Pentose Phosphate Pathway Metabolism and Glutathione in the Host Mucosal Response to *Helicobacter pylori* Infection.

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By

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ABSTRACT

PENTOSE PHOSPHATE PATHWAY METABOLISM AND GLUTATHIONE IN THE HOST MUCOSAL RESPONSE TO *HELICOBACTER PYLORI* INFECTION.

Helicobacter pylori is the primary cause of gastritis and peptic ulcer disease. Recent studies have suggested a major role of reactive oxygen species (ROS) in the mediation of *H. pylori* associated disease. Hence, the severity of mucosal damage during *H. pylori* infection is likely to be dependent on the ability of mucosal cells to counteract the ROS load. It was the aim of this thesis to investigate both the activity of the oxidative pentose pathway (OPP) of glucose metabolism, and glutathione levels in the host response to *H. pylori* infection in both a mouse model and in adult symptomatic patients. Novel agents (N-acetylcysteine and oxythiamine) were assessed for their effects on the activity of the OPP and the levels of intracellular glutathione in host mucosa in the mouse. Concomitantly, measures of neutrophil infiltration, myeloperoxidase activity (MPO) were also carried out. Studies in *H. pylori* (SS1) infected mice were carried out to assess host mucosal responses at different times after infection. Studies in *H. pylori* infected adult humans assessed gastric mucosal G6PDH activity, GSH levels and MPO activity. The ability of the ^{13}C-urea breath test to non-invasively determine the level of *H. pylori* infection was also investigated. G6PDH activity and GSH levels were both significantly increased in the gastric mucosa of *H. pylori* infected mice after one month of infection. A small increase in MPO activity was also observed but this returned to normal levels by six months. Oxythiamine treatment inhibited the up-regulation of G6PDH activity and

decreased GSH levels, while NAC administration significantly decreased OPP activity and GSH levels in *H. pylori* infected mice. No difference in G6PDH activity or GSH levels were observed between *H. pylori* infected and non-infected patients. However, MPO activity was significantly increased in *H. pylori* infected patients. Results of the ^{13}C-urea breath test significantly reflected the severity of *H. pylori* associated antral gastritis as measured by histological scoring and MPO activity. These results suggest that an up-regulation of the OPP and increased GSH levels may protect the mucosa against the oxidant load during *H. pylori* infection in the mouse. However, in the adult patient (chronic infection) this proposed mechanism may have be overcome allowing the onset of pathological changes associated with the infection.

DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

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Geoffrey Mark Matthews.

September 2000

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1.0 INTRODUCTION

1.1: *Helicobacter pylori*

Helicobacter pylori is a gram-negative microaerophilic bacterium that was discovered in 1983 in the presence of active chronic gastritis and is now considered the primary cause of gastritis and peptic ulcer disease (Suzuki *et al.*, 1999; Marshall and Warren, 1984; Warren, 1983). Marshall and Warren (1984) observed the presence of *H. pylori* infection in greater than 90% of patients with duodenal ulcers and 70 to 80% of patients with gastric ulcers. It has since been shown to infect more than 50% of the world's population with an incidence of up to 80% in developing countries (Delvin *et al.*, 1999; Catherton, 1997) and has been linked to the onset of gastric cancer (Meining *et al.*, 1998; Asaka *et al.*, 1997; International Agency for Research on Cancer, 1994). It is also accepted that initial infection with *H. pylori* is acquired in childhood and that primary infections in adults are rare (Rowland *et al.*, 1999). Furthermore, it seems that unless treated, *H. pylori* infection persists throughout life in most individuals.

Like many other gastrointestinal bacteria, *H. pylori* secretes urease, an enzyme that catalyses the hydrolysis of urea to NH_3 and CO_2 (Smoot, 1997; Moran, 1996; Bauerfiend *et al.*, 1997). The action of urease on its substrate results in the production of an ammonium cloud encompassing the bacteria protecting it from the highly acidic conditions of the stomach. *H. pylori* urease is also thought to play a role in the pathogenicity of the bacteria (Takahashi *et al.*, 1998).

H. pylori infection is resistant to host-immunity resulting in a failure to eliminate the pathogen, and instead, facilitating its further colonisation (Sipponen and Hyvarinen, 1993). A characteristic of this unsuccessful immune response is the active chronic gastritis that is now considered to be indicative of the infection in all cases (Smoot, 1997). Little is yet known regarding the mechanisms allowing *H. pylori* to evade the aggressive host immune response.

