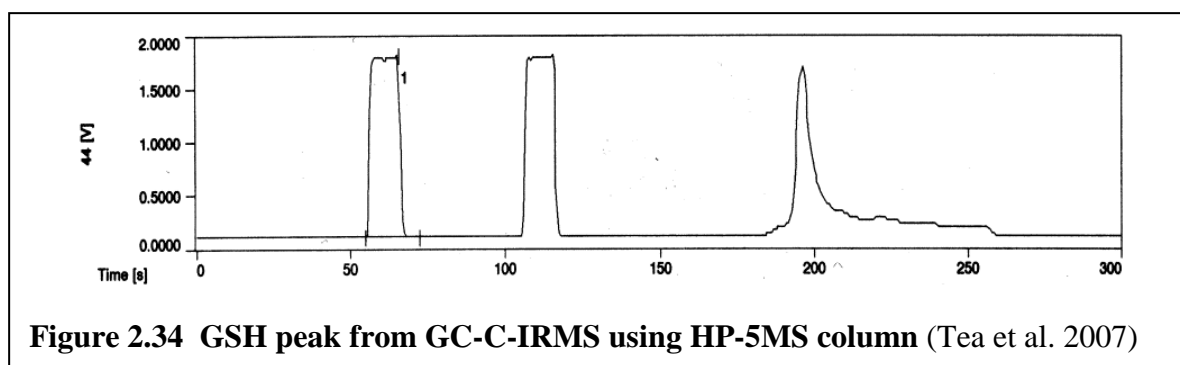
Even though derivatization with chloroformate plus alcohol blocks the hydroxyl groups, GSH derivatives still retain some polarity due to the secondary amine functions (Figure 2.10). Hence, using a more polar column, such as the ZB-50 column (50% phenyl polysilphenylene-siloxane) which I used, GSH chloroformate-derivatives are eluted more clearly and reproducibly. When the polar column was used for analysis of GSH in GC-C-IRMS, both GSH and glycine, as a

tracee and a tracer, could be analysed in a single chromatographic run because of a good peak separation. Tea et al. also had good results for their stability study, confirming my findings that glycine and GSH chloroformate derivatives have good stability properties up to 10 days.

Shierbeek, et al. performed a study of GSH kinetics using LC-IRMS (Schierbeek et al. 2007). LC-IRMS has been recently developed and became commercially available in 2004 (Krummen et al. 2004). Shierbeek et al. used LC-IRMS for analysis of GSH and GSSG in low birth weight infants who were infused ^{13}C-glycine intravenously for 6 hours. They were able to measure ^{13}C-enrichment of GSH by LC-IRMS without the need for derivatization, and were also able to measure GSH concentration by the use of two internal standards. Their method has two disadvantages. Firstly, underivatised ^{13}C-glycine was not clearly separated from other amino acids in their LC-IRMS analysis, so that it was necessary to measure ^{13}C-glycine enrichment independently by GC-C-IRMS. This requirement for two separate measurements would increase the amount of sample needed for analysis. Secondly, LC-IRMS cannot be used for deuterated tracers, as all HPLC solvents contain hydrogen, which would overwhelm the IRMS signal.

Very recently, another method for measuring GSH synthesis using deuterated tracers has been described (Cabral et al. 2008). In this method, animals are given deuterated water over a period of several days to reach a target enrichment of 5% in water. Repeated blood samples are taken, and the incorporation of deuterium into GSH is measured by LC-MS after derivatisation to the *N*-ethylmaleimade derivative of GSH. Analysis of the GSH synthesis rate depends on a complex isotopomer analysis. Administration of deuterated water

over a period of several days is clearly not easily applicable to patients, although the derivatisation and analytical method could also be applied when deuterated amino acids are administered.

The method I have developed in this chapter has the advantages that it can be used with either ^{13}C or ^{2}H tracers, and that glycine and GSH can be analysed in a single sample. In theory, my method could also be used with ^{15}N- or ^{18}O- labelled amino acids, although I did not test this. It does, however, have the disadvantages that (i) I was unable to find a suitable internal standard to allow GSH concentration to be determined, (ii) although linearity is good at medium-high enrichments, linearity is not so good at low enrichments so cannot currently be used for analyses close to natural abundance, (iii) unlike the method of Shierbeek et al, GSH and GSSG cannot both be analysed, (iv) the derivative used adds several carbon and hydrogen atoms, thus diluting the enrichment of ^{13}C or ^{2}H in the IRMS analysis.

2.6   Conclusions

GC-C/TC-IRMS is suitable for compound specific isotope analysis (CSIA), and the derivatization method which I established is compatible with IRMS and does not chemically attack the mass spectrometer or the combustion/high temperature conversion interfaces. The newly-developed derivatization method is suitable for both chemical standards and physiological samples, is simple and is able to derivatise tri-peptides such as GSH as well as single amino acids. Using either ^{13}C-GSH standard, or in vitro-generated ^{2}H-GSH, acceptable calibration curves were generated. Thus, the new method can be used for *in vivo* studies, although it has the disadvantage that GSH concentration is not simultaneously measured.

Chapter 3

Measurement of glutathione synthesis

in septic & non-septic critically ill infants

Chapter 3: _____

3.1 Introduction

Despite recent advances in the care of critically ill children, systemic inflammatory response syndrome (SIRS) and sepsis, which cause multiple organ dysfunction syndrome (MODS), remain a major source of morbidity and mortality (Edited by: Deitch et al. 2002). Systemic inflammatory response syndrome (SIRS), sepsis and MODS are major issues in the paediatric intensive care unit (Despond, Proulx, Carcillo, & Lacroix 2001). Until now there has been no definitive treatment of MODS patients, therefore, control of infections, conventional treatments and total parenteral nutrition (TPN) support is necessary. A systemic inflammatory response induces cytokines, which includes pro-inflammatory and anti-inflammatory mediators, and these activated cytokines also induce production of reactive oxygen (ROS) and nitrogen species (RNS). ROS and RNS damage cellular structures and can be a trigger of organ dysfunction (Goode, Cowley, Walker, Howdle, & Webster 1995;Tanjoh, Shima, Aida, Tomita, & Kurosu 1995;Thomas & Balasubramanian 2004).

Antioxidant defences play a key role in critical illness as they can protect tissues and cells from reactive oxygen free-radicals which are induced from inflammation. One of the major important antioxidant functions is GSH. GSH, a tripeptide, is synthesized *de novo* within many cells from the precursor amino acids cysteine, glutamate and glycine, and is a major low molecular weight thiol in mammalian cells (Meister & Anderson 1983). The study of antioxidant defences,

especially GSH, in critically ill paediatric patients can help understanding of the involved pathophysiology and potential therapies. When patients are critically ill, GSH must be continually synthesised and the oxidised form re-converted to the reduced form to protect cells from ROS and RNS.

3.2 Aim

The aim of this study was to measure erythrocyte GSH synthesis, using deuterated glycine as a tracer, in septic and non-septic infants and children, to determine the GSH synthesis rate in both groups.

I hypothesised that GSH synthesis rate would be decreased in septic patients compared with non-septic patients.

3.3 Methods

This study was approved by Great Ormond Street Hospital and Institute of Child Health joined ethics committee.

3.3.1 Study groups

All patients in this study were in neonatal and paediatric intensive care units (NICU and PICU). There were two groups, one group with evidence of sepsis and the other group (control) was in the ICU but had no evidence of infection or inflammation at the time of the study. Patients were defined as septic on the basis of a clinical diagnosis based on two or more of the following conditions being present: (1) Hyperthermia ($> 38^{\circ}$C) and/ or hypothermia ($< 36^{\circ}$C); (2) Age-related tachycardia (Heart rate higher than the normal range for age: beats/min); (3) Age-related tachypnoea (Respiratory rate higher than the normal range for age: breaths/min); (4) White blood-cell count (x 10^9 cells/l) higher or lower than the normal range for age; (5) Platelet count less than 150 x 10^9 cells/l. (American & Society 1992;Turi et al. 2001)

Infants and children requiring ventilation for airway management but with no clinical signs of sepsis were used as controls as there is no reason to suspect that they might have a reduced rate of GSH synthesis. (Table 3.1 shows clinical characteristics of patients in this study.)

Table 3.1 Clinical characteristics of patients

	Age at study (months)	Gender	Weight (kg)	Clinical Diagnosis	Nutrition	Ventilation
Control	1	M	4.2	Head injury	saline/dextrose	PRVC
	6	M	5	Stable post-surgery	saline/dextrose	Pressure support
	8	M	7	Subglottic stenosis	saline/dextrose	SIMV
	8	M	4.5	Subglottic stenosis	100% EN (formula)	CPAP
	9	F	7	Stable post-surgery	80% PN, 20% EN	Y
	11	F	10	Seizures	saline/dextrose	SIMV-extubated
	15	F	8	Stable post-surgery	saline/dextrose	SIMV
	20	M	12	Head injury	EN/saline/dextrose	PRVC
	23	M	14	Head injury	EN	PRVC
	32	M	12	Stable post-surgery	saline/dextrose	Y
	73	M	20	Head injury	EN/saline	Y
	108	M	25	Head injury	EN	Y
	108	M	43	Asthma	saline/dextrose	Y
Sepsis	0.25	F	3	Bilateral pneumonia	EN	HFOV
	1	M	5	Meningitis/ Enterovirus encephalitis	EN	SIMV
	2	M	5	Bronchiolitis	EN	SIMV
	4	M	5.3	Bronchiolitis	EN	Y
	5	M	6.5	Septicaemia	saline/dextrose	SIMV
	6	M	6	RSV Bronchiolitis and pulmonary pseudomonas	TPN/EN	PRVC
	7	F	7.3	RSV Bronchiolitis	EN	SIMV
	11	F	10	Meningitis	saline/dextrose	PRVC
	18	M	11	Epiglotitis	EN	Y
	51	M	16	Asthmatic exacerbation with suspected sepsis	EN	Mask O2
	99	M	33	Severe chest infection	EN	PRVC

PRVC: pressure regulated volume control; SIMV: Synchronized Intermittent Mandatory Ventilation; HFOV: high frequency oscillatory ventilatio

3.3.2 Infusion and sampling protocol

Administrated d_2-glycine was sterile and pyrogen-free, and prepared to pack in ampoules by Department of Pharmacy at Northwick Park Hospital in Harrow and Department of Pharmacy at Royal Victoria Infirmary in Newcastle. D_2-glycine infusion was intravenously given at 15 μmol/kg/h after a priming dose of 15 μmol/kg to all patients. All patients were given d_2-glycine for 8 hours. 0.2 - 1 ml of blood samples were taken into EDTA tubes from at baseline and then both of groups every 2 hours until 8 hours. Blood samples were centrifuged and separated erythrocyte was kept at -80°C until analysis.

3.3.3 Sample preparation, derivatization and analysis

Glycine analysis in preliminary study using GC-MS was used heptafluorobutyric anhydride (HFBTA) for derivatization; 300 μl of H_2O and 600 μl of 1M perchloric acid (PCA) was added into 100 μl of erythrocytes, and mixed vigorously. The mixed sample was centrifuged at 13000 rpm for 15 min. Supernatant added through AG-50W-X8 column H^+ form. Pellet was washed with 400 μl of H_2O and 600 μl of 1M PCA, and the supernatant was also added through the column. The column was washed by 2 ml acetic acid and 6 ml of water until elution was pH >4. The retaining sample was eluted with 3 ml of 30% ammonia water (NH_4OH), and the eluted sample was evaporated to dryness under N_2. 0.5 ml of esterification agent (freshly mixed by propanol:acetyl chloride = 5:1) was added and heated for 1h at $110°C$. Excess agent was evaporated under N_2, and 100 μl of

HFBTA was added and heated at $60\,^\circ$C for 20 min. Excess derivatization agent was dried under N_2. 200 µl of ethylacetate was added to the dry sample and well resolved, and the sample was transferred to an autosampler vial. 2 µl of injection volume was on split/splitless injector in splitless mode, and initial temperature of injector was at $200\,^\circ$C. GC-MS temperature was from $80\,^\circ$C to $280\,^\circ$C at the rate of $10\,^\circ$C/min and helium flow rate was 2 ml/min. HP-5MS column (Agilent Technologies) was used. Samples were monitored in selected ion monitoring (SIM) mode for the derivative of glycine (272 for unlabelled and 274 for d_2-labelled).

50 µl of erythrocytes form each sample was reduced, derivatised and analysed as described in section 2.3.7. Duplicate injections were made from each sample.

3.3.4 Data analysis

^{2}H/^{1}H-glycine and ^{2}H/^{1}H-GSH in RBC patient samples were analysed by GC-TC-IRMS. The atom percent excess (APE) of both glycine and GSH were calculated using the baseline, RBC sample as time zero (t_o).

Using the following formula:

$$APE = 100 \times \frac{(r_{ta} - r_{t0})}{(r_{ta} - r_{t0} + 1)}$$

Where r_{ta} is observed ^{2}H/^{1}H at time a. (Wolfe 1992)

The fractional synthesis rate (FSR) of GSH was calculated from the steady

$$\text{FSR (\%/h) of GSH} = 100 \times \frac{(\text{GSH}_{\text{APE t8}} - \text{GSH}_{\text{APE t4}})}{\text{Gly}_{\text{ss}} \times 4_{\text{h}}}$$

state enrichment of glycine and the rate of increase of GSH enrichment in both groups. The FSR (%/h) of GSH was calculated following formula:

Gly_{ss} = APE of glycine of steady state from 4 to 8 hr.

The FSR of GSH between groups was also statistically analysed by Student *t*-test. Values were means ± SE. The calculated method of FSR was based on (Wolfe 1992;Wolfe & Chinkes 2004).

3.3.5 Statistics

Data were compared by t-tests (two samples), one-way ANOVA (more than two samples) or by linear regression analysis. $p < 0.05$ was regarded as significant.

3.4 Results

3.4.1 Preliminary study

In a preliminary study to determine whether plasma glycine enrichment and erythrocyte glycine enrichment are equivalent, a healthy adult male volunteer was infused with deuterated glycine for 6 hours. Blood samples were separated to plasma and erythrocytes immediately, and then both samples were analysed by GC-MS.
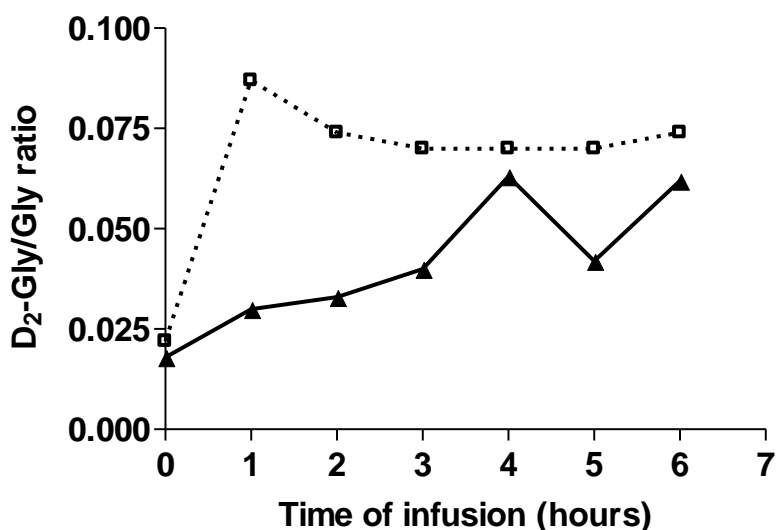
Figure 3.1 The ratio of d$_2$-glycine to glycine in plasma and erythrocyte
The dotted line (□) is plasma and the straight line (▲) is erythrocytes.

The result showed that the enrichment of deuterated glycine in plasma was higher than in erythrocytes. Therefore, in order to calculate the erythrocyte synthesis rate of GSH, it is necessary to measure erythrocyte glycine enrichment,

as this is the true precursor for erythrocyte GSH synthesis and is lower than the enrichment in plasma (Figure 3.1). Therefore, in all further experiments, I analysed only erythrocyte glycine enrichment.

3.4.2 A sample graph of enrichment of glycine and GSH

One septic patient sample showed a typical graph of enrichment of erythrocyte both glycine and GSH by GC-TC-IRMS analysis. On the figure 3.2, the glycine enrichment achieved steady state at 4 to 8 hours, and the GSH enrichment was linearly increased during the steady state of glycine at 4 to 8 hours.
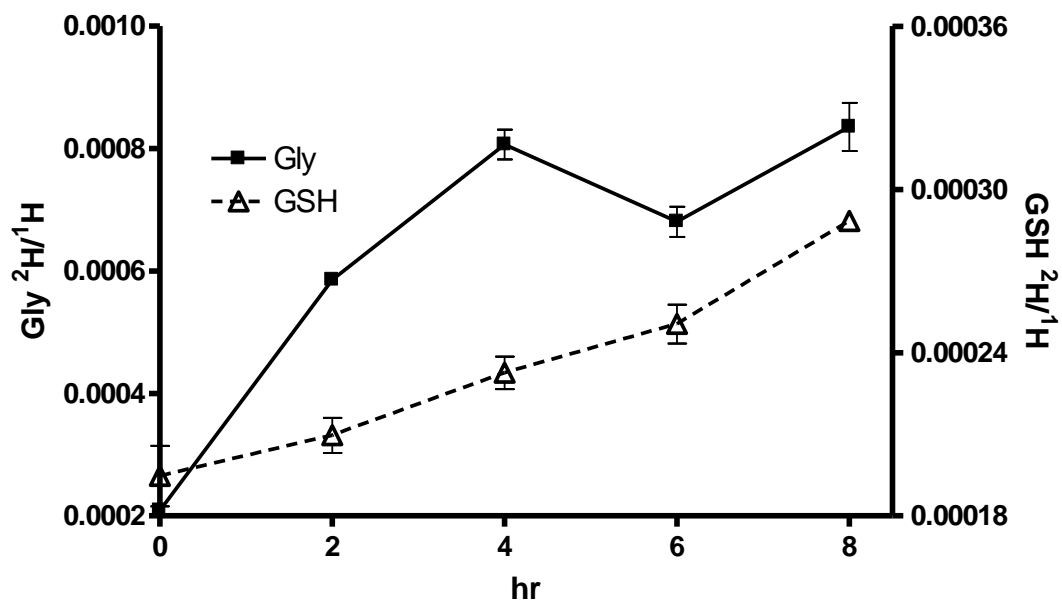
Figure 3.2 A sample graph of enrichment of both glycine and GSH of a septic patient

Calculation of APE of glycine and GSH, and FSR of GSH was described using this septic patient; APE of glycine and GSH was calculated from the baseline

at time 0 to each time point. For example, glycine and GSH APE at time 4 h was calculated the following formula.

Glycine APE (at time 4h)

$$= 100 \times (0.0008069_{t4} - 0.0002090_{t0}) / (0.0008069_{t4} - 0.0002090_{t0} + 1)$$

GSH APE (at time 4h)

$$= 100 \times (0.0002326_{t4} - 0.0001947_{t0}) / (0.0002326_{t4} - 0.0001947_{t0} + 1)$$

During glycine steady state of 4 to 8 hours, GSH FSR was calculated from glycine and GSH APE at time 4 h and 8 h.

GSH FSR (%/h)

$$= 100 \times (0.009369_{GSHt8} - 0.003795_{GSHt4}) / (0.06119 \times 4_{h})$$

Gly_{ss}: $0.06119 = (0.062621_{Glyt8} + 0.059759_{Glyt4}) / 2$

GSH FSR = 2.28 %/h: This is GSH FSR of this septic patient.

3.4.3 Glycine enrichment between control and septic group

Glycine enrichment in erythrocytes increased to steady state in both septic and control patients. Between 4 and 8 hours, There was no significant difference in glycine enrichment in either group. There was no significant difference between control and septic patients at any time point. These data are shown as enrichment (see Figure 3.3) and as atom percent excess (APE) (see Figure 3.4).