1.2: *H. pylori and gastric inflammation in humans*

H. pylori was discovered by Warren (Warren, 1983) in the presence of active chronic gastritis. This initially led Marshall *et al.* (1984) to hypothesise that the bacterium colonised the epithelial surface of inflamed gastric mucosal cells. The hypothesis was tested by studying the outcomes of the ingestion of live bacteria by two human subjects (Marshall *et al.*, 1985). It was observed that the ingestion of approximately 1 x 10^9 colony forming units of *H. pylori* led to the development of an acute gastritis of the antrum, the distal portion of the stomach, by the tenth day after ingestion. Antral biopsies showed polymorphonuclear cells within the lamina propria along with *H. pylori* on the mucosal surface and adhering to the epithelial surface. Gastritis and *H. pylori* infection had diminished by the fourteenth day post-ingestion. Thus, Marshall *et al.*, (1985) had shown that the gastric inflammation associated with *H. pylori* infection was a result of the infection and, therefore, did not precede the colonisation, as initially proposed.

Rauws *et al.* (1988) studied 303 patients with peptic ulcers and non-ulcer dyspepsia to investigate the association between *H. pylori* infection and gastritis and to observe gastric

responses to drug therapies. Of these patients, 235 were found to have histologically evident gastritis of which 233 were *H. pylori* positive while only 5% of patients with normal histology were *H. pylori* positive. Anti-*H. pylori* drug therapy, consisting of colloidal bismuth citrate and amoxicillin, led to an immediate disappearance of *H. pylori* along with an improvement in the level of histologically evident gastritis within this study. It was noted that in the cases where *H. pylori* was completely eradicated, an entire disappearance of gastric inflammation one year after eradication therapy had occurred. This again provides strong evidence of the role of *H. pylori* in the aetiology of active chronic gastritis.

1.3: *The mouse model of H. pylori infection*

Lee *et al.*, (1997) first described the isolation of a strain of *H. pylori*, known as the Sydney Strain 1 (SS1), that could heavily colonise the mouse stomach for long periods. This provided the scientific community with an animal model of *H. pylori* infection that could be used in studies evaluating treatments for *H. pylori* associated disease and also host-pathogen interactions. However, as with many other animal models of *H. pylori* infection, the pathology of the infection does not completely mimic the human disease.

Some mouse strains show a complete absence of gastritis for nearly the whole life of the infected animals (Sakagami *et al.*, 1996). However, the C57BL/6 strain of mouse becomes heavily colonised with the SS1 *H. pylori* strain and has been observed to slowly develop a mild active chronic gastritis, by 6 months of infection (Lee *et al.*, 1997) . This inflammation was found particularly within the transitional zone between the antrum and

the body of the stomach closely resembling the gastritis observed in *H. pylori* infected humans. Further, a more severe body (corpus) gastritis with atrophy is observed at 8 months post-infection. However, this model still lacks the early active chronic gastritis and the development of peptic ulcers seen in humans.

Lee (1998) suggested that the lack of an early active chronic gastritis in the mouse model may indicate that there is an inherently human-specific factor that induces an active gastritis that is not seen within animal models of this disease. Perhaps, a factor controlling the inflammatory process in the gastric mucosa of both humans and the mouse may react differently to *H. pylori* infection. Little work has been undertaken in this area and it is therefore the aim of this study to investigate the role of an important redox modulating pathway of glucose metabolism, the pentose phosphate pathway, in the host mucosal response to *H. pylori* infection in the human and the mouse.

1.4: *Role of neutrophils, cytokines and reactive oxygen species in H. pylori-associated gastric inflammation*