Gly
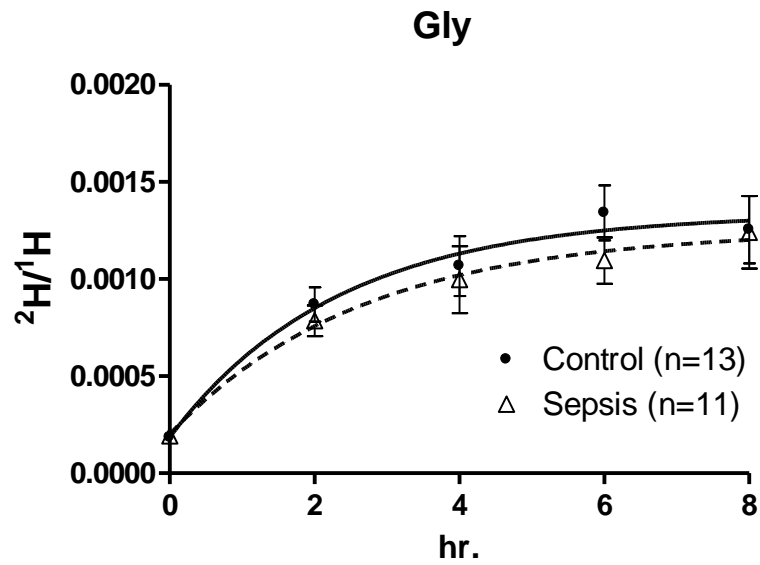
Figure 3.3 Glycine enrichment of control and septic group for 8 hours

Gly
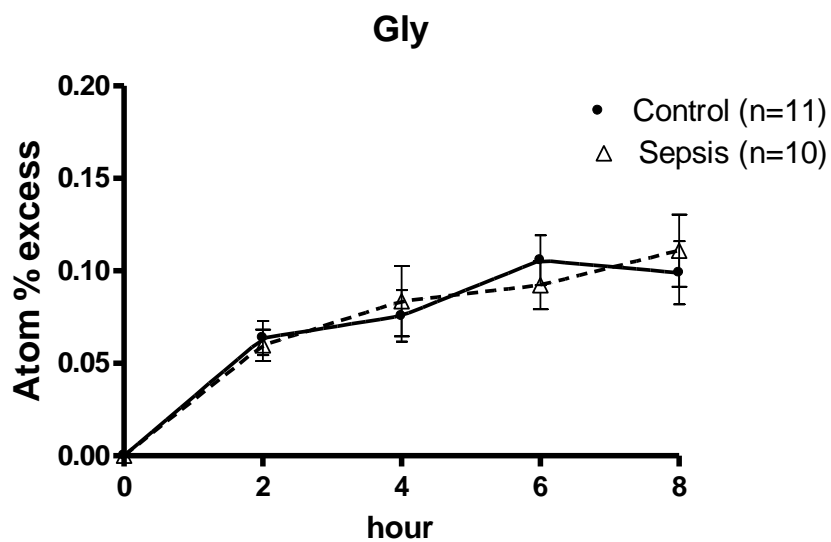
Figure 3.4 Glycine APE in two groups for 8 hours: The dotted line is septic group and the straight line is control.

3.4.4 GSH enrichment between control and septic group

During steady state of glycine enrichment at 4 to 8 hours, there was a linear increase in GSH enrichment in both control and sepsis, but there was a big variation of GSH enrichment in septic group. Although the septic group showed a higher enrichment, there was, however, no statistically significant difference between control and septic patients at any time point. The results are shown in figure 3.5 as enrichment, and in figure 3.6 as APE.
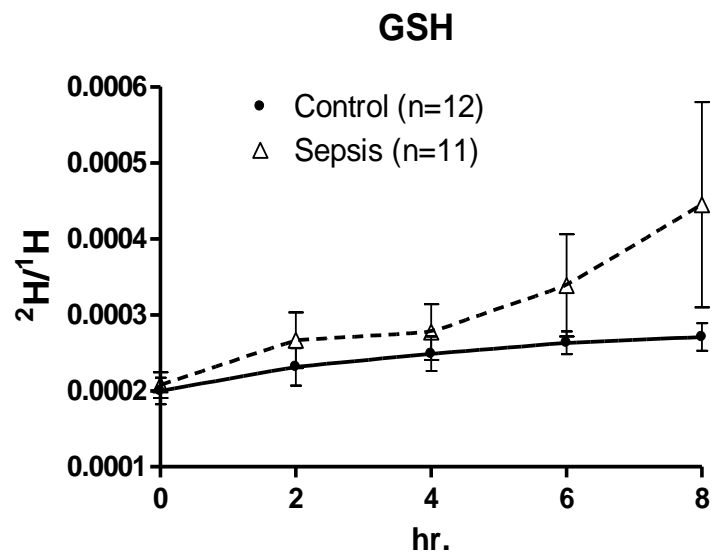
Figure 3.5 GSH enrichment of control and septic group for 8 hours: The dotted line is septic group and the straight line is control.
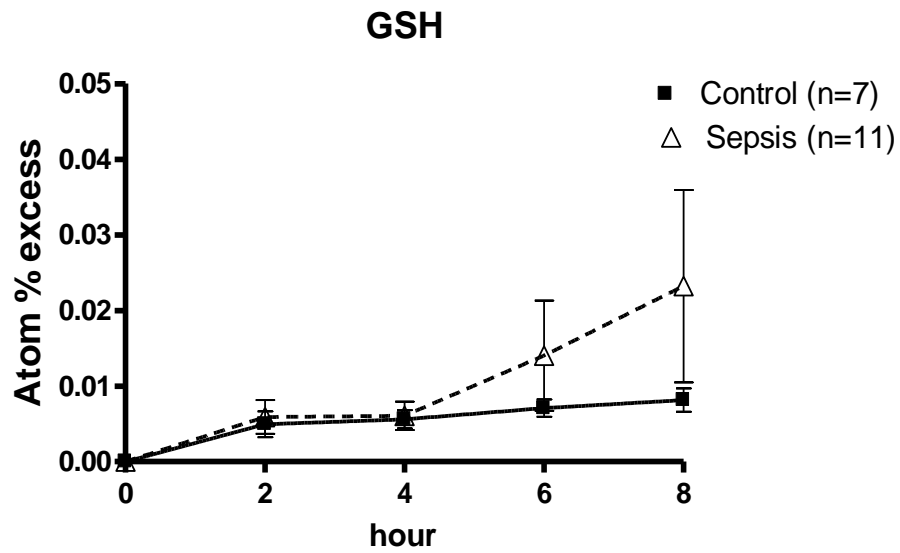
Figure 3.6 GSH APE for 8 hours in two groups: The dotted line is septic group and the straight line is control.

3.4.5 The FSR of GSH between control and septic group

The FSR (%/h) of GSH was calculated from APE of both glycine and GSH during steady state of glycine enrichment. The FSR of GSH in control group was higher than septic group, but this difference was not significant. The FSR of GSH in control group was 1.096 ± 1.173 %/h and the FSR of GSH in septic group was 0.246 ± 0.618 %/h (see Figure 3.7). There was, however, a very wide variation in FSR in each group.
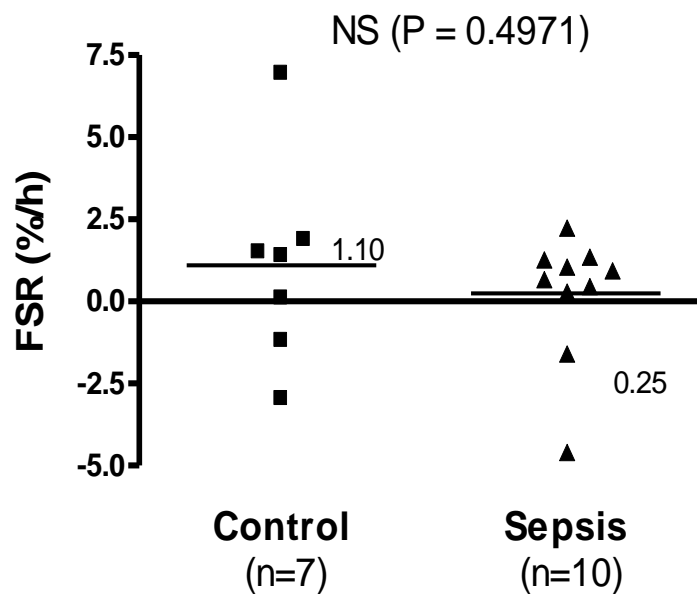
Figure 3.7 The FSR (%/h) of GSH between control and septic group:
The blank column is control and the cross line column is septic group.

3.5 Discussion

In the study of GSH synthesis in critically ill patients, I used the method of GC-TC-IRMS analysis which I had developed. Using this method, it was possible use a single 50 µl erythrocyte sample for measurement of $^2H/^1H$ enrichment of both glycine and GSH. This was an important consideration for performing *in vivo* GSH synthesis studies in infants, where blood samples must be as small a volume as practical. Erythrocyte GSH was analysed in this study, rather than plasma, because erythrocyte GSH is a much bigger GSH pool than plasma (Meister & Anderson 1983), and has been thought of as representative of other tissues (Humbert et al. 2001;Lyons et al. 2001;Reid et al. 2000).

The enrichment of deuterated glycine in plasma was higher than in erythrocytes. Therefore, in order to calculate the erythrocyte synthesis rate of GSH, it is necessary to measure erythrocyte glycine enrichment, as this is the true precursor for erythrocyte GSH synthesis and is lower than the enrichment in plasma. Enrichments of both erythrocyte glycine and GSH were measured in this study. Lyons, et al. commented that the enrichment of the "true" intercellular precursor pool is correspondingly lower than that which can be determined from the apparent cysteine plateau (Lyons et al. 2000). My preliminary study showed that the erythrocyte enrichment was lower than plasma. When erythrocyte GSH synthesis rate from a precursor amino acid would be calculated, erythrocytes precursor enrichment must be measured.

The GSH enrichment between both groups was not significantly different, although the GSH enrichment of the septic group was higher than control group.

The APE of GSH in septic group seemed to show a delayed response compared to the control group. During steady state of glycine, the GSH APE in control group showed a linear increase, whereas GSH APE in the septic group started to increase only after 6 hours. The wide variation in the GSH FSR in the septic group suggests that GSH re-synthesis might be subject to other variables, such as ventilation or inotropic support, or whether patients have acute sepsis or are in the recovery phase. Interestingly, in children with severe protein-energy malnutrition, concentration and fractional synthesis rate (FSR) of erythrocyte GSH was different between groups of each stage (admission: Study 1, postadmission: Study 2 and recovery: tudy 3, see Figure 3.8) (Reid et al. 2000).

The fractional synthesis rate (FSR) of GSH between the two groups was not significantly different, although the FSR of the control group was higher than the septic group. Although 11 septic patients and 13 non-septic patients were analysed in this study, a larger study group may have shown a significant difference.
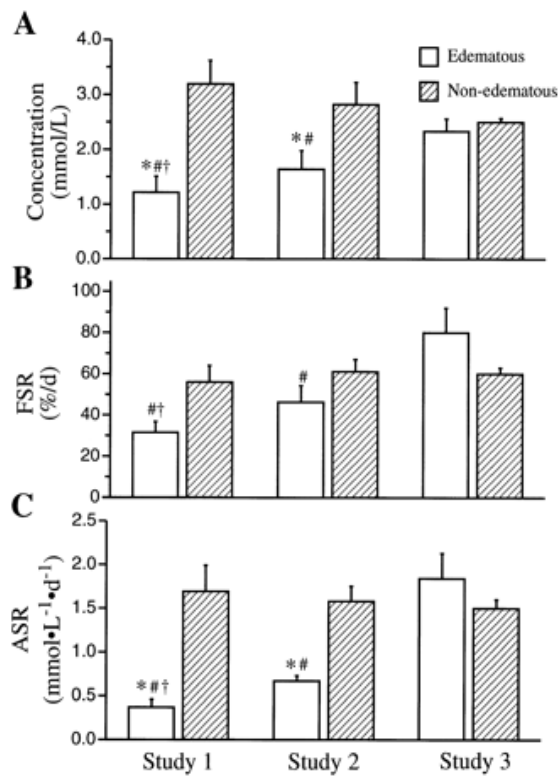
Figure 3.8 from Reid, et al.: Erythrocyte GSH concentration. †*P* < 0.05 vs. *Study 2*, #*P* < 0.01 vs. *Study 3* and *P < 0.01 oedematous vs. non-oedematous.

Using ^{13}C-cysteine, Lyons, et al. showed that septic children have a significantly lower FSR of GSH than control (see Figure 3.9) (Lyons et al. 2001).

As well as the fact that Lyons, et al. used ^{13}C-cysteine as a tracer, there are other differences between their study and mine. Firstly, whole blood measurement
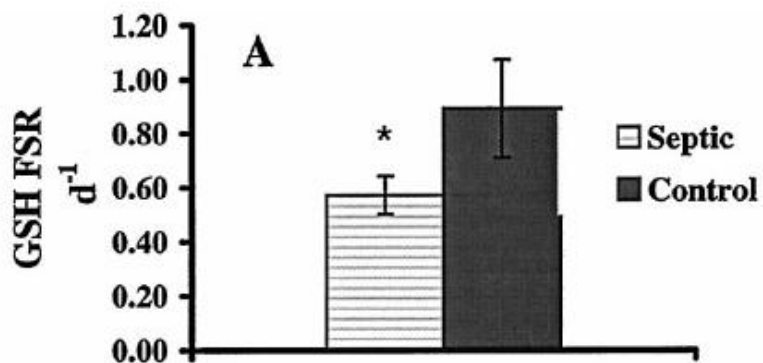
Figure 3.9 from Lyons, et al., 2001: Whole blood glutathione fractional synthesis rate (day[-1]) in septic patients and control (*p < 0.02)

of cysteine and GSH enrichment were made in their study. This may give incorrect estimates of FSR, as the true precursor for erythrocytes GSH synthesis is intracellular cysteine, which may have a different enrichment from plasma cysteine as was measured in their study (Lyons et al. 2001). Indeed, the authors comment on this in a previous paper (Lyons et al. 2000). Secondly, the clinical status of the patients may have been different between the two studies: in Lyons et al., many of the patients were in septic shock, whereas in my study, they were more stable. Additionally, the control group were different, as Lyons et al. studied apparently healthy patients admitted for elective procedures, whereas in my study, patients were critically ill but with no evidence of inflammation (Lyons et al. 2001). These differences could also have contributed to the differences between our results and theirs.

There was no significant difference in glycine enrichment between the control and septic groups. The APE of glycine was also not different between the two groups. This could suggest that glycine turnover of erythrocytes in both groups as a probe of GSH synthesis is independent of sepsis. The control group in this study was not septic, but did consist of critically ill infants and children. The uptake of glycine into erythrocytes might be also depend on the stage of sepsis. Glycine is one of precursor amino acids of GSH, however it is not though to be the rate-limited factor as much as cysteine in the pathway of GSH synthesis (Griffith & Meister 1979).

Although ideally, a healthy control group should have been studied, it was not possible to submit healthy age-matched children to an infusion protocol, cannulation and multiple blood sampling. A non-inflammatory control group was

therefore studied. Although this group can be criticised as they were also receiving intensive care, they were not in an inflammatory state, as evidenced by the significantly lower cytokine profile in the control group (Figure 3.10).
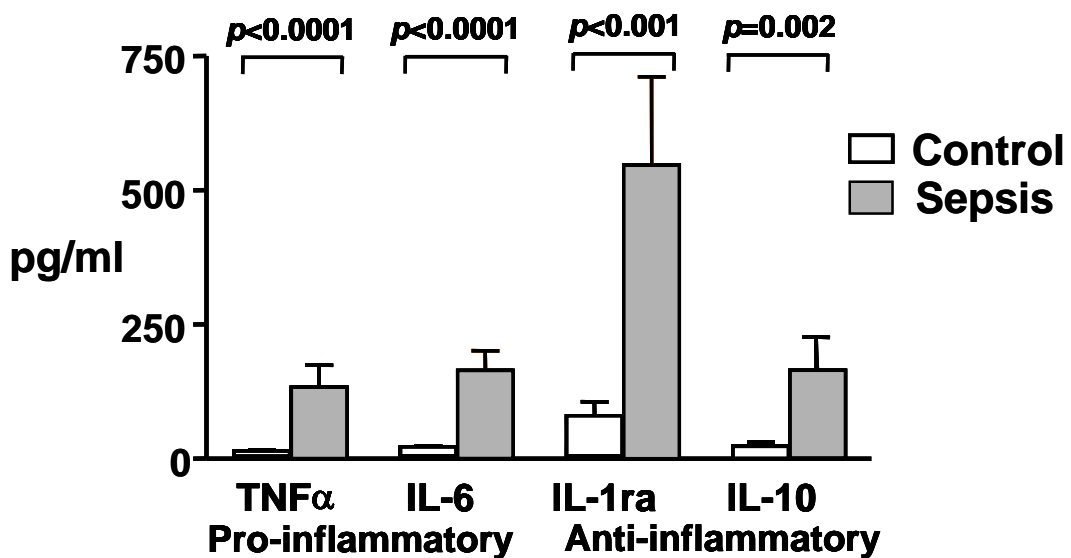
Figure 3.10 Pro- and anti- inflammatory cytokine profile of control and septic patients from the current study (Data of Mr Moti Chowdhury)

Unfortunately, absolute synthesis rate (ASR) of GSH could not be measured in my study as there was insufficient volume of erythrocytes for a separate measurement of GSH concentration, and I had been unable to find a suitable internal standard for GC-IRMS to allow measurement of GSH concentration as well as enrichment.

3.6 Conclusion

GSH synthesis, as fractional synthesis rate, in septic children was lower but not significantly so, compared to non-septic critically ill children. However, the results in each group showed very wide variability, suggesting that the septic and control groups may not have been homogeneous in terms of GSH synthesis. It is possible that a study either on more homogeneous patient groups, or on a lager number of patients, may have shown a difference.

Chapter 4

Effect of glutamine infusion on

erythrocyte glutathione synthesis in

septic infants and children

Chapter4:

4.1 Introduction

Glutamine is the most abundant amino acid in the body (Neu et al. 2002). It is particularly important in cells of the immune system (Ardawi & Newsholme 1990;Newsholme 2001) and in enterocytes (Kimura 1996). In some cell types, such as erythrocytes, extracellular glutamine is used as a source of glutamate, as glutamate uptake by human erythrocytes is extremely low and inadequate to maintain erythrocyte GSH levels (Ellory et al. 1983). The major source of glutamate for GSH synthesis by erythrocytes is thought to be glutamine (Ellory et al. 1983), taken up largely by the Na^+-dependent system N.

Although glutamine is a non-essential amino acid, the nutritional requirement for this amino acid in catabolic illness may differ greatly from normal healthy condition (Neu et al. 2002). During starvation or stress, the concentration of free glutamine in the intracellular amino acid pool of skeletal muscle rapidly decrease (Roth 2007). Thus, some people consider glutamine to be a "conditionally essential" amino acid during sepsis, trauma or after surgery. Historically, glutamine has not been included in parenteral nutrition, firstly because it was thought to be non-essential, and secondly because glutamine is not stable in aqueous solution and changes the formation to pyroglutamic acid and ammonia during heat sterilisation. In addition, glutamine is not very water soluble so a large infusion volume is needed. Recently, however, glutamine containing dipeptides, such as alanyl-glutamine, have become available, which have the advantages that they are much

more water soluble than free glutamine, and can be heat sterilised and thus are stable when added to parenteral nutrition (Furst et al. 1997).

As GSH is very important during oxidative stress, it is possible that glutamine supply for GSH synthesis also becomes very important during oxidative stress. Thus, provision of glutamine can maintain intracellular GSH levels *in vitro* (Babu et al. 2001) and in several *in vivo* studies where oxidative stress and/or GSH depletion takes place (Denno et al. 1996;Flaring et al. 2003;Hong et al. 1992). Glutamine supplemented total parenteral nutrition (TPN) has been shown to prevent intestinal atrophy and necrosis in critically ill adult patients (Jian et al. 1999), and to improve gut immune function in animal models (Calder 1994). Studies have also shown the clinical benefits of glutamine supplemented TPN in adult patients undergoing bone marrow transplantation (Furst & Stehle 1993;Schloerb & Amare 1993;Young et al. 1993;Ziegler et al. 1992).

Although it is classified as a *"non-essential"* amino acid, in states of critical illness or trauma it has been described as by some authors as *"conditionally essential"* (Lacey & Wilmore 1990), and it has even been described as "a life saving nutrient" in critically ill patients (Preiser & Wernerman 2003). However, critically ill patients do not routinely receive glutamine, and although there is evidence that glutamine supplementation may be beneficial during critical illness, there is also evidence that glutamine may have no effect.

Glutamine is able to participate in many biological activities which are crucial to maintenance of homeostasis during critical illness: scavenging of ammonia, acid-base regulation, nitrogen transport between tissues and organs, as a fuel and nucleic acid precursor for rapidly dividing cells such as enterocytes and

cells of the immune system, and as a precursor for GSH. Proliferating cells, such as enterocytes and cells of the immune system, which are particularly important in critical illness, require a continual supply of purines and pyrimidines, dependent on glutamine, for DNA and RNA synthesis. Supply of amino sugars for mucin synthesis, together with other components of the extracellular matrix, also seems to be crucially dependent on glutamine for maintenance of intestinal integrity (Khan et al. 1999;Panigrahi et al. 1997). Oxidation of glutamine as a fuel is thought to be particularly important for enterocytes (Kimura 1987;Windmueller & Spaeth 1974), and glutamine can also be used as a respiratory substrate by cells of the immune system, although the rate of glutamine oxidation is low compared to the total rate of glutamine utilisation (Newsholme et al. 2003). Although, circulating glutamine levels decrease during critical illness, it is not known whether this is due to increased oxidation of glutamine as a fuel, or due to increased utilisation of glutamine in other pathways. There is very little information available on whether critical illness increases the utilization of glutamine as a fuel.

Heat shock proteins (HSPs) are essential to cellular survival under a variety of conditions not limited to heat shock. There are many different heat shock proteins with differing functions and subcellular localisation, but HSP70 is of particular interest both during critical illness and glutamine supplementation. HSPs frequently act as chaperones, protecting misfolded proteins from degradation. During critical illness, HSP70 expression is impaired in a variety of cell types (Durand et al. 2000;Schroeder et al. 1999;Weingartmann et al. 1999) and serum levels are lower in those patients who do not survive trauma, suggesting that the ability to up-regulate HSP70 is associated with improved ability to withstand stress

(Pittet et al. 2002). Interestingly, *in vitro* and animal studies (reviewed in (Roth 2008)) have suggested that glutamine increases HSP70 expression, and it has been shown that parenteral glutamine enhances plasma HSP70 levels in critically ill patients (Ziegler et al. 2005). In this study, the increases in HSP70 inversely correlated with length of ICU stay. It has been suggested that increased HSP70 expression by glutamine renders monocytes better able to withstand high temperatures induced by fever (Pollheimer et al. 2005).

Glutamine can potentially act as a gluconeogenic substrate, so it is important to know, in the era of tight glucose control in the ICU, whether glutamine supplementation is likely to cause hyperglycaemia or worsen glucose control. In fact, the available evidence suggests that the converse is true; i.e. incidence of hyperglycaemia and insulin requirement are decreased by glutamine in critically-ill patients (Dechelotte et al. 2006), and insulin sensitivity is improved in multiple trauma patients (Bakalar et al. 2006).

Glutamine has a great number of effects within the immune system. Although many of these are directly related to metabolism and proliferation of cells of the immune system, glutamine also appears to directly influence cell signalling and cytokine production. Of particular interest is the ability of glutamine to affect expression of monocyte HLA-DR, a cell-surface molecule thought to reflect global immune function, which is usually expressed on more than 90% of monocytes. HLA-DR expression falls following surgery or other trauma, and expression on less than about 60% of monocytes is predictive of ICU patients getting infections (Allen et al. 2002;Cheadle et al. 1991;Hershman et al. 1990). This immune hyporesponsiveness (termed "*immunoparalysis*") is also reflected in a reduced

ability of whole blood to produce TNF-α in response to lipopolysaccharide. Glutamine is able to prevent this immunoparalysis in surgical patients (Exner et al. 2003;Spittler et al. 2001) and trauma patients (Boelens et al. 2002). The effects of glutamine on cytokine production are complex, and different studies have yielded different results.

During critical illness, whole body glutamine economy is altered, such that glutamine release from muscle and lung is increased due to increased proteolysis, and utilization by other organs, such as kidney, liver and gut may increase; ultimately consumption exceeds production and glutamine may become "conditionally essential" (Jackson et al. 1999;Souba & Austgen 1990). Glutamine rapidly becomes depleted in muscle following surgery, sepsis or trauma (Blomqvist et al. 1995;Hammarqvist et al. 1989;POWELL et al. 1994;Roth et al. 1982;Stehle et al. 1989;Vinnars, Bergstom, & Furst 1975), and of all the amino acids, low muscle glutamine concentration was found to be most predictive of mortality in patients with abdominal sepsis (Roth et al. 1982). Muscle glutamine deficiency also results in depletion of muscle GSH (Hammarqvist et al. 1997), which has also been shown to a prognostic marker for poor outcome (Brealey et al. 2002). Muscle glutamine deficiency is prolonged, even in patients undergoing uncomplicated elective liver surgery (Petersson et al. 1992), but can be prevented by supplementation with glutamine (Hammarqvist et al. 1989), alanyl-glutamine (Hammarqvist et al. 1990;Stehle et al. 1989), or even by alpha-ketoglutarate, which forms the carbon skeleton of glutamine (Hammarqvist et al. 1991). Glutamine supplementation also prevents the fall in muscle GSH (Flaring et al. 2003). Depletion of muscle glutamine appears to be due to increased glutamine efflux

from muscle in critical illness, possibly due to a decreased ability of glutamine transporters to maintain the normal 25-30:1 ratio of intracellular:extracellular glutamine (Biolo et al. 2005). This also explains why muscle glutamine is only slowly repleted by exogenous supplementation. The hypothesis that glutamine efflux from muscle during critical illness is due to dysregulation of transport mechanisms is supported by the finding that patients with elevated creatine kinase levels in plasma, suggestive of muscle damage, also have elevated plasma glutamine (Oudemans-van Straaten et al. 2001). Despite the increases in glutamine efflux from muscle, increased utilization of glutamine in plasma results in glutamine depletion, which is characteristic of plasma amino acid changes during critical illness (Jackson et al. 1999). Decreases in plasma glutamine have been observed following burns (Parry-Billings et al. 1990), major surgery (Parry-Billings et al. 1992;POWELL et al. 1994), sepsis (Planas et al. 1993;Roth et al. 1982), pancreatitis (Roth et al. 1985) and in nutritional depletion due to cancer (vanderHulst et al. 1997). Plasma glutamine at ICU admission has also been found to be a prognostic indicator for mortality in emergency admissions; having a plasma glutamine of <0.42mM at admission carried a relative risk of mortality of 1.2 compared to patients having a plasma glutamine of >0.42mM (Oudemans-van Straaten et al. 2001). Given the importance of glutamine for the gastrointestinal system, limited data are available on glutamine content of intestinal mucosa during critical illness. Mucosal glutamine levels are decreased by chronic malnutrition and/or inflammation (Hulsewe et al. 2004;vanderHulst et al. 1994), but other studies have indicated that glutamine content is *increased* during critical illness or following abdominal surgery (Ahlman et al. 1995a;Ahlman et al. 1995b).

If glutamine is administered parenterally, it is usually given as a dipeptide, either alanyl-glutamine or glycyl-glutamine. These dipeptides are rapidly hydrolysed to constituent amino acids and do not accumulate to any great extent, even in critically-ill patients (Berg et al. 2005). In order not to increase total fluid administered, or the amount of nitrogen administered, the amount given of other parenteral amino acids is usually decreased. This limits the amount of glutamine that can be given parenterally, as sufficient of the other amino acids has to be given in order to prevent deficiency of those amino acids occurring during the period of parenteral feeding.

4.1.1 Supplementation in critical illness

Several studies have been conducted with glutamine-supplementation of either enteral or parenteral nutrition (EN or PN) in critical illness. The most recently available systematic reviews and meta-analyses both suggest that there is no significant effect of glutamine-supplemented EN on mortality, infection or organ failure when all studies on critical illness are aggregated (Avenell 2009;Heyland et al. 2009a). Thus, the European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines for EN state that there is insufficient evidence to recommend routine is of glutamine supplementation in EN for critically-ill adults (Kreymann et al. 2006). However, both the Canadian Clinical Practice Guidelines, the ESPEN guidelines and the American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines all recommend the use of glutamine-supplemented EN for patients with trauma and burns (Heyland et al. 2009b;Kreymann et al. 2006;McClave et al. 2009), where the evidence for the use of glutamine is stronger,

as highlighted by previous systematic reviews (Windle 2006). Glutamine supplementation of PN, on the other hand, may be associated with benefit, although there is a discrepancy between the two recent meta-analyses on whether this effect is significant: Avenell's meta-analysis suggests that there is no significant benefit of glutamine supplemented PN on mortality, although she found a significant decrease in infection rate and organ failure (Avenell 2009), whereas Heyland's meta-analysis found a significant benefit for glutamine in decreasing mortality as well as infectious complications and length of hospital stay (Heyland et al. 2009c). These differences appear to be due to the studies included in the meta-analyses. The Canadian Clinical Practice Guidelines, the ESPEN PN guidelines and the ASPEN guidelines all recommend glutamine supplementation of PN for critically-ill adults, but with differing grades of evidence (Heyland et al. 2009d;McClave et al. 2009;Singer et al. 2009).

Small studies in premature infants showed that intravenous or enteral glutamine supplementation decreased duration of ventilation and decreased incidence of sepsis (Lacey et al. 1996;Neu et al. 1997;Thompson et al. 2003). Despite these promising results from small studies, larger scale studies of enteral (Vaughn et al. 2003) or parenteral (Poindexter et al. 2004) glutamine supplementation showed no effect on the incidence of sepsis. One randomised controlled trial in surgical infants found that parenteral glutamine supplementation had no significant effect on intestinal permeability or nitrogen balance, although this study was not powered to detect differences in clinical endpoints such as incidence of sepsis or duration of parenteral nutrition (Albers et al. 2005). Despite these studies on glutamine supplementation in parenteral nutrition of infants, there

have been no studies on the effect of glutamine supplementation in septic infants and children, and no studies on the effect of glutamine on GSH synthesis in septic infants and children.

4.2 Aim

The aim of this study was to measure erythrocyte GSH synthesis, using deuterated glycine as a tracer, in septic infants and children who were randomly allocated to receive either glutamine dipeptide or placebo, to determine the GSH synthesis rate in both glutamine and placebo groups.

I hypothesised that intravenous glutamine supplementation would increase GSH synthesis rate in septic patients compared to placebo.

4.3 Methods

4.3.1 Study groups

All patients in this study were in neonatal or paediatric intensive care units (NICU and PICU) and were aged from 3 days to 9 years. All patients were defined as septic on the basis of a clinical diagnosis based on two or more of the following conditions being present: (1) Hyperthermia ($> 38^{\circ}$C) and/ or hypothermia ($< 36^{\circ}$C); (2) Age-related tachycardia (Heart rate higher than the normal range for age: beats/min); (3) Age-related tachypnoea (Respiratory rate higher than the normal range for age: breaths/min); (4) White blood-cell count (x 10^9 cells/l) higher or lower than the normal range for age; (5) Platelet count less than 150 x 10^9 cells/l. (American & Society 1992;Turi, Petros, Eaton, Fasoli, Powis, Basu, Spitz, & Pierro 2001).

4.3.2 Study design and interventions

Patients were randomly allocated to receive either glutamine or placebo for 76h. *L*-alanyl-*L*-glutamine (Dipeptiven®; 0.6 g/kg/day), equivalent to 0.4 g/kg/day of glutamine, was given to 9 septic patients, and Vaminolact® as isonitrogenous placebo which did not contain glutamine (See Table 4.1) was given to 7 septic patients. The clinician administering the study intervention was blinded to which arm the patients were assigned to, and I was likewise not aware of the group allocation whilst performing the analyses. The only people who knew the group allocations were (i) Dr Eaton who performed the randomisation and (ii) the study pharmacist who prepared the glutamine/placebo infusions in identical bags

marked only with the patient name and the nitrogen content. These supplements were intravenously infused. GSH synthesis was measured twice in all patients, using a deuterated glycine infusion from 0 to 8 hours and again from 20 to 28 hours (Figure 4.1). D_2-glycine infusion was given intravenously at 15 µmol/kg/h after a priming dose of 15 µmol/kg.

Table 4.1 Composition of placebo infusion (Vaminolact)

Vaminolact	Composition	g/l
	Alanine	6.3
	Arginine	4.1
	Aspartic acid	4.1
	Cysteine	1
	Glutamic acid	7.1
	Glycine	2.1
	Histidine	2.1
	Isoleucine	3.1
	Leucine	7
	Lysine	5.6
	L-methionine	1.3
	Phenylalanine	2.7
	Proline	5.6
	Serine	3.8
	Taurine	0.3
	Threonine	3.6
	Tryptophan	1.4
	Tyrosine	0.5
	Valine	3.6
Total amino acid	65.3 g/l	
Nitrogen	9.3 g/l	
Osmolality	510 mOsmol/kg	
Energy	240 kCal	

4.3.3 Blood samples

Blood samples of the two septic groups were taken every 2 hours during the first 8 hours (i.e. during the first d_2-glycine infusion) and then at 20, 24, 28 hours (second d_2-glycine infusion) and at 52 and 76 hours (See Figure 4.1). Erythrocytes (RBC) were immediately separated and kept at -80°C until analysis.
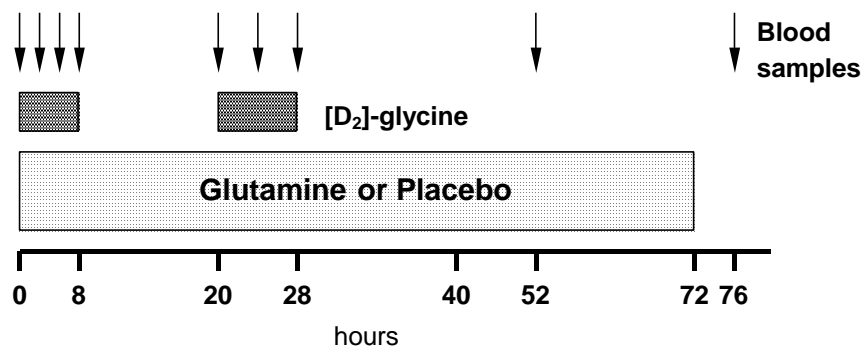
Figure 4.1 Infusion protocol of glycine and either glutamine or placebo

4.3.4 Sample preparation and derivatization

Blood samples were prepared, derivatised and analysed by the same procedures as described in Chapter 3 (section 3.3.3).

4.3.5 Data analysis

$^2H/^1H$-glycine and $^2H/^1H$-GSH in RBC patient samples were analysed by GC-TC-IRMS. Each time point of $^2H/^1H$-glycine and $^2H/^1H$-GSH was statistically analysed by Student *t*-test. Values were means ± SEM.

The atom percent excess (APE) of both glycine and GSH were calculated from time at 0 (base line) to 8 hours and 20 (2nd base line) to 28 hours. Each APE was calculated using the following formula (Wolfe 1992):

$$APE = 100 \times \frac{(r_{tx} - r_{t0})}{(r_{tx} - r_{t0}) + 1}$$

Where r_{t0} and r_{tx} are the observed hydrogen ratios ($^2H/^1H$) at baseline (t_0) and time x (t_x) respectively.

Each time point of APE of both glycine and GSH was statistically analysed by Student *t*-test. Values were mean ± SEM.

The fractional synthesis rate (FSR) of GSH was calculated from the steady state enrichment of glycine and the rate of increase of GSH enrichment in both groups. The FSR (%/h) of GSH was calculated following formula (based on (Wolfe 1992;Wolfe & Chinkes 2004)):

FSR (%/h) of GSH:

$$FSR\ (\%/hr) = 100 \times \frac{(GSH_{APEt2} - GSH_{APE\ t1})}{(Gly_{APEt1} + Gly_{APEt2})/2} \Big/ \Delta t$$

Where: Gly_{APEt1} and Gly_{APEt2} are the glycine atom percent excess values at t1 and t2, GSH_{APEt1} and GSH_{APEt2} are the GSH atom percent excess values at t1 and t2, and Δt is the difference in time in hours between t1 and t2. The FSR of GSH between groups was also statistically analysed by Student t-test. Values were means ± SEM.

4.4 Results

4.4.1 Study groups

Patients were allocated by weighted minimisation, balancing groups for age, inotropic support, ventilation, and whether immunosupressed. The allocations were well matched, as shown in Table 4.2.

Table 4.2 Patient allocations

	Vaminolact	**Dipeptiven**
Age		
0-6 months	2	3
6 months – 3 years	3	4
over 3 years	3	3
Ventilated?		
Yes	8	8
No	0	2
Inotropes?		
Yes	4	5
No	4	5
Immunosupression?		
Yes	0	1
No	8	9
TOTAL	**8**	**9**

One patient in each group subsequently dropped out.

4.4.2 Glycine enrichment and APE in two septic groups

D$_2$-glycine was infused twice in the two groups during 76 hours. Erythrocyte glycine enrichment declined between the two d$_2$-glycine infusions. There was a significant difference in erythrocyte glycine enrichment between the glutamine group and the placebo group at 8 hours (p = 0.013), but enrichment was similar at all other time points (see Figure 4.2).
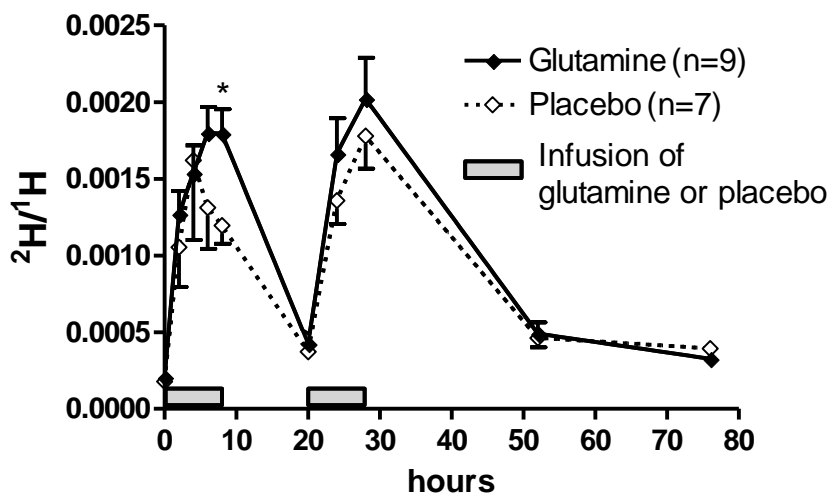
Figure 4.2   Glycine enrichment (^{2}H/^{1}H) in glutamine and placebo groups over 76 hours * p=0.013 between glutamine and placebo

The same data, expressed as APE, are shown for the first 8 hours in Figure 4.3. After 4 hours, glycine APE achieved steady state. There was a significant difference in glycine APE only time at 8 hours (P = 0.012).
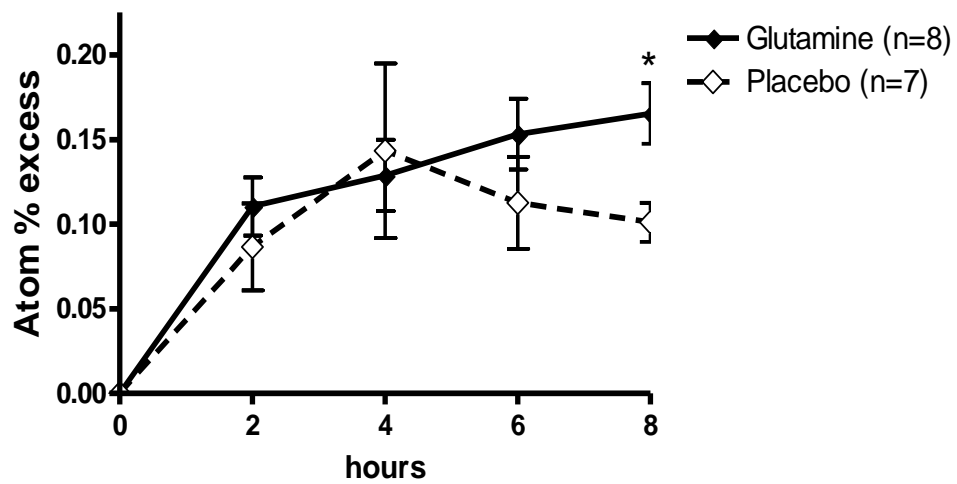
Figure 4.3 Glycine APE in glutamine and placebo groups during the first 8 hours; * p=0.012 between the two groups

The glycine enrichment declined to near normal level at 20 hours (Figure 4.4) and then the second d_2-glycine infusion was given. Figure 4.4 shows the glycine enrichment in the two groups between 20 and 76 hours. The glycine enrichment achieved the second steady state during 24 to 28 hours. The glycine enrichment started to decrease after 28 hours, and at 52 hours it was back to the same level as at 20 hours. There was no significant different at any time point after the second d_2-glycine infusion.
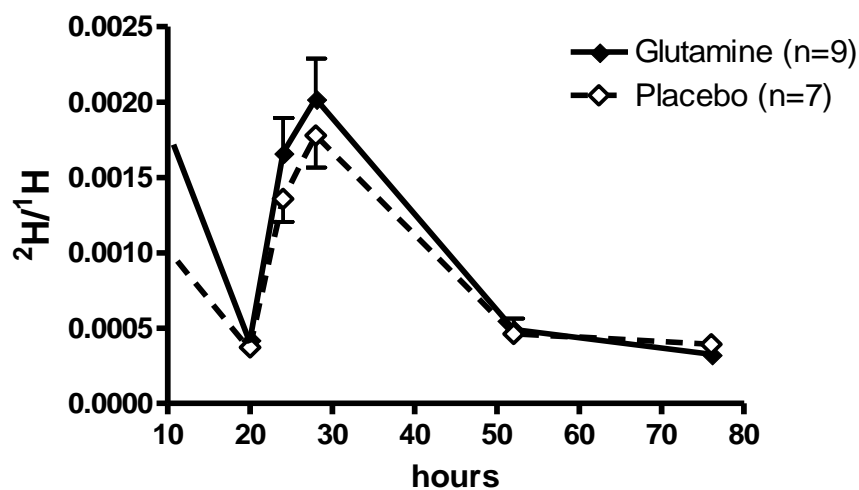
Figure 4.4 Glycine enrichment in glutamine and placebo groups during 20 to 76 hours after the second d_2-glycine infusion. There were no significant differences at any time point

During 20 to 28 hours, glycine APE in two septic groups was also calculated. It was thought to be more appropriate to use the glycine enrichment at t_0 as the baseline for the APE calculations, so there is an APE of approximately 0.02% at t_{20}, when the second d_2-glycine infusion was started. There was no significant difference between two groups at any time point (see Figure 4.5).
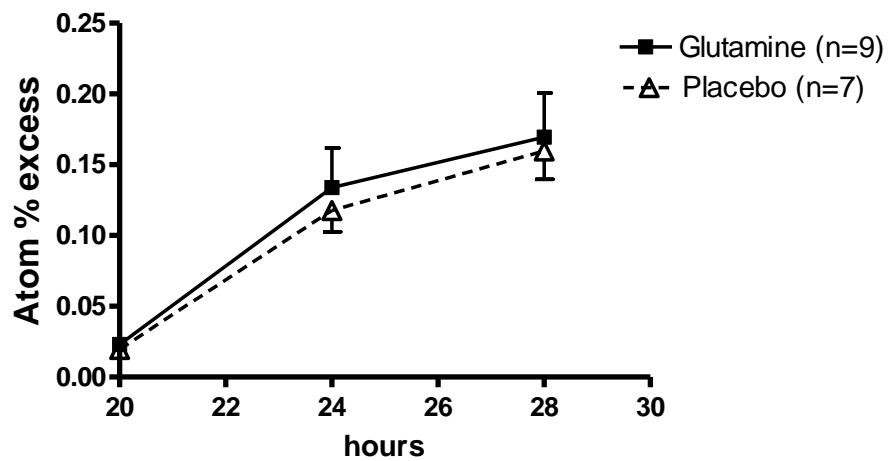
Figure 4.5 Glycine APE in two septic groups during 20 to 28 hours

4.4.3 GSH enrichment and APE in two septic groups

The enrichment of GSH in the two septic groups showed no change between the two d_2-glycine infusions (see Figure 4.6).
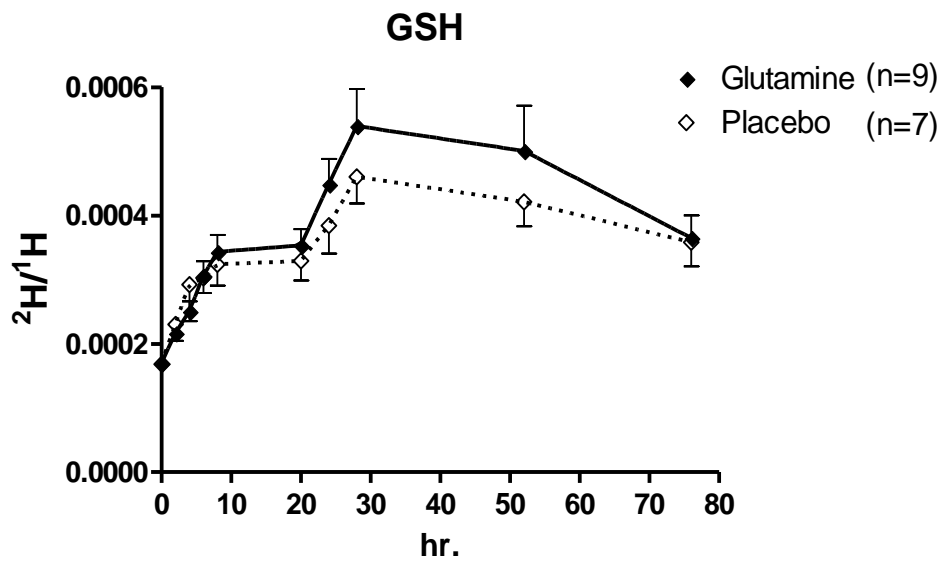
Figure 4.6 GSH enrichment in two septic groups for 76 hours

GSH$_{APE}$ was calculated during the first 8 hours. GSH$_{APE}$ linearly increased during 4 to 8 hours and was no difference between two groups at any time point. Placebo group was increased but not as much as glutamine group during that time (see Figure 4.7).
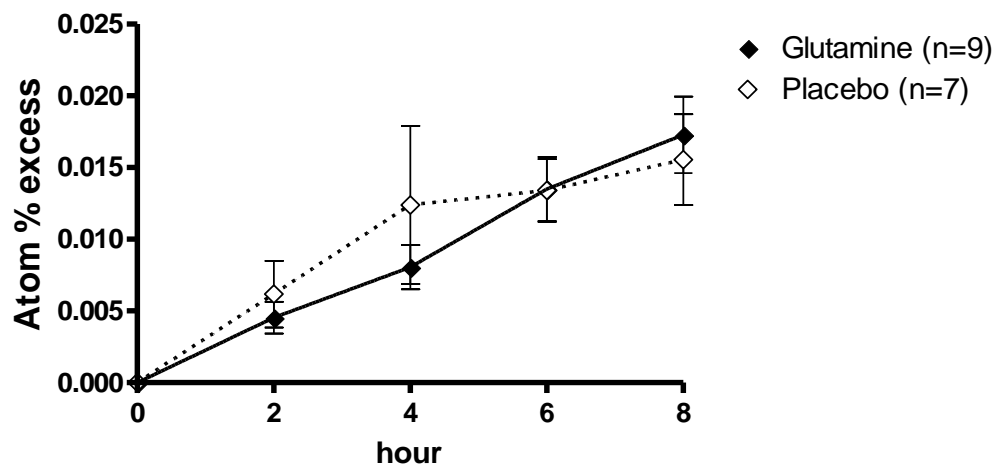
Figure 4.7 GSH APE in two septic groups for the first 8 hours

The enrichment of GSH was maintained until the second d$_2$-glycine infusion. After the second d$_2$-glycine infusion, GSH enrichment was increased and well maintained in both groups by 76 hours. Although the enrichment was always higher in glutamine group, this difference was not statistically significant at any time point (see Figure 4.8).
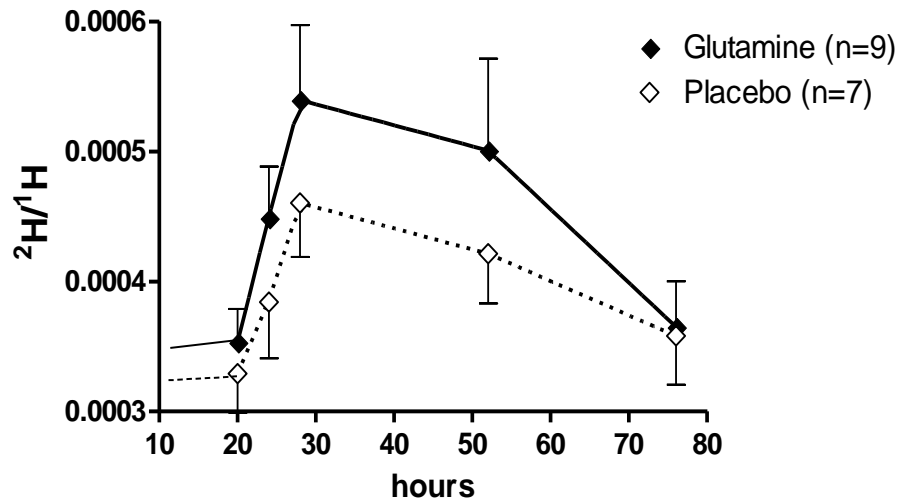
Figure 4.8 GSH enrichment in two septic groups during 20 to 76 hours after the second d$_2$-glycine infusion

During 20 to 28 hours, GSH$_{APE}$ was also calculated, again using t$_0$ as the baseline. GSH$_{APE}$ in both septic groups linearly increased from 24 to 28 hours. Glutamine group was slightly higher than placebo group but there was no significant difference at any time point in two groups (see Figure 4.9).
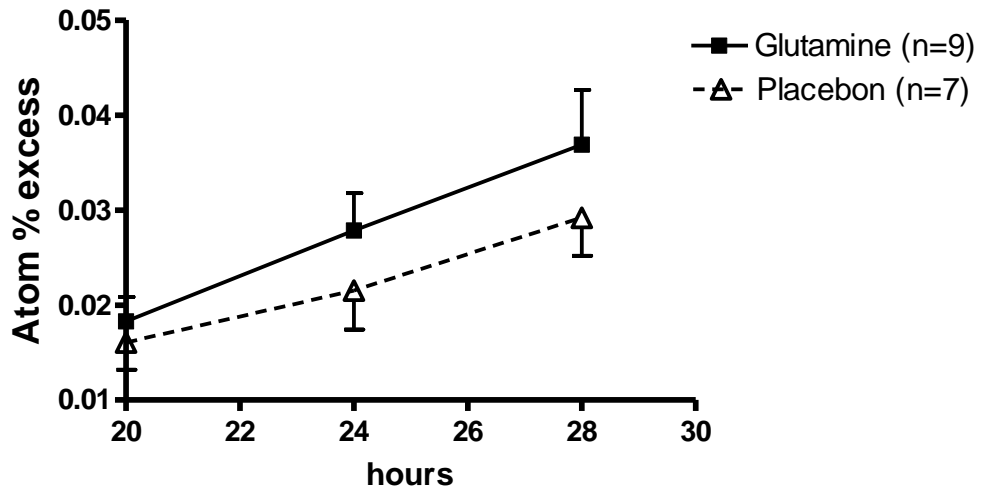
Figure 4.9 GSH APE in the two septic groups during 20 to 28 hours

4.4.4 The FSR of GSH in the two septic groups

During steady state of glycine enrichment at 4 to 8 hours and 24 to 28 hours, The fractional synthesis rate (FSR) (%/h) of GSH in the two septic groups was calculated from APE of both glycine and GSH, respectively.

The FSR (%/h) of GSH for the first 8 hours in two groups showed the glutamine group was slightly higher than placebo group but there was no significant difference between two groups (p = 0.61; Figure 4.10).
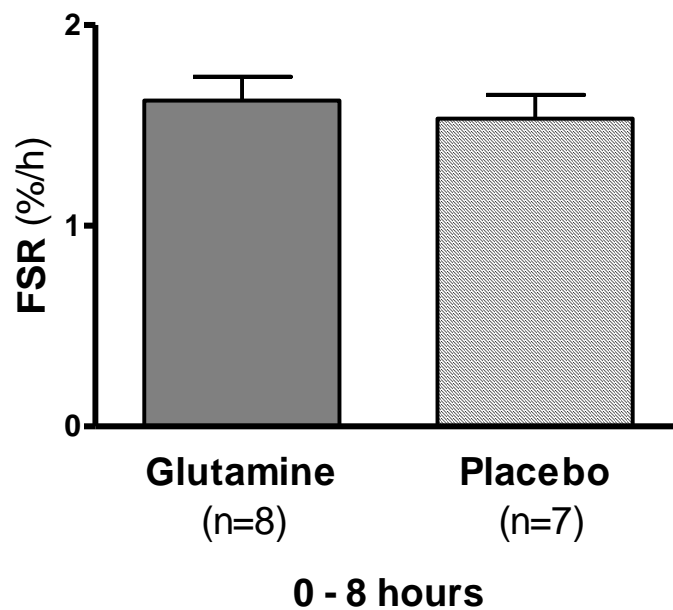
Figure 4.10 The FSR (%/h) of GSH in two septic groups for the first 8 hours

After the second d_2-glycine infusion, the FSR (%/h) of GSH during the 24 to 28 hours in two groups showed the glutamine group was still slightly higher than placebo group but again, there was no significant difference between two groups (p = 0.39; Figure 4.11)
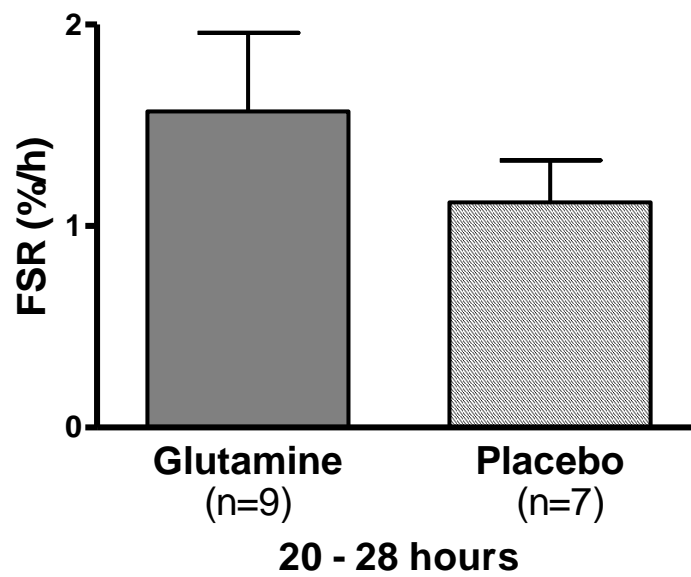
Figure 4.11 The FSR (%/h) of GSH in two septic groups during 24 to 28 hours

4.4.5 Comparison between the non-supplementation groups and supplementation groups

I also compared the glutamine and placebo septic groups (this Chapter) with the two groups studied with the same infusion and sampling protocol as Chapter 3 (i.e. control and sepsis).

The glycine APE of all groups for the 8 hours showed that the glutamine group and placebo group achieved a higher steady state at 4 hours than the control and septic group, but the placebo group was gradually decreased at 8 hours to the similar level to the control and septic group (see Figure 4.12). There was no significant difference at any time point between the 4 groups, except between the glutamine and placebo group at 8 hours (discussed previously in section 4.4.2).
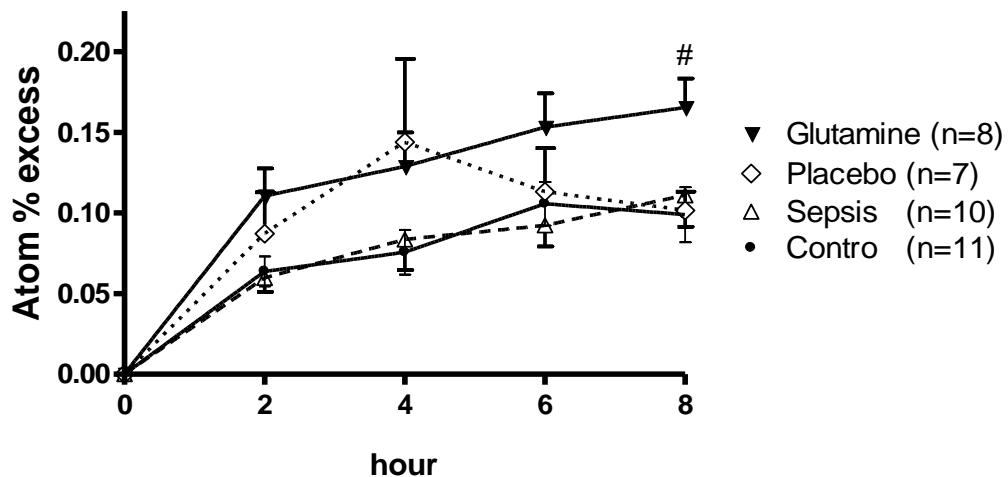
Figure 4.12 The glycine enrichment between all groups for the first 8 hours; There was a significant difference between glutamine and placebo time at 8 hours. (#p < 0.05)

The GSH$_{APE}$ in the 4 groups showed that all groups increased during steady state of glycine at 4 to 8 hours. The glutamine and placebo groups both linearly increased and had a higher GSH$_{APE}$ than control and septic group during 4 to 8 hours (see Figure 4.13). There were significant differences in GSH$_{APE}$ between sepsis and both the glutamine and placebo groups ($p < 0.05$) at 6 hours.
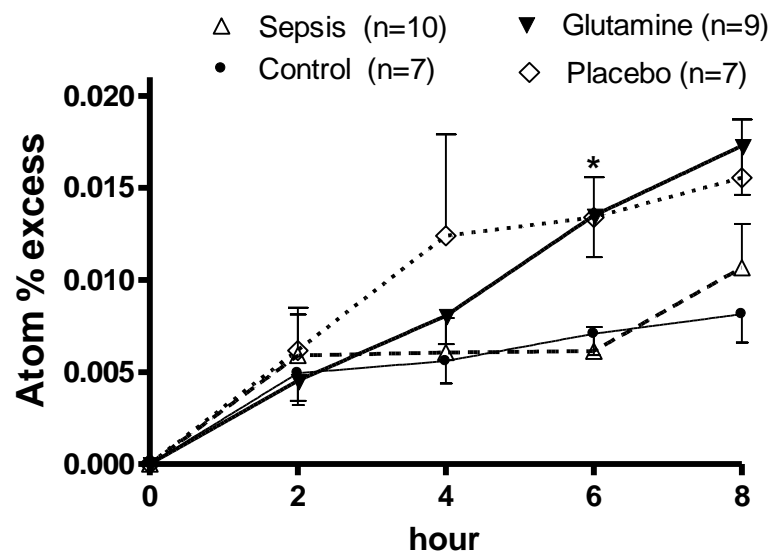
Figure 4.13 GSH$_{APE}$ in all groups for the first 8 hours;
At 6 hours, the sepsis group had a significantly lower GSH$_{APE}$ than both the placebo and glutamine groups (* $p < 0.05$).

The FSR (%/h) of GSH was also compared between all four groups during 4 to 8 hours. There was a lower FSR in the septic group than all three others, but this difference was not significantly different. The FSR of GSH of both glutamine and placebo groups was slightly higher than control group, but again there was not a statistically significant difference (see Figure. 4.14).
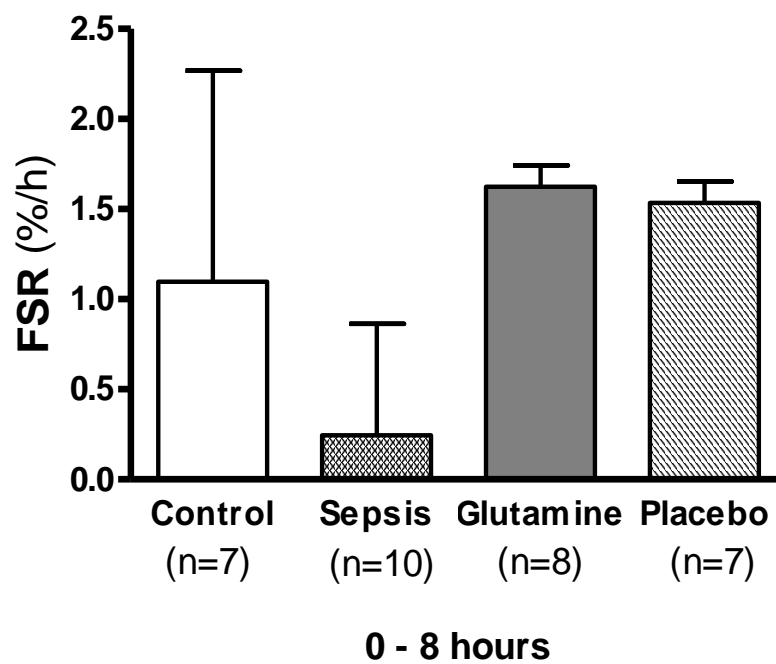
Figure 4.14 FSR (%/h) of GSH in all four groups during 4 to 8 hours

4.5   Discussion

I hypothesised that glutamine infusion during sepsis in infants and children would increase erythrocyte GSH synthesis. My data presented in this chapter suggest that, on the contrary, glutamine infusion has no effect on GSH synthesis.

Free glutamine is not usually added to parenteral nutrition, for reasons of stability, as it forms either free glutamate or pyroglutamic acid. With the advent of stable glutamine dipeptides, it is possible to add glutamine into parenteral nutrition as either alanyl-glutamine or as glycyl-glutamine. However, in this study, a stand-alone infusion of either alanyl-glutamine or Vaminolact (isonitrogenous placebo) was used. The reason for this is that when identified as being septic, infants and children may be receiving some enteral nutrition, may already be receiving parenteral nutrition, or may be receiving only intravenous glucose whilst intravenous nutrition is initiated. Thus, in order to maximise patient recruitment, and to make the results more widely generalisable, it was decided to give alanyl-glutamine or Vaminolact as a separate infusion, in addition to whatever other nutrition was being received by the patient.

GSH synthesis was measured on two occasions during the intervention with glutamine or placebo. Thus, I was able to test whether glutamine was able to increase erythrocyte GSH synthesis under two conditions: (i) initiation of glutamine/placebo infusion and (ii) after 20 hours of glutamine/placebo infusion.

Plasma glutamine concentration in glutamine and placebo groups, and the control group previously referred to in chapter 3, are shown below:
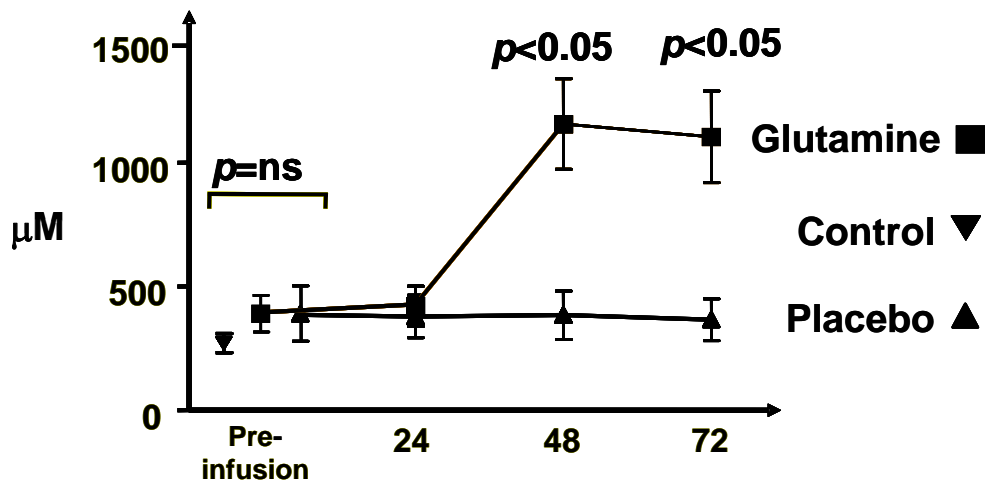
Figure 4.15

Figure 4.15, Plasma glutamine concentration in the glutamine and placebo groups studied in this chapter, and the control group studied in chapter 3 (data of Mr Moti Chowdhury).

As seen in this graph, there was no significant difference in plasma glutamine concentration between the glutamine group and the placebo group at the time of the second d_2-glycine infusion (GSH synthesis measurement). Thus, it could be argued that there might be a difference in GSH synthesis when measured after at least 48 hours of infusion. However, it is also possible that plasma glutamine levels are not increased during the first 24 hours of infusion because utilisation of glutamine could be increased. This is indeed a possibility, as cells of the immune system are known to be major consumers of glutamine during sepsis (Newsholme 2001). In order to determine whether glutamine utilisation is increased during sepsis, it would be necessary to undertake a glutamine turnover

study, using labelled glutamine. Interestingly, although a d_2-glycine infusion was not performed during the 48-76 hours during which we know that plasma glutamine is elevated, I did measure the enrichment of deuterated GSH at 52 and 76 hours. If GSH turnover was increased during this time period due to elevated plasma glutamine levels, one would expect the enrichment of deuterium in erythrocyte GSH to decrease more rapidly than that of the placebo, because glycine incorporated into newly-synthesised GSH would be unlabelled. If the decrease in GSH enrichment between 52 and 76 hours is compared between the glutamine and placebo groups (Figure 4.6), it appears that the decrease in the glutamine-supplemented group is indeed more rapid than that of the placebo group. However, this possible difference would require further study to be confirmed. Unfortunately, in my study due to lack of sample, and lack of an internal standard to measure GSH concentration in the same sample as GSH enrichment, I was unable to measure erythrocyte GSH concentration.

To my knowledge, there are no studied that have studied the effect of glutamine supplementation on GSH synthesis in humans. In a study of adult intensive care unit patients, Flaring et al. showed that glutamine supplementation attenuates the fall in muscle GSH concentration which is induced by surgery. However, in this study, GSH synthesis was not measured, and the effects of glutamine were demonstrated in muscle rather than in erythrocytes (Flaring et al. 2003). Using ^{15}N-glutamic acid to measure GSH synthesis adult beagle dogs, Humbert et al. showed that erythrocyte GSH synthesis was not affected by enteral glutamine supplementation (Humbert et al. 2007). However, in their study, the dogs were not septic but instead had been starved for 3 days to mimic the

malnutrition frequently observed in intensive care unit patients. Interestingly, in their study glutamine supplementation caused a *decrease* in intestinal GSH synthesis, which they speculated could be due to glutamine utilisation to provide NADPH, thus allowing reconversion of oxidised GSH back to the reduced form, and thus a decreased need for *de novo* GSH synthesis.

It is possible that at the rates of GSH synthesis taking place in erythrocytes, factors other than glutamate supply are limiting. For instance, some authors have considered cysteine supply to be rate limiting for erythrocyte GSH synthesis (Badaloo et al. 2002). However, in the current study, the placebo group received cysteine supplementation (at 4.3μmol/kg/h as a component of Vaminolact), so that if cysteine were rate-limiting for GSH synthesis in these patients, one would expect to see an increase in GSH synthesis in the placebo group compared to the glutamine supplemented group. This is not the case, so cysteine is probably not rate-limiting. In addition, erythrocyte GSH concentration may have been adequate in these children, so that GSH synthesis rate was limited by feedback inhibition of the pathway, rather than substrate limitation. Indeed, there appears to be no difference in GSH fractional synthetic rate between any of the four patient groups studied (i.e. control non-septic, sepsis without supplementation, sepsis plus glutamine and sepsis plus placebo). Despite this lack of difference in GSH synthesis, sepsis and glutamine supplementation had marked effects on immune function in these patients in the same timescale over which GSH synthesis was measured (Chowdhury et al. unpublished). As glutamine is a very important fuel for cells of the immune system, and GSH is also important as an anti-oxidant for cells of the immune system during infection, where it protects against cytotoxic

reactive oxygen species produced in the bacterial killing process, it is possible that glutamine may have increased GSH synthesis in leukocytes in this study, but unfortunately I did not measure GSH synthesis in this cell type. Another tissue where it would be of great interest to measure the effect of glutamine supplementation during sepsis would be muscle. During sepsis and other critically-ill states, amino acids are released from muscle in order to provide amino acids to the liver. As a consequence, muscle GSH becomes depleted (Brealey et al. 2002;Flaring et al. 2003) and levels may be relatively preserved by glutamine supplementation in adults (Flaring et al. 2003). Ethically, I was of course unable to obtain muscle biopsies from the infants and children in this study.

Deuterated glycine was used in this study as a probe of GSH synthesis. Glycine is also one of the precursor amino acids of GSH, but in this study deuterated glycine infusion was given at tracer amounts (15μmol/kg/h after a priming dose of 15μmol/kg). This can be compared with the glutamine supplementation rate of 114μmol/kg/h (in the glutamine supplemented group), and 14.5μmol/kg/h glycine plus 4.3μmol/kg/h cysteine and 25μmol/kg/h glutamate received as a component of Vaminolact in the placebo group; however the glutamine/Vaminolact infusion was in addition to whichever nutrition was being administered to the patients. One advantage of using glycine as a precursor in these studies is that glycine is incorporated at the final step of GSH synthesis, so the contribution of recycling of γ-glutamyl cysteine is included in the measurement, whereas this would not be included if labelled glutamate or cysteine were to be used as the precursor.

One possibility for the negative results observed is that an inadequate dose of glutamine was given (0.6k/kg/d dipeptide, equivalent to 0.4g/kg/d glutamine). This dose was chosen on the basis of previous experience of glutamine supplementation (Allen et al. 1993) and is comparable to that used in other studies in infants and children (Albers et al. 2005;Lacey et al. 1996;Ong et al. 2007;Scheltinga et al. 1991;Ziegler et al. 1992). Although a similar dose of glutamine is frequently used in adult studies (e.g. (Heyland et al. 2007)), some studies in adults have suggested that 0.4g/kg/d is in the middle of the a dose-response curve and that higher doses are well tolerated (Tjader et al. 2004). Although glutamine supplementation in parenteral nutrition is theoretically limited because the amounts of other amino acids given are decreased and amino acid deficiency must be avoided, in the current study glutamine was given as an standalone infusion so was independent from any parenteral amino acids given.

The enrichment of GSH in each group was maintained at an elevated level between the two glycine infusions, whereas that of glycine fell rapidly between the two infusions, as would be expected as the turnover of intracellular glycine is much faster than that of GSH. The fractional synthesis rates (FSR) for GSH which I measured in this study, approximately 1.5% per hour, are lower, but comparable to, the rates obtained by Lyons et al. and Reid et al. in children (Lyons et al. 2001;Reid et al. 2000) and Humbert et al. in dogs (Humbert et al. 2007), whereas the fractional synthesis rate (FSR) of GSH in the rat is much higher, approximately 20% per hour (Lauterburg & Mitchell 1981). The FSR which I obtained equates to 36% per day, so one would potentially expect the enrichment of GSH to fall by 18% during the 12 hours between the end of the first glycine infusion and the start

of the second. However, the enrichment of glycine does not fall to 0 APE immediately after stopping the infusion, so presumably the continued incorporation of glycine at a gradually lower enrichment is enough to prevent a fall in GSH enrichment over these 12 hours. The estimated curves of GSH and glycine enrichments are shown in Figure 4.16.
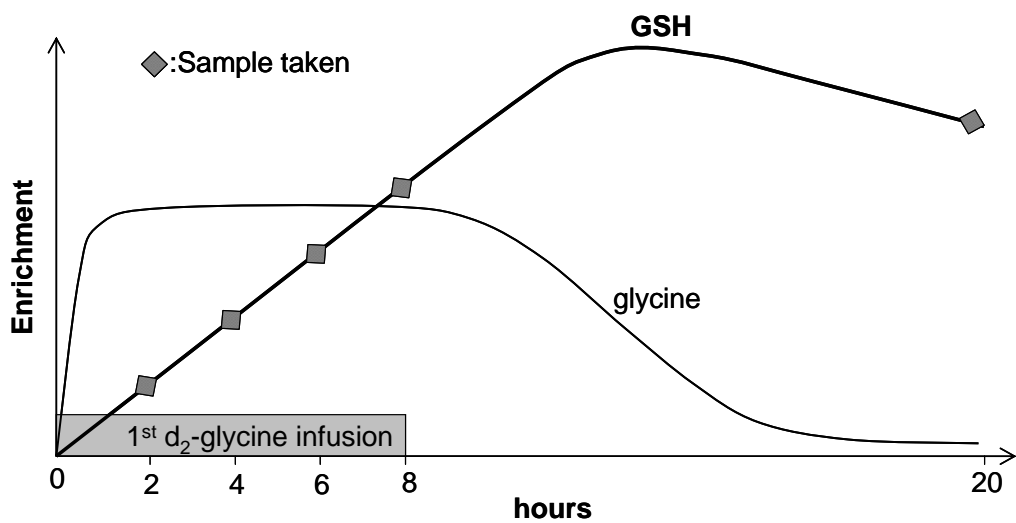
Figure 4.16 Estimated curves of enrichment glycine and GSH

The finding that the FSR over the first 8 hours was different between the placebo group in this study and the sepsis group in chapter 3 is surprising, as these groups should be comparable. Possible explanation for this discrepancy are: (i) the small numbers of patients in each study meant tat there was a clinical difference between the two groups, resulting in a difference in FSR; (ii) there was an effect of acute infusion of placebo; (iii) there was a systematic difference in the analyses (samples were analysed in different batches, and differences small in analysis between the two studies could have accounted for the apparent difference in FSR).

Other work on these patients (performed by Mr Moti Chowdhury) indicated that glutamine attenuated the immune response to sepsis by decreasing pro- and anti- inflammatory cytokines and decreasing the fall in HLA-DR expression. In addition, glutamine seemed to improve organ failure scores compared to placebo, although the number of patients was small.

4.6 Conclusion

Glutamine supplementation did not significantly enhance erythrocyte GSH synthesis in septic paediatric patients in comparison to placebo. This could be due to several factors:

(i) not enough patients were studied

(ii) other amino acids were rate-limiting

(iii) GSH synthesis is already maintained at adequate levels, even during sepsis,

(iv) there could be differences in absolute synthesis rates which it was not possible to determine in this study

(v) there may be differences in other tissues.

(vi) the dose of glutamine may have been inadequate

Despite this negative finding, my results show the utility of my method for measuring GSH synthesis *in vivo* in paediatric patients where sampling of large blood volumes is a problem.

Chapter 5

Glutathione synthesis in a rat model of
Intestinal Ischaemia-Reperfusion

Chapter 5:

5.1 Introduction

Intestinal ischaemia and reperfusion (I/R) injury represents a major clinical problem in infants, children and adults. Diseases associated with this condition include necrotizing enterocolitis, midgut volvulus acute mesenteric arterial occlusion and haemodynamic shock (Schoenberg & Beger 1993). Intestinal I/R can also occur as a consequence of surgical procedures such as cardiopulmonary bypass, aortic aneurysm repair and intestinal transplantation (Schoenberg & Beger 1993). Sepsis can be both a cause and a consequence of intestinal I/R injury (Fink 1993;Schoenberg & Beger 1993). The intestinal ischaemia and reperfusion rat model, consisting of superior mesenteric artery occlusion (ischaemia) followed by de-occlusion (reperfusion) is a widely-used and reproducible model of intestinal I/R (Iglesias, LaNoue, Rogers, Inman, & Turnage 1998;Poggetti et al. 1992;Stefanutti, Vejchapipat, Williams, Pierro, & Eaton 2004;Stefanutti, Pierro, Vinardi, Spitz, & Eaton 2005).

During ischaemia and subsequent reperfusion, there is a great increase in production of reactive oxygen and nitrogen species, such as hydrogen peroxide (H_2O_2) and the superoxide radical anion ($O_2^{\bullet-}$), which reacts with the nitric oxide radical ($NO^{\bullet}$) to produce the potentially cytotoxic peroxynitrite anion ($ONOO^-$) (Edited by: Halliwell & Gutteridge 1999;Granger & Kubes 1994). This production of reactive oxygen and nitrogen species is thought to be important in causing tissue

damage after I/R of several tissues, such as heart (Stefanutti et al. 2004) kidney (LaNoue, Jr. et al. 1996), and the intestine (Stefanutti et al. 2005). The actions of these deleterious reactive species can be prevented by antioxidants, either given exogenously, or with endogenous antioxidants such as GSH. GSH levels are depleted in liver (Turnage et al. 1991) and intestine (Bhaskar et al. 1995;Gibson et al. 1993) following intestinal I/R.

Our group has shown that hypothermia prevents liver bioenergetic failure and mortality during experimental intestinal I/R injury (Vejchapipat et al. 2001). Hypothermia is beneficial not only in reducing local damage (Hassoun et al. 2002;Kalia et al. 2002;Vejchapipat et al. 2002) but also in decreasing lung neutrophil infiltration (Vinardi et al. 2003), alterations in heart mitochondrial function (Stefanutti et al. 2004) and the effect on mortality has been confirmed (Kalia et al. 2001). Some studies suggested that hypothermia may protect the gut in intestinal I/R injury by induction of heme oxygenase-1 above the levels observed at normothermia (Attuwaybi et al. 2003) and prevents an increase in nuclear factor Kappa-B (NF-kappaB) and inducible nitric oxide synthase (iNOS) expression (Hassoun et al. 2002). Interestingly, in a study of the effects of moderate hypothermia on oxidative stress following intestinal ischaemia-reperfusion injury, it has been shown that GSH levels were maintained by moderate hypothermia, and were even higher than control normothermic animals in control hypothermic animals (Stefanutti et al. 2005) (Figure 5.1).
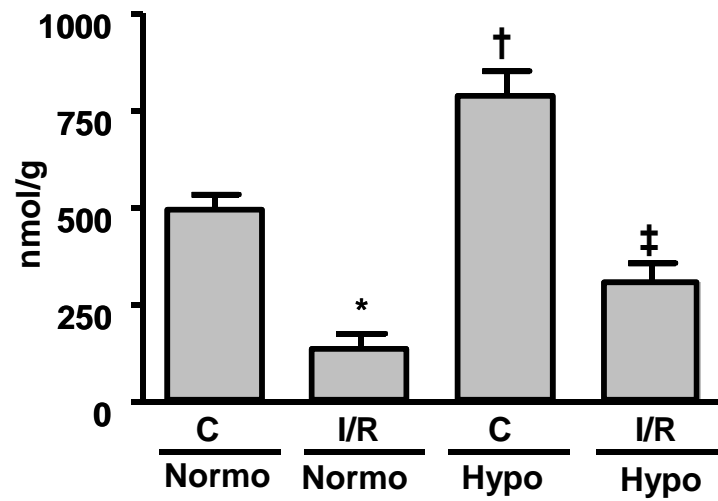
Figure 5.1 Concentration of total glutathione in rats undergoing sham (C) operation or intestinal ischaemia reperfusion injury (I/R) at normothermia (Normo) or hypothermia (Hypo), from (Stefanutti et al. 2005).

This could be due to decreased export/destruction of GSH in hypothermic animals, or even due to increased GSH synthesis due to hypothermia.

5.2 Aim

The aim of this study was to investigate the consequences of intestinal ischaemia and reperfusion (I/R) and the effects of whole-body moderate hypothermia on systemic oxidative stress in the rat, by measuring the GSH synthesis rate with deuterated glycine intravenous infusion. The hypothesis was that GSH synthesis in hypothermia I/R could increase more than normothermia I/R, and could be maintained the same as control (sham operation).

5.3 Methods

5.3.1 Experimental animals

This study was approved under the United Kingdom Home Office regulations for Animals (Scientific Procedures) Act 1986, after obtaining appropriate project and personal licenses. Adult male Sprague-Dawley rats (210 to 340g) were used. The following five experimental groups (n = 8 per group) were studied:

(1) baseline for harvesting samples without operative procedure;

(2) sham operation for 150 minutes at normothermia;

(3) intestinal ischaemia for 60 minutes, followed by reperfusion for 90 minutes at normothermia;

(4) sham operation for 150 minutes at hypothermia;

(5) intestinal ischaemia for 60 minutes, followed by reperfusion for 90 minutes at hypothermia.

5.3.2 Anaesthesia and body temperature

Anaesthesia was induced with ~3% isoflurane, following which spontaneously breathing animals were maintained under anaesthesia with oxygen/nitrous oxide (1:1) and 2.0% to 2.5% isoflurane inhalation, delivered via a nose cone, as described in previous studies (Stefanutti et al. 2004;Stefanutti et al. 2005;Vejchapipat et al. 2001;Vinardi et al. 2003). Baseline samples were taken immediately after induction of anaesthesia.

Normothermia (rectal temperature between 36 and 38°C) was maintained by use of a heating blanket and lamp. Moderate hypothermia (rectal temperature between 30 and 32°C) was induced at the beginning of the experiment by exposing the rats to an environmental temperature of 22 to 23°C, with warming by a heating blanket and lamp to prevent excessive cooling. Anaesthetised rats lose body temperature rapidly if not kept with a heating blanket, so active cooling was not required. All animals were allowed to stabilise for period of 30 minutes after anaesthesia and monitoring had been established, in order to achieve the desired rectal temperature. During this time, a cannula was placed in the femoral vein, and an intravenous infusion of d_2-glycine (0.592μmol/kg/h) initiated, as a tracer of GSH synthesis, until the experiment was terminated.

5.3.3 Operative procedure

In sham groups (group (2) and (4)), laparotomy was performed and the origin of the superior mesenteric artery (SMA) identified and dissected only, the abdominal wound was sutured and the animal monitored until the end of the experiment. Intestinal ischaemia (group (3) and (5)) was induced by occluding the SMA by a vessel clip (8 × 1.5mm jaws vessel clip, World Precision Instruments, UK), and the abdomen sutured. After 60 minutes the clip was removed and reperfusion was confirmed by visualization of pulsation in the vascular arcades of the mesentery, and the abdomen sutured. Reperfusion was continued for a further 90 minutes when the experiment was terminated.

5.3.4 Sample collection

Rats from all groups were killed by exsanguination, and liver and terminal ileum (5 to 10cm from the ileocecal valve) were removed. Blood was taken via cardiac puncture into an EDTA tube, and erythrocytes were immediately separated from whole blood by centrifugation at 3000rpm for 15 min at 4°C, following which samples were stored at -80°C. One lobe of liver was immediately stored at -80°C, and the rest of the liver used to prepare mitochondrial and cytosolic fractions (see below). Intestine sample was gently washed with saline and then stored at -80°C until analysis.

5.3.5 Hepatic mitochondria and cytosol

Liver was placed in a Teflon-on-glass Potter-Elvejheim hand homogenizer with ice cold Medium B (250mM sucrose, 2mM HEPES (4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid), 0.1mM EGTA (ethyleneglycol-bis-β-aminoethylether)-N,N,N',N'-tetraacetic acid), pH 7.4), and hand-homogenized by 10 strokes. The homogenate was transferred into centrifuge tubes (Round-bottom plastic tubes) and centrifuged at 1500g for 10 minutes at 4°C. The supernatant was gently transferred into fresh tubes. Medium B was added into the residue, which was hand-homogenized again and re-centrifuged. The supernatant was combined with the first supernatant, which were then centrifuged at 13500 rpm for 10 minutes at 4°C, to obtain a primary mitochondrial pellet, and a cytosolic supernatant, which was transferred into a capped tube and stored at -80°C until analysis. The primary mitochondrial pellet was re-homogenised with 2-3ml of Medium B using a plastic disposable pipette. The resuspended mitochondria were combined into microcentrifuge tubes, and samples centrifuged at 13500 rpm for 10 minutes at 4°C. The supernatant was discarded. The pellets were washed with Medium B again and centrifuged at 13500rpm for 5 minutes at 4°C. The supernatant was discarded with care and the pellets stored at -80°C until analysis.

5.3.6 Sample preparation and derivatization

Erythrocytes (50μl) were extracted and derivatised with *n*-Propyl chloroformate and methanolic hydrochloric acid as described earlier in this thesis (section 2.3.7). Whole liver (~60mg) and ileum (~100mg) were homogenised with 200μl TCEP buffer (7.5mM TCEP (tris-(2-carboxyethyl)-phosphine), 0.2M KH$_2$PO$_4$, 5mM EDTA, pH 7.5) in a hand-held glass-glass homogeniser. The homogenate was transferred to a microcentrifuge tube and the homogeniser tube and pestle washed with 200μl TCEP buffer, which was combined with the first

homogenate. The homogenate was mixed for 15 minutes at room temperature, and the samples then protein precipitated and derivatised as for erythrocyte samples. Cytosol (500µl) and mitochondria (200µl) were mixed with 400µl of TCEP buffer, protein precipitated with 400µl of 10% sulphosalicylic acid. The samples were then centrifuged and derivatised as for erythrocytes. After derivatisations, all samples were resuspended in 100µl of ethyl acetate and analysed by GC-TC-IRMS.

5.3.7 GC-TC-IRMS analysis

11.25µl of sample was analysed by GC-TC-IRMS using a Programmable Temperature Vaporization (PTV) inlet, as described in section 2.4.5. Each sample was injected twice, and ^{2}H/^{1}H isotope ratios of glycine (Rt. 6.8 min) and GSH (Rt. 20.5 min) were measured by isotope ratio mass spectrometry. H_3^+ factor calculation was performed before each batch of samples.

5.3.8 Data analysis

^{2}H/^{1}H-glycine and ^{2}H/^{1}H-GSH in all rat samples were analysed by GC-TC-IRMS. The each atom percent excess (APE) of both glycine and GSH were calculated from the average of base line group (r_B) to the end point of the experiment ($r_{t\Delta}$), as described in section 4.3.5. Glycine and GSH APE of each group was compared using one-way analysis of variance (ANOVA); data are shown as mean±SEM. Fractional synthesis rate could not be calculated, as this relies on samples from multiple time points, which was not possible using this model. In order to make an estimation of GSH synthesis, data were expressed as a ratio of GSH APE: glycine APE according to the formula:

$$GSH/Gly = \frac{GSH_{APE} \times 33/2}{Gly_{APE} \times 13/2}$$

The factors of 33 and 13 are because 2/13 hydrogen atoms in the glycine derivative are labelled, whereas only 2/33 hydrogen atoms in the GSH derivative can become labelled by incorporation of d_2-glycine. GSH/Glycine of each group was compared using one-way analysis of variance (ANOVA); data are shown as mean±SEM.

5.4 Results

5.4.1 Erythrocytes

Glycine APE was similar in all groups (Figure 5.2).

Figure 5.2 Erythrocyte glycine APE

Sham N: sham normothermia; sham H: sham hypothermia; I/R N:
ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion
hypothermia

GSH APE was significantly different between normothermia I/R group and hypothermia I/R group (p< 0.05; Figure 5.3).

Figure 5.3 Erythrocyte GSH APE

Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. p<0.05: Normothermia I/R group significantly higher than hypothermia I/R group

The GSH to glycine APE ratio was also significantly different between normothermic I/R and hypothermic I/R ($p < 0.05$), and between the hypothermic sham group and normothermic I/R group ($p < 0.05$; Figure 5.4).

Figure 5.4 Erythrocyte GSH$_{APE}$/Glycine$_{APE}$

Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. $p<0.05$ normothermia I/R group significantly higher than hypothermia I/R group and hypothermia sham group

5.4.2 Whole liver

Glycine APE was significantly higher in hypothermia sham group the than both the normothermia sham group ($p < 0.01$) and the normothermic intestinal I/R group ($p < 0.05$, Figure 5.5).

Figure 5.5 Glycine APE of whole liver
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Hypothermia sham group significantly higher than normothermia sham ($p < 0.01$) and normothermia I/R group ($p < 0.05$)

The APE of GSH was not significantly different between the groups, although there was a non-significant trend to higher enrichment values in both hypothermic groups (Figure 5.6).

Figure 5.6 GSH APE of whole liver
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. No significant differences between the groups

The ratio of GSH APE to glycine APE was significantly different between hypothermia sham group and normothermia I/R group ($p<0.05$, Figure 5.7).

Figure 5.7 GSH$_{APE}$/glycine$_{APE}$ of whole liver
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. $p<0.05$ normothermia I/R group significantly higher than hypothermia sham group

5.4.3 Hepatic cytosol

Glycine APE was significantly higher in the hypothermia sham group than in the normothermic sham group ($p < 0.05$; Figure 5.8). This significant difference, and values of glycine APE in the cytosolic fraction was similar to that of whole liver (section 5.4.2, Figure 5.5).

Figure 5.8 Glycine APE of hepatic cytosol
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Hypothermia sham group significantly higher than normothermia sham ($p < 0.05$)

The APE of GSH was significantly different between normothermia sham and hypothermia sham groups (p<0.001), hypothermia sham and normothermia I/R groups (p< 0.01), and normothermia sham and hypothermia I/R groups (p< 0.05; Figure 5.9).

Figure 5.9 GSH APE of hepatic cytosol

Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Hypothermia sham significantly higher than normothermia sham (p < 0.001) and normothermia I/R group (p<0.01). Hypothermia I/R group was significantly higher than normothermia sham group (p< 0.01)

The ratio of GSH_{APE} to $glycine_{APE}$ was similar in all groups (Figure 5.10).
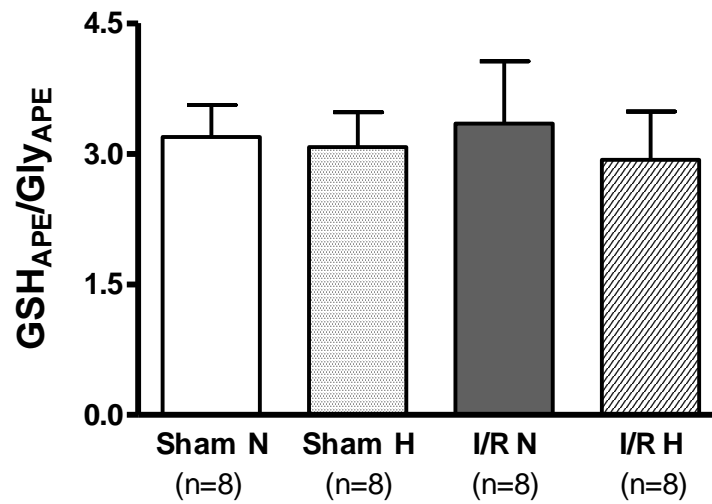
Figure 5.10 GSH_{APE} / glycine$_{APE}$ of hepatic cytosol

Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. No significant differences between the groups

5.4.4 Hepatic mitochondria

Glycine APE of liver mitochondria was significantly different between normothermia sham and hypothermia sham ($p<0.05$; Figure 5.11).
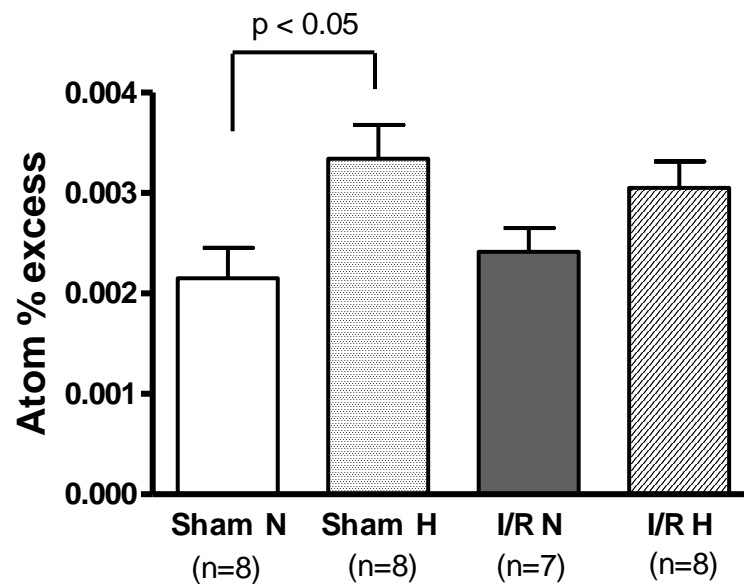
Figure 5.11 Glycine APE of hepatic mitochondria
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Hypothermia sham group significantly higher than normothermia sham ($p<0.05$)

GSH was only detectable in a few of the mitochondrial samples. There was no significant difference between any of the groups in those samples where GSH enrichment was measurable (Figure 5.12).

The ratio of GSH_{APE} to $glycine_{APE}$ was also calculated from these samples and there was no significant difference between the groups (Figure 5.12). However, as discussed below, mitochondrial GSH is probably entirely synthesised within the cytosol and then transported to the mitochondria, so the true precursor for the mitochondrial GSH pool is cytosolic glycine. The GSH_{APE} / $glycine_{APE}$ data were therefore recalculated using cytosolic $glycine_{APE}$; the results are shown in figure 5.13.
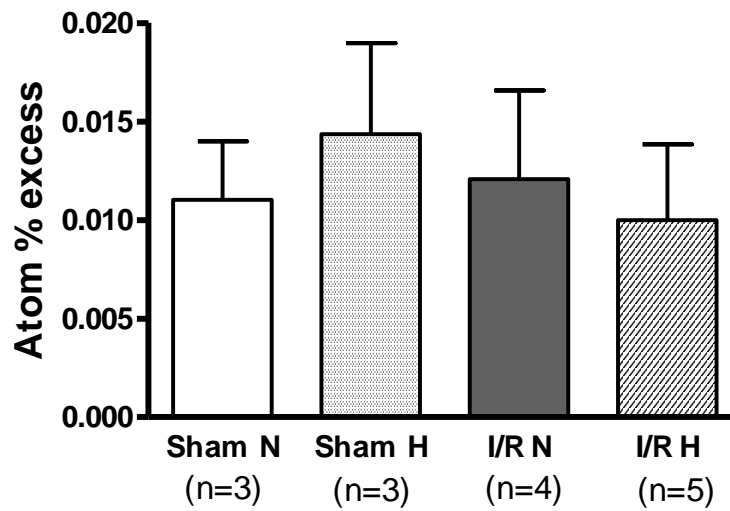
Figure 5.12 GSH APE of hepatic mitochondria
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia
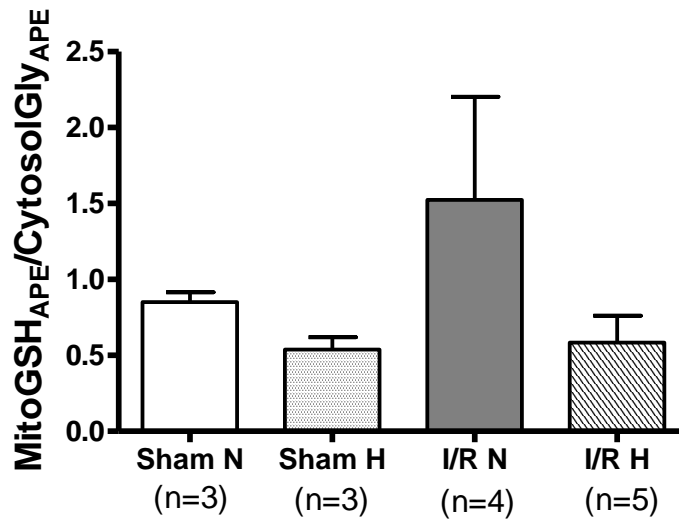
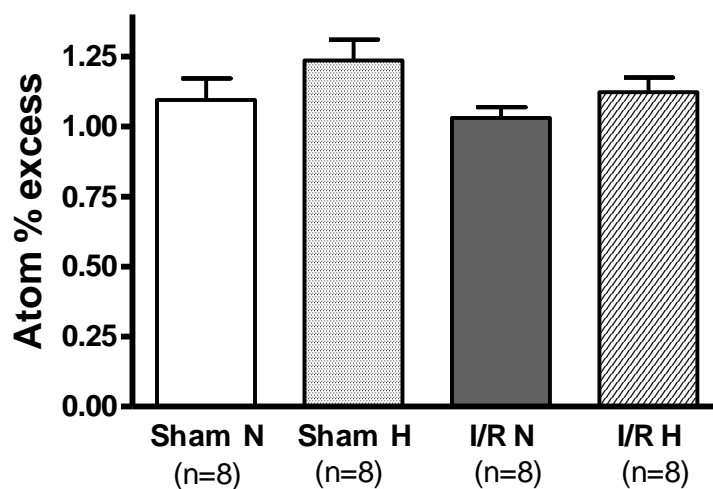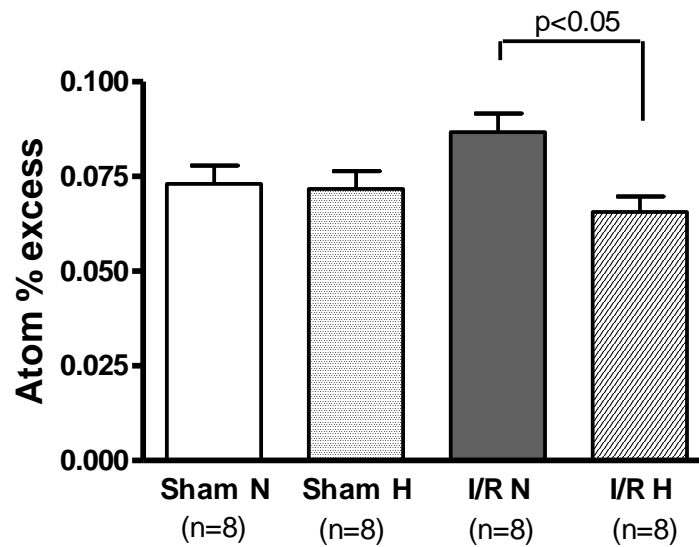**Figure 5.13 GSH$_{APE}$ / glycine$_{APE}$ of hepatic mitochondria, using cytosolic glycine$_{APE}$**

Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. No significant differences between the groups

5.4.5 Small intestine (ileum)

Glycine enrichment of the normothermia intestinal I/R group was significantly higher than that of all the other groups, and the glycine APE of hypothermia intestinal I/R group was significantly higher than that of both the hypothermia sham group and the normothermia sham group (Figure 5.14).
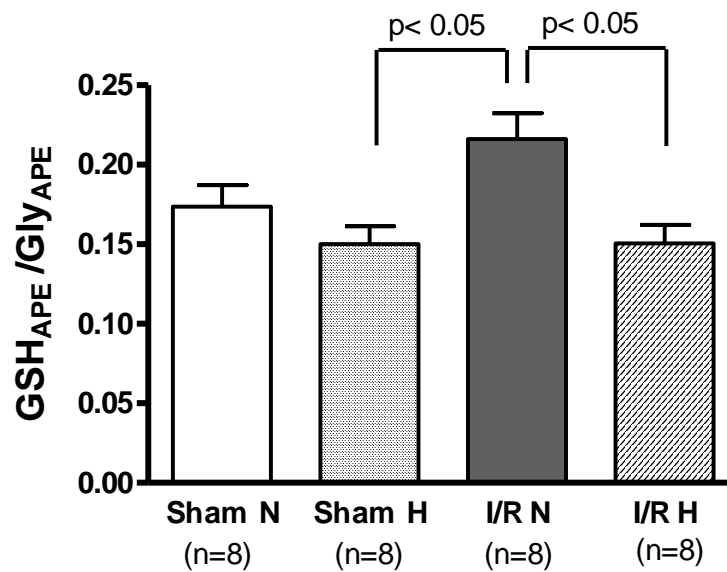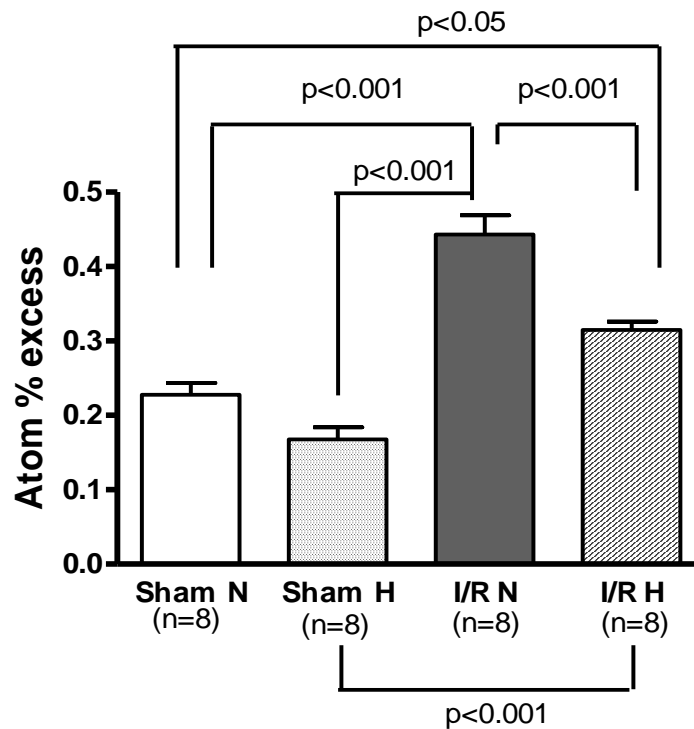

Figure 5.14 Glycine APE of ileum
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Normothermia I/R group was significantly (p<0.001) higher than all the other groups. Hypothermia I/R group was significantly higher than hypothermia sham group p<0.001 and normothermia sham group (p < 0.05)

For GSH APE in ileum, there was an overall significant difference between the groups (one-way ANOVA $p < 0.05$). However, the post-tests did not show any significant differences between the groups. GSH APE showed a non-significant trend to be lower in the sham hypothermic group compared to both the sham normothermic and the I/R hypothermic groups (Figure 5.15).
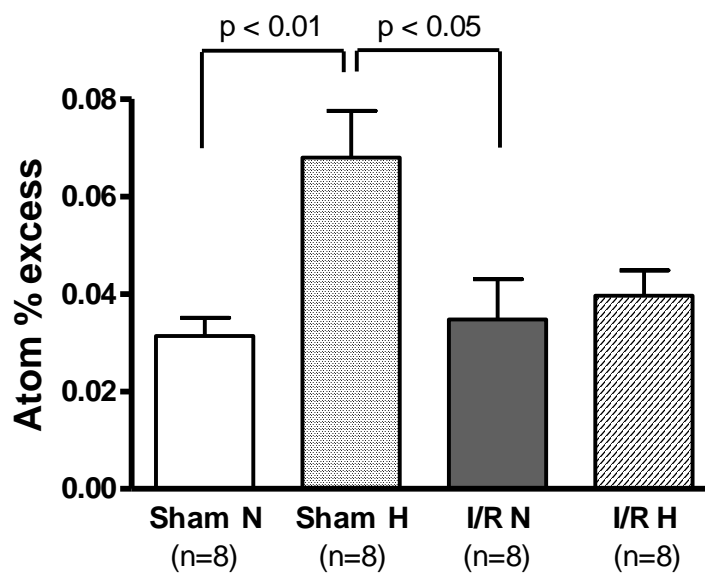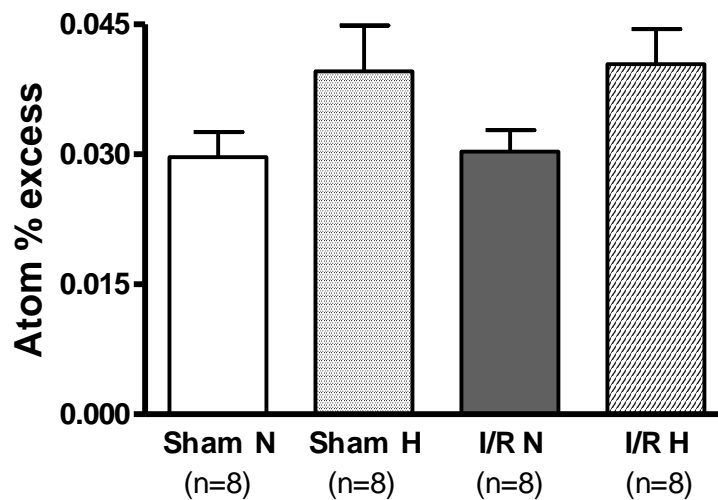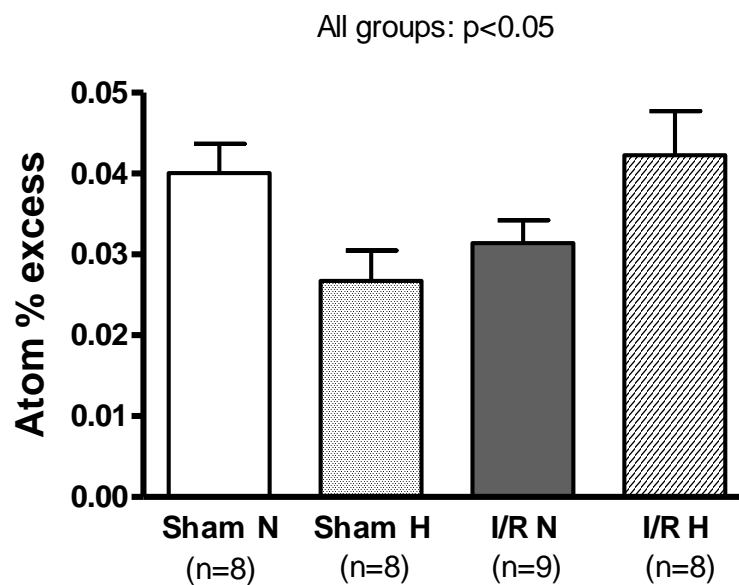

Figure 5.15 GSH APE of ileum
Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Although there was an overall significance on one-way ANOVA, there were no individually significant differences between the groups

The ratio of GSH to glycine was significantly different between normothermia sham and normothermia I/R ($p<0.001$), hypothermia sham and normothermia I/R ($p<0.01$), and normothermia I/R and hypothermia I/R ($p<0.05$; Figure 5.16).
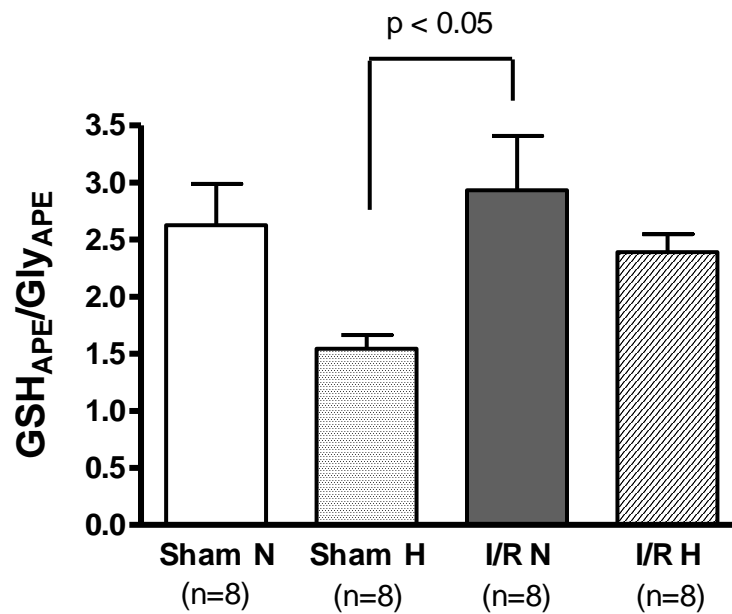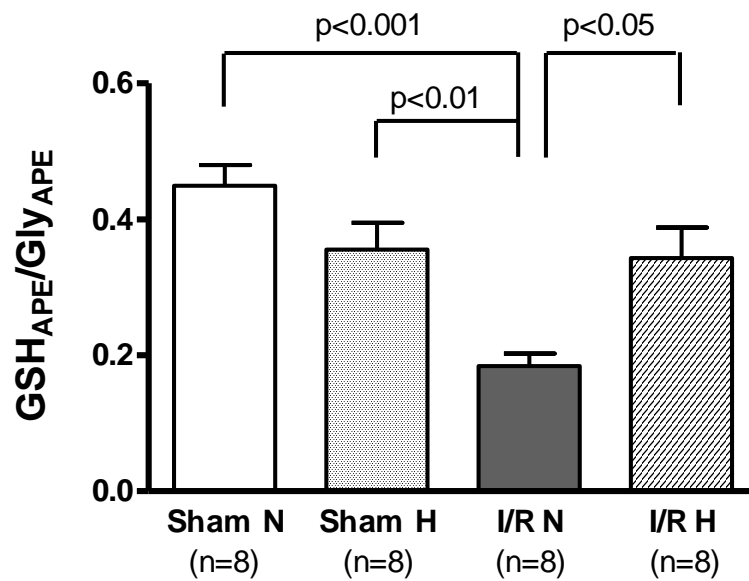

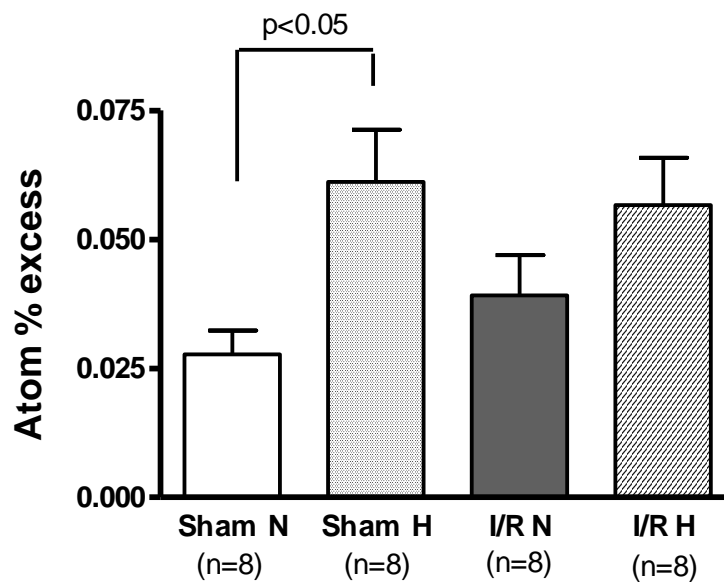**Figure 5.16 GSH$_{APE}$ / glycine$_{APE}$ of ileum**
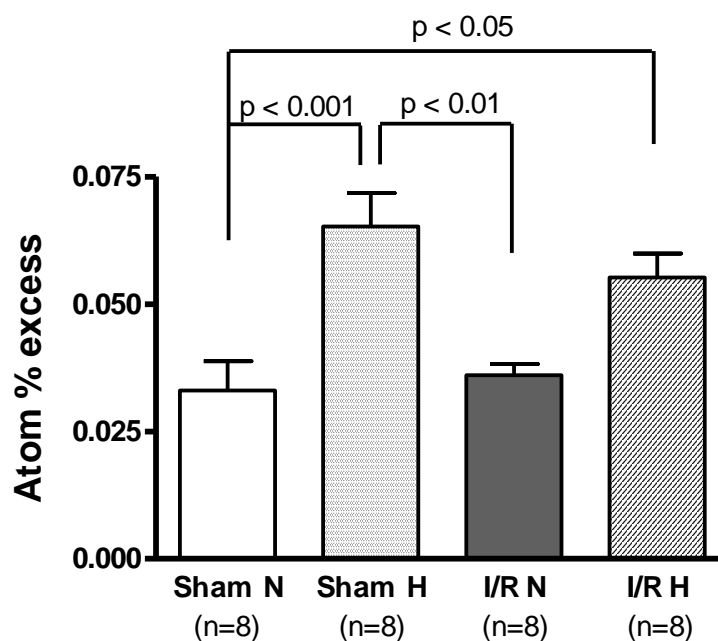Sham N: sham normothermia; sham H: sham hypothermia; I/R N: ischaemia-reperfusion normothermia; I/R H: ischaemia-reperfusion hypothermia. Ischaemia-reperfusion normothermia significantly lower than normothermia sham ($p<0.001$), hypothermia sham ($p<0.01$) and ischaemia-reperfusion at hypothermia ($p<0.05$)

5.5 Discussion

When the local tissue injury associated with ischaemia-reperfusion (I/R), distant organs can also be affected, particularly if the intensity of the inflammatory reaction in post-ischaemic tissue, for example intestine, is great (Carden & Granger 2000). The remote effects of I/R are mostly frequently observed in the lung and cardiovascular system, and can result in the development of systemic inflammatory response syndrome (SIRS) and MODS, both of which account for 30 to 40% of the mortality in tertiary referral intensive care units (Neary & Redmond 1999). Reperfusion of ischaemic tissues can induce reactive oxygen (ROS) and nitrogen species (RNS) as consequences of inflammatory reaction. GSH is the most important factor of antioxidant defences during I/R injury. In this study, GSH synthesis during I/R was analysed using a rat intestinal I/R model, in which failure of liver, lungs and heart occurs secondary to I/R of the intestine. In this model, moderate hypothermia has been shown to have a beneficial effect, and this appears to be associated with a better preservation of intestinal GSH levels (Stefanutti et al. 2005).

In erythrocytes, the ratio of GSH_{APE} to $glycine_{APE}$ was higher in the normothermia I/R group than in both hypothermia sham and I/R groups, suggesting that GSH synthesis is faster in this condition. Although reactive oxygen and nitrogen species were not measured in the current study, previous studies have shown that lipid peroxidation (measured as plasma malondialdehyde) and reactive nitrogen species (measured as nitric oxide products) are significantly higher in normothermic ischaemia reperfusion than in sham animals, and are also decreased

by hypothermia (Stefanutti et al. 2005). Hence, my data suggest that GSH synthesis could be stimulated by reactive oxygen and nitrogen species, possibly originating from endothelial dysfunction (Banda et al. 1997). There are three possibilities for an increase in GSH synthesis: (i) an increase in supply of precursors (glycine, glutamate, cysteine or γ-glu-cys); (ii) an increase in erythrocyte activity of γ-glutamyl cysteine ligase, the rate-limiting step of GSH synthesis, and (iii) an increase in erythrocyte activity of GSH synthetase. Although activity of γ-glutamyl cysteine ligase has been reported to be increased by various reactive oxygen and nitrogen species (Wild & Mulcahy 2000), and that of GSH synthetase has been reported to be increased in vitamin E and folate deficiency (Tchantchou et al. 2004) and by *tert*-butylhydroquinone (Huang et al. 2000), these changes are transcriptional changes mediated by increased expression of enzyme protein and so cannot account for the changes observed in erythrocytes. The pathway of GSH synthesis is mediated by feedback control: γ-glutamyl cysteine ligase is strongly inhibited by GSH (Griffith & Mulcahy 1999;Wild & Mulcahy 2000), so it is much more likely that in erythrocytes during intestinal I/R injury, there is utilisation of GSH (e.g. detoxification of hydroperoxides) or conversion to oxidised GSH (GSSG). This would, by decreasing intracellular GSH amount, relieve the product inhibition of γ-glutamyl cysteine ligase and therefore cause an increase in the rate of GSH synthesis.

In erythrocyte, the ratio of GSH_{APE} to $glycine_{APE}$ in hypothermia I/R group was decreased to similar levels to the sham hypothermia group and the sham normothermia group. This finding supports the hypothesis that the increase in normothermic I/R is due to reactive oxygen and nitrogen species causing a

decrease in GSH and thus relieving the product inhibition on γ-glutamyl cysteine ligase, as these have been shown to be decreased by hypothermia after intestinal I/R. To confirm this, it would be necessary to make parallel measurements of GSH and GSSG concentration, measure reactive oxygen and nitrogen species and determine GSH synthesis. It is also important to note that in my analyses, glycine incorporation into the *total* GSH pool (i.e. GSH plus GSSG) was measured because of the TCEP reduction step before derivatization. Further experiments could be designed to try to measure the rate of GSH oxidation by measuring glycine incorporation into the GSH and GSSG pools differentially, although it is difficult to measure GSSG accurately in erythrocytes because of the high artefactual oxidation of GSH during sample preparation procedures (Rossi et al. 2006).

Glycine$_{APE}$ of erythrocytes was similar between all groups. Our Home Office license did not allow repeated blood sampling, so we were not able to establish glycine steady state in these animals. The similar results obtained for glycine$_{APE}$ between the experimental groups, suggests, however, that steady state was obtained.

Glycine$_{APE}$ in whole liver was similar in sham normothermia, I/R normothermia and I/R hypothermia groups, but was significantly higher in the sham hypothermia group. This suggests that either the endogenous production rate of glycine was significantly *lower* in the hypothermic group, or an alteration in the routes of glycine utilisation. Glycine metabolism is based on two enzymes; serine hydroxymethyltransferase reversible interconverts serine and glycine, whereas glycine is catabolised by the mitochondrial glycine cleavage enzyme system (GCS). In mammalian liver, this reaction is reversible, so that glycine can be synthesised

from CO_2 + ammonia + N^5,N^{10}-methylene-tetrahydrofolate. However, in addition, glycine is used for several synthetic purposes: GSH, protein, nucleotide and bile acid synthesis. It can also provide a gluconeogenic precursor via serine and pyruvate. In order to investigate the biochemical basis for the increased glycine APE in hypothermia, it would be interesting to measure each of these processes. Interestingly, glycine has recently been shown to have hepatoprotective effects during inflammation and hepatic I/R injury, although the mechanism of this is not known (Habib et al. 2006;Schemmer et al. 1999).

The ratio of GSH_{APE} to $glycine_{APE}$ in whole liver was significantly higher in the normothermia I/R group compared to hypothermia sham group, but that there was no differences between the other groups. This isolated difference is difficult to interpret, as there was no difference with the corresponding normothermic sham group. It is possible that there were differences in the production of reactive oxygen and nitrogen species between the groups, but this difference was only of sufficient magnitude to cause an difference in GSH synthesis rate between normothermic I/R (assumed to be the highest oxidative stress of all 4 groups) and hypothermic sham (assumed to be the lowest oxidative stress of all 4 groups). However, this would need further experiments to verify. In addition, for all the hepatic samples (whole liver, cytosol and mitochondria), $GSH_{APE}/glycine_{APE}$ values in excess of 1 were obtained, which does not make sense for a product precursor relationship. The reason for this is unknown, but means that the hepatic results must be interpreted with caution.

In order to increase understanding of hepatic GSH synthesis inI/R, I studied hepatic mitochondria and cytosol separately. Unfortunately, mitochondria

samples, however, had severe protein interferences, and GSH could not be detected by GC-IRMS in many samples, although the concentration of GSH in hepatic mitochondria was approximately 3.5 nmol/mg protein by HPLC analysis.

Glycine$_{APE}$ in hepatic cytosol, unsurprisingly, showed a similar pattern to that of whole liver, in that hypothermic sham animals had higher glycine enrichment than normothermic sham animals. The magnitude of the glycine$_{APE}$ measurements was also similar to that of whole liver, as most of the hepatic glycine pool is probably cytosolic. Despite the finding that there was a significant difference in GSH$_{APE}$/glycine$_{APE}$ on whole liver, there were no significant differences between any of the groups in this ratio in liver cytosol. This observation is a little unexpected, as cytosol is thought to contain ~90% of hepatic GSH, and mitochondria only about 10% (Fernandez-Checa et al. 1998;Jocelyn & Kamminga 1974;Meredith & Reed 1982). However, this lack of difference between the groups may be related to distribution of GSH between different compartments: if the GSH$_{APE}$ values are compared between cytosol and whole liver, they are very similar numerically between the normothermic groups (~ 0.03%). However, there is a large difference between the numeric values for GSH$_{APE}$ for the hypothermic groups between cytosol and whole liver (~0.04% for whole liver hypothermic sham, ~0.06 % for cytosol hypothermic sham). The reason for this discrepancy is unclear, but could possibly reflect a higher concentration of slowly turning over GSH in the mitochondria of the hypothermic animals, which would have the effect of lowering GSH$_{APE}$ in whole liver. To understand the basis of this difference would require further experiments, including precise measurements of GSH concentration in each compartment.

The GSH$_{APE}$/glycine$_{APE}$ ratio of hepatic cytosol was similar between all the groups. This is despite there being markedly significant differences in both glycine$_{APE}$ and GSH$_{APE}$ between the groups; however as all differences were of a similar magnitude between groups (compare Figures 5.8 and 5.9), there is no difference in the GSH$_{APE}$/glycine$_{APE}$ ratio. This is also, unexpectedly, in contrast to the results of whole liver, in which there was a significantly lower ratio in the hypothermic sham group. The reason for this discrepancy is unknown but could be connected to different subcellular compartments and/or cell types: whole liver contains Kupffer cells, erythrocytes, stellate cells and other cell types in addition to hepatocytes. It is not known whether all these cell types are equally represented in the "cytosolic fraction"; certainly erythrocytes are not present as they are removed in the low speed centrifugation step.

Hepatic mitochondrial glycine$_{APE}$ was significantly higher in sham hypothermia than the other groups; this is a similar finding to both whole liver and to cytosol, although the APE obtained was approximately 10-fold lower than cytosol or whole liver, suggesting either that glycine does not equilibrate quickly between cytosol and mitochondria or that glycine is being produced endogenously with the mitochondria, via reversal of the glycine cleavage system. Unfortunately, mitochondrial GSH was not detectable is several of the samples, so data on GSH$_{APE}$ and GSH$_{APE}$/glycine$_{APE}$ is based on just a few animals. I calculated GSH$_{APE}$/glycine$_{APE}$ for mitochondria using both mitochondrial glycine$_{APE}$ and cytosolic glycine$_{APE}$. Using mitochondrial glycine$_{APE}$ as the precursor, GSH$_{APE}$/glycine$_{APE}$ values greatly in excess of 1 were obtained, because of the very low glycine$_{APE}$ values of mitochondria. This does not make biological sense, as the

product cannot have a higher enrichment than the precursor. In fact, as mitochondrial GSH is almost certainly synthesed entirely in the cytoplasm and then imported into the mitochondria (Fernandez-Checa et al. 1998;Griffith & Meister 1985). I therefore also calculated $GSH_{APE}/glycine_{APE}$ using the cytosolic $glycine_{APE}$ value, as this is probably the true precursor pool. The results obtained were considerably lower and consistent with those obtained for whole liver and for hepatic cytosol. There were, however, no significant differences between the groups. This may be due to the small number of mitochondrial samples in which it was possible to successfully measure GSH.

Glycine$_{APE}$ in ileum was significantly higher in the normothermic I/R group than the other groups. This may reflect a decrease in endogenous glycine production in the liver during normothermic I/R. Interestingly, glycine has been shown to have cytoprotective effects on the intestine during intestinal I/R injury (Jacob et al. 2003;Kallakuri et al. 2003;Lee et al. 2001;Lee et al. 2002;Mangino, Kotadia, & Mangino 1996). Thus it is plausible that the increase in glycine$_{APE}$ in the I/R normothermia group could be due to a decreased glycine production rate during I/R. However, in their studies on the protective effect of glycine on intestinal I/R injury at normothermia, Lee et al. did not detect any differences in intestinal glycine concentration (Lee, McCauley, Kong, & Hall 2002).

The ratio of GSH$_{APE}$ to glycine$_{APE}$ in the ileum resulted in a very different profile from that in the liver and in erythrocytes. $GSH_{APE}/glycine_{APE}$ in normothermic I/R was significantly lower compared to all the other groups, suggesting that ileum GSH synthesis is impaired by normothermic I/R. This could contribute to the marked decrease in intestinal GSH concentration which has been

previously observed in intestinal I/R injury (Bhaskar et al. 1995;Gibson et al. 1993;Stefanutti et al. 2005). Usually, loss of GSH under conditions of oxidative stress is thought to be either (i) oxidation of GSH to oxidized GSH, followed by its loss from cells (Rahman et al. 1995;Srivastava & Beutler 1969) or (ii) loss of GSH by reaction with hypochlorous acid (Pullar, Vissers, & Winterbourn 2001) or degradation to cystine (Bhaskar, Mathan, & Balasubramanian 1995). My results are novel in that they suggest a possible further mechanism by which intestinal GSH concentration is decreased: i.e. slower GSH synthesis. The reason for impaired GSH synthesis in the ileum during I/R injury is unknown. This could be due to (i) a decrease in supply of precursors (glycine, glutamate, cysteine or γ-glu-cys); (ii) inhibition of the activity of glutamyl cysteine ligase or GSH synthetase by reactive oxygen or nitrogen species. However, as I have shown that GSH synthesis is increased in erythrocytes, it appears to be unlikely that the enzymes of GSH synthesis are inhibited by reactive oxygen and nitrogen species, although it is possible that the degree of oxidative stress is much greater in ileum than in erythrocytes during I/R and the two enzymes could be inhibited. Alternatively, glycine, glutamate, cysteine or γ-glu-cys concentration could be limiting for GSH synthesis in the ileum during I/R. To clarify these issues would require careful measurement of the concentration of GSH, glycine, cysteine, and γ-glu-cys, together with measurement of the activity of γ-glutamyl cysteine ligase and GSH synthetase.

Hypothermia, on the other hand, appeared to relatively preserve GSH synthesis, as $GSH_{APE}/glycine_{APE}$ was significantly higher in hypothermic I/R ileum than normothermic I/R ileum, and was not significantly different from either

normothermic or hypothermic sham animals. The reason for this preservation of GSH synthesis is unknown. As the synthesis of GSH from glycine, cysteine and glutamate is enzymatic, one would expect that the activity of the enzymes γ-glutamyl cysteine ligase and GSH synthetase would be decreased by hypothermia. I was not able to find any literature on the temperature dependence of either of these enzymes, but it appears unlikely that the activity of either of these enzymes is increased by lowering the temperature from 37°C to 30-32°C, although there are some mammalian enzymes which are stimulated at lower temperatures, e.g. during hibernation (Andrews 2007). Although the body temperature of hibernating animals is much lower than the hypothermia induced in my experiments, it is interesting to note that the intestinal total GSH pool is larger in hibernating animals than active animals (Carey, Rhoads, & Aw 2003) and that hibernating animals are markedly resistant to intestinal I/R injury (Kurtz et al. 2006).

Again, clarification of the mechanism by which hypothermia preserves GSH synthesis in the intestine during I/R should involve careful measurement of the concentration of GSH, glycine, cysteine, and γ-glu-cys, together with measurement of the activity of γ-glutamyl cysteine ligase and GSH synthetase at different temperatures.

5.6 Conclusion

In this study, I have shown that:

(i) erythrocyte GSH synthesis is stimulated by normothermic intestinal I/R, probably due to relieving product inhibition of γ-glutamyl cysteine ligase. Hypothermic I/R animals had a similar rate of GSH synthesis to sham animals.

(ii) in the liver, the results of GSH synthesis are difficult to interpret, because the ratio $GSH_{APE}/glycine_{APE}$ is greater than 1, suggesting that there may be compartmentation of glycine/GSH metabolism and that whole liver glycine is not the true precursor for whole liver GSH.

(iii) intestinal GSH synthesis is markedly decreased by normothermic intestinal I/R, and this may contribute to the low GSH concentrations observed in the intestine following I/R injury. Interestingly, hypothermia during I/R appeared to prevent the impairment in GSH synthesis, which is in keeping with previous observations that hypothermia causes preservation of ileal GSH levels during I/R. However, the mechanisms of these alterations are not known and require further investigation along the directions indicated above.

Chapter 6

General Discussion

and

Further work

During sepsis and critical illness, a systemic inflammatory response can significantly produce reactive oxygen and nitrogen species, which can damage host tissues. Usually, several different antioxidant defences protect host cells from these reactive species. One of the important antioxidant defences is GSH, a tripeptide synthesised from the precursor amino acids cysteine, glutamate and glycine. The aim of this study was to measure GSH synthesis *in vivo*, using deuterated glycine as a tracer, and to use this method to measure GSH synthesis *in vivo* in both animal studies and in humans

In order to do this, it was necessary to develop a method to measure GSH synthesis using stable isotope precursors. Previous methods for measuring GSH synthesis have used radioactive precursors (not suitable for use in human patients), or have separated GSH by liquid chromatography, hydrolysed to its precursor amino acids, and analysed isotopic enrichment by GC-MS. This would therefore require large volumes of blood samples, and at least two separate aliquots of blood, one for measurement of precursor enrichment, the other for purification of GSH and subsequent enrichment measurement. I chose to try to develop a method with the characteristics:

(i) isotopic enrichment of the precursor amino acid and GSH should be possible from a single aliquot of blood sample, preferably in a single chromatographic separation

(ii) enrichment should be measurable from both deuterated and ^{13}C-labelled amino acid precursors, to allow wide applicability

(iii) low levels of enrichment should be measurable, so that low rates of isotopic tracer infusion are able to be used.

In order to develop a method with these characteristics, I chose to develop a method which was based on GC-IRMS. GC-IRMS is capable of obtaining highly accurate and specific isotopic ratios, provided that adequate chromatographic separation is obtained before conversion of target analyte to $^{13}CO_2/^{12}CO_2$ or $^{2}H_2/^{1}H_2$. However, care must be taken during derivatisation not to introduce materials which are incompatible with IRMS. Hence, silylated or fluorinated derivatives, commonly used in GC-MS, were not used as they may chemically react with parts of the GC-IRMS. Glycine and GSH were effectively derivatised by *n*-propyl chloroformate and methanolic hydrochloric acid. This yielded esters which were compatible with both GC-IRMS and GC-MS. The newly developed method can be used to analyse both glycine and GSH as a tracer and a tracee in a single chromatogram, which means more exact estimate isotopic traceability. However, the method which I developed had the disadvantage that as I could not identify a suitable internal standard for GSH, I was unable to quantify GSH in the same GC-IRMS analysis as enrichment was measured. As I was limited by the blood volume available, in the further studies I undertook using this method, only fractional synthesis rates rather than absolute synthesis rates could be measured. In order to establish general suitability of the method, I constructed calibration curves using ^{13}C-GSH/^{12}C-GSH. A very good linear relationship between theoretical/calculated enrichment was obtained, showing that the method was suitable for use with ^{13}C-labelled tracers. However, ^{2}H-GSH is not commercially available, so in order to construct calibration curves for ^{2}H-GSH/^{1}H-GSH, I first had so generate ^{2}H-GSH by incubating erythrocytes with ^{2}H-glycine. This was successful, so I was able to construct calibration curves for ^{2}H-GSH/^{1}H-GSH.

These curves generally showed a good linear relationship, except at deuterium enrichments close to natural abundance. This showed that my method was also suitable for measurement of GSH synthesis from deuterated amino acids precursors.

As GC-IRMS is inherently less sensitive in deuterium mode than in carbon mode, in order to obtain enough signal to reliably measure GSH in erythrocyte samples from human infants, it was not sufficient to inject 2μl in splitless mode on a split/splitless injector. I therefore developed a technique of large volume injection, using a programmable temperature vaporization (PTV) injector, which allowed injection of up to 12μl. Using this method, I was able to measure both glycine and GSH enrichment in a single 50μl blood sample, thus my method was suitable for the measurement of GSH synthesis in infants in children, where repeated blood samples must, by necessity, be very small.

Sepsis is the most common cause of MODS in the paediatric population. Despite recent advances in the care of critically ill children, sepsis and MODS remain a major source of morbidity and mortality in paediatric patients (Edited by: Deitch, Vincent, Windsor, & Editors 2002). Until now there has been no definitive treatment of MODS patients, therefore, control of infections, conventional treatments and TPN support is necessary. Antioxidant defences play a key role in critical illness as they can protect tissues and cells from reactive oxygen and nitrogen species which are induced from a systemic inflammatory response. GSH is a very important intracellular antioxidant during sepsis, however, details of synthesis and metabolism of GSH in particularly children and infants are still not clear. I hypothesised that GSH synthesis could be impaired by sepsis in infants in

children, owing to decreased supply of precursor amino acids (e.g. cysteine and glutamine/glutamate). However, the fractional synthesis rate of GSH was similar in critically-ill controls and septic patients. This could have been due to the wide variation in calculated FSR, or could also have been due to the fact that the controls which were studied were critically ill, and may therefore have also had an impairment in GSH synthesis. In order to study this further, it would be useful to study a further group of infants and children, with a lesser degree of illness. However, this is difficult to undertake, as it is not ethical to expose relatively healthy children to two venepunctures (one for isotope administration, one for sampling) and an 8-hour infusion. In addition, it would have potentially also been beneficial to measure GSH synthesis in more critically ill and septic patients, as this might have showed a significant difference. It is also possible that if the GSH concentration had been measured, there may have been a difference in GSH pool size and/or absolute synthesis rate between the two groups.

Glutamine, as a precursor for GSH, has been suggested to become conditionally essential during sepsis, so, using my newly-developed method for GSH synthesis, I undertook a study to determine whether glutamine supplementation would increase GSH synthesis in septic infants and children. Free glutamine is not usually added to parenteral nutrition, for reasons of stability, as it forms either free glutamate, or pyroglutamic acid. With the advent of stable glutamine dipeptides, it is possible to add glutamine into parenteral nutrition as either alanyl-glutamine or as glycyl-glutamine. However, in this study, a stand-alone infusion of either alanyl-glutamine or Vaminolact (isonitrogenous placebo) was used, allowing the study to be initiated quickly in a wide range of septic

patients, rather than being restricted only to those receiving TPN. GSH synthesis was measured on two occasions during the intervention with glutamine or placebo. Thus, I was able to test whether glutamine was able to increase erythrocyte GSH synthesis under two conditions: (i) initiation of glutamine/placebo infusion and (ii) after 20 hours of glutamine/placebo infusion. The results showed that glutamine supplementation did not significantly enhance erythrocyte GSH synthesis in septic paediatric patients in comparison to placebo. In addition, it would appear that cysteine may not be rate-limiting for GSH synthesis under the conditions studied, as the placebo arm would have received more cysteine than the glutamine arm (cysteine is a component of Vaminolact). Although these results showed that there was no significant effect of glutamine on GSH synthesis during sepsis, glutamine could have other benefits such as modulation of the inflammatory response, or could have improved GSH synthesis in other organs, such as the intestine, muscle of liver, which it is not possible to ethically sample in children.

In order to study GSH synthesis in tissues other than whole blood, it was necessary to undertake studies in an animal model. I chose to use the rat model of intestinal ischaemia-reperfusion (I/R), which easily induces systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome. Intestinal GSH has been shown to be important in this model, so I aimed to measure GSH synthesis from deuterated glycine infusion in whole blood, intestine and liver. I did these studies under normothermic conditions, and during hypothermia, as hypothermia has previously been shown the have beneficial effects on intestinal GSH levels but it was not known whether these effects were due to an effect on

GSH synthesis. To summarise the data from these experiments, in erythrocytes, intestinal I/R injury increased to the rate of GSH synthesis – possibly as a result of reactive oxygen and nitrogen species causing a depletion in GSH concentration, thus relieving the product inhibition of the enzymes of GSH synthesis. In liver, there was an apparent increase in GSH synthesis compared to the hypothermic sham group, but not to other groups. This is difficult to understand, and further experiments would be necessary to further explore this effect.

The rate of GSH synthesis in whole liver was significantly higher in the normothermia I/R group compared to hypothermia sham group, but that there was no differences between the other groups. This isolated difference is difficult to interpret, as there was no difference with the corresponding normothermic sham group. It is possible that there were differences in the production of reactive oxygen and nitrogen species between the groups, but this difference was only of sufficient magnitude to cause an difference in GSH synthesis rate between normothermic I/R and hypothermic sham. However, this would need further experiments to verify. This finding is also complicated by the finding that the enrichment in GSH appeared to by higher than that of glycine, which makes the liver data difficult to interpret. In the intestine, GSH synthesis was significantly decreased by normothermic I/R, which could contribute to the marked decrease in GSH concentration observed by other workers. This could be due to inhibition of the enzymes of GSH synthesis by reactive oxygen and nitrogen species. Interestingly, hypothermia led to a significant improvement in the rate of GSH synthesis, suggesting that the enzymes of GSH synthesis are relatively preserved

by hypothermia, allowing GSH synthesis to continue, even though the body temperature is lower (30 - 32ºC).

In conclusion, the newly developed method in my study was used for an assay of patient samples and several tissue samples from the animal model. The preparation of samples and the analytical procedure in this method was simple and straight forward. The derivatised samples obtained were relatively stable and gave good quality results. From the results of my studies, GSH synthesis shows a rather complex relationship with sepsis and systemic inflammation. Although therapies such as glutamine supplementation and hypothermia could have benefits on GSH synthesis, further work is necessary, both in humans and animals, to understand whether they could have a useful clinical role.

Further work

In my study, I was unable to analyse the concentration of blood GSH in patient samples because of lack of volume. Therefore, absolute synthesis rate was not measured. Future work should be aimed to further develop the method by finding an appropriate internal standard so that concentration of blood GSH can be analysed in the same sample as the enrichment. This would allow calculation of the absolute synthesis rate of GSH from the GSH concentration and enrichment. Although I had tried to find an appropriate internal standard for measurement of GSH concentration in this study, this was limited to commercially available di- and tri- peptides. Organic synthesis of other di- and tri-peptides would potentially allow a suitable internal standard to be developed.

In the patient studies, glutamine supplementation was used to try to enhance the GSH synthesis rate. Further studies in these patient groups could also be aimed to try cysteine supplementation, either directly or as *N*-acetylcysteine, to try to enhance GSH synthesis. In further studies, it would be interesting to measure γ-glutamylcysteine ligase activity, and γ-glutamylcysteine concentration to obtain an overview of the control of GSH synthesis in sepsis.

The preliminary data from my animal experiments on ischaemia-reperfusion injury suggested some very interesting changes in GSH synthesis. These data could be greatly expanded. For instance, it would be important to measure the activity of the enzymes γ-glutamylcysteine ligase and GSH synthetase, and also the mitochondrial import of GSH in this model. In addition, the model that I used was limited by being a short-term model, in which steady state for glycine

isotopic enrichment could not be reached, as the clinical status of the rats changed too rapidly. These experiments could be repeated in other animal models of sepsis/ inflammation, such as the caecal ligation and puncture model, where animals typically survive for at least 24h. It would be possible to measure GSH synthesis after peritonitis is established, but infusion of labelled glycine could be continued for long enough to obtain a true steady state, thus allowing measurement of a true fractional synthetic rate. In addition, it would be of great interest to extend these studies to infant rats.

List of publications, presentations and prizes arising from this work

Publication:

Kimura Y, Pierro A, Eaton S: Glutathione synthesis in intestinal ischaemia-reperfusion injury: effects of moderate hypothermia.
J Pediatr Surg 2009;44:353-357.

Presentations at international meetings:

1. Kimura, Y., Chowdhury, M, Eaton, S., et al.: Glutamine Supplementation Does Not Increase Glutathione Synthesis in Septic Children*, British Association of Paediatric Surgeons 54th Annual International Congress, Edinburgh, 2007*

2. Kimura, Y., Eaton, S., et al., Hypothermia Increases Glutathione Synthesis in Intestinal Ischaemia-Reperfusion Injury, *British Association of Paediatric Surgeons 55th (BAPS) Annual International Congress, Salamanca, Spain, 2008*

3. Kimura, Y., Eaton, S., et al., Development of a method to measure glutathione synthesis by GC-combustion/high temperature conversion-IRMS, *Annual Meeting of Stable Isotope Mass Spectrometry User Group (SIMSUG) in Newcastle, UK, 2007*

4. Kimura, Y., Eaton, S., et al., Development of a method to measure glutathione synthesis by GC-combustion/high temperature conversion-IRMS, *Annual Meeting of The Pharmaceutical Society of Japan*, Toyama*, Japan 2007*

Prize:

Best oral presentation prize awarded at SIMSUG, 2007 for presentation 3 listed above.

Appendix

Materials & Procedures

1. Mechanics

GC-MS:

Agilent 6890 Series Gas Chromatograph coupled to an Agilent 5973 Series Mass Spectrometer

GC-IRMS:

Thermo Finnigan^{TM} GC Combustion III and Thermo Finnigan^{TM} DELTA^{Plus} XP with ISODAT software

GC Column:

10m, ZB-50 column (ID 0.25mm × film thickness 0.25μm)

Phenomenex, Macclesfield, Cheshire, UK

PTV inlet: Programmable temperature vaporization inlet (Gerstel CIS4) on the Agilent 6890 GC

Liner: Siltek-deactivated baffled inlet liner plugged with Siltek-deactivated glass wool

both Thames Restek, Saunderton, Bucks, UK

2. Chemicals and Procedures

L-glycine: Sigma-Aldrich, Poole, Dorset, UK

Reduced glutathione: Sigma-Aldrich, Poole, Dorset, UK

$^{13}C_2$-glycine: Sigma-Aldrich, Poole, Dorset, UK

$Gly^{13}C(C_2)$-^{15}N-glutathione: Cambridge Isotope Laboratories, Inc.

D_2-glycine: Cambridge Isotope Laboratories, Inc.

D_2-glycine saline ampoule (11.55 mg/ml): packed in ampoules by Department of Pharmacy at Northwick Park Hospital in Harrow and Department of Pharmacy at the Royal Victoria Infirmary in Newcastle.

Water: Analytical (HPLC) grade water (MiliQ water)

Reduction; 200 µl of TCEP buffer added 50 - 200 µl of erythrocytes, and mixed up for 15 min at room temperature.

TCEP buffer (pH 7.5):

    7.5 mM TCEP (tris-(2-carboxyethyl)-phosphine)

    0.2 M KH_2PO_4 (potassium dihydrogen orthophosphate)

    5 mM EDTA (ethylendiaminetetraacetic acid)

    These chemicals: Sigma-Aldrich, Poole, Dorset, UK

Protein precipitation; 200 µl of 10% (v/v) SSA (sulphosalicylic acid) added into the reduction sample and mixed vigorously. The sample was centrifuged at 1200g for 13 min at 4°C.

    SSA (sulphosalicylic acid): Sigma-Aldrich, Poole, Dorset, UK

Derivatization; 200 µl of the supernatant was made to pH > 10 with 2M NaOH (sodium hydrochloride). 50 µl of *n*-PCF (*n*-Propyl chloroformate) added the sample and mixed for 5 min at room temperature. The sample was

acidified (<pH 2) with 1M HCl (hydrochloric acid) and saturated with 0.5g of NaCl (sodium chloride). The sample was extracted twice with 3 ml diethyl ether and the combined organic phase was dried under N_2 (nitrogen gas) at 60°C. The sample was methylated with 200 μl of 1M methanolic HCl. The samples was heated to 80°C for 10 min, and the evaporated under N_2. The sample was resolved in 100 μl ethyl acetate and transferred to an autosampler vial.

1M methanolic HCl; 340 μl of 6M HCl added into 10 ml methanol.

2M NaOH (sodium hydrochloride)

n-PCF (*n*-Propyl chloroformate)

1M HCl (hydrochloric acid)

NaCl (sodium chloride)

Ethyl acetate

Methanol

These chemicals: Sigma-Aldrich, Poole, Dorset, UK

Diethyl ether (peroxide free): VWR, Lutterworth, Leics., UK

All other chemicals and solvents were analytical grade or other from Sigma-Aldrich, Poole, Dorset, or VWR, Lutterworth, Leics., UK.

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