Recent investigations have focussed on the role of various cytokines in the host response to *H. pylori* infection (Imanishi, 2000; Yamaoka *et al.*, 1999; Yamaoka *et al.*, 1998; Sasayama *et al.*, 1997). It has been observed that levels of interleukin-6 (IL-6), IL-7, IL-8 and IL-10 and tumour necrosis factor-alpha (TNF-α) are significantly higher in *H. pylori* infected gastric mucosa (Imanishi, 2000) compared to non-infected controls. However, the role of IL-8 has been of most interest to researchers due to its ability to activate

neutrophils *in vitro*. Crabtree *et al.*, (1994) observed an increase in IL-8 levels in *H. pylori* infected mucosal samples and this has since been supported by the findings of various studies, including Crowe *et al.*, (1995). Crowe *et al.*, (1995) noted that expression of IL-8 occurred within three hours of *H. pylori* infection *in vitro*. This provides further evidence that the gastric epithelium has the ability to directly evoke an inflammatory response during *H. pylori* infection. Furthermore, Sasayama *et al.*, (1997) demonstrated that gastric mucosal IL-8 levels were correlated to myeloperoxidase (MPO) activity, a biochemical measure of the number of neutrophils present in the tissue. This suggests that *H. pylori* infection enhances the production of IL-8 in gastric mucosal tissue resulting in the neutrophil infiltration.

The presence of neutrophils within the gastric mucosa is proposed to be the main cause of the mucosal damage seen with *H. pylori* infection in humans (Crabtree, 1994). Indeed, it is well documented that mucosal damage associated with *H. pylori* infection is partly caused by the production of excessive levels of reactive oxygen species (ROS) by neutrophils (Suzuki *et al.*, 1999; Yajima *et al.*, 1999; [b]Suzuki *et al.*, 1998). Neutrophil activation results in the release of myeloperoxidase (MPO), an enzyme that catalyses the oxidation of electron donors by hydrogen peroxide (Krawisz *et al.*, 1984). This leads to the generation of ROS that include the superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH^-$). More importantly, MPO catalyses the oxidation of chloride to hypochlorous acid (HOCl) by H_2O_2. Chemiluminescence studies have shown HOCl content in *H. pylori* infected mucosa to be increased ([b]Suzuki *et al.*, 1998). Suzuki

et al., ([b]1998) have proposed that the HOCl reacts with NH_3, produced by *H. pylori* urease, to eventually form the highly reactive and toxic monochloramine (NH_2Cl). NH_2Cl is a lipophilic compound that is able to penetrate biological membranes leading to the oxidation of intracellular components. *H. pylori* was shown to directly evoke the infiltration of neutrophils leading to gastric mucosal injury mediated by NH_2Cl ([b]Suzuki *et al.*, 1998).

A recent study has suggested that ROS may be important modulators of IL-8 expression in gastric mucosal cells (Shimada *et al.*, 1999). Shimada *et al.*, (1999) examined the sensitivity of IL-8 to an antioxidant (N-acetylcysteine) and oxidative stress (H_2O_2). It was observed that IL-8 expression in MKN28 gastric cells was inhibited by the administration of the antioxidant and significantly increased by externally applied H_2O_2. These results suggest that a mechanism controlling the expression of IL-8 exists within gastric epithelial cells that is redox sensitive. Previous studies have implicated the involvement of the transcription factor NF-κB in redox regulated gene expression (Sato *et al.*, 1996; Marui *et al.*, 1993). Many NF-κB-dependent genes are reported to be redox sensitive. This suggests that ROS are not only a major causative agent of gastric mucosal cell damage but are also able to control IL-8 expression in these cells. It is, therefore, hypothesised that the severity of inflammation and damage caused by *H. pylori* infection would be dependent on the ability of mucosal cells to reduce the ROS load.

1.5: *Regulation of cellular redox status*

Role of glutathione

Reduced glutathione (GSH) is an important antioxidant found in most mammalian cells (Potter and Tran, 1993; Mehta *et al.*, 1998). It is known that the ability of cells to maintain their redox status lies, in part, in their ability to synthesise GSH (Potter and Tran, 1993). GSH protects the cell from free-radical induced damage by either protecting cells from lipid peroxidation or by protecting sulfhydryl groups from becoming irreversibly oxidised after oxidant injury (Mehta *et al.*, 1998).

Previous studies have shown that GSH depletion causes cells to become sensitive to a flux of ROS (Szabo *et al.*, 1992; Mehta *et al.*, 1998). Mehta *et al.*, (1998) observed that intestinal epithelial cells, isolated from 24-hr food-deprived animals, had decreased GSH stores and were shown to be more susceptible to the oxidising effects of cumene hydroperoxide, a toxic agent.

This study also investigated the functional status of the antioxidants in enterocytes during *Salmonella typhimurium* infection (Mehta *et al.*, 1998). The authors hypothesised that *S. typhimurium* infection would affect the antioxidant system of the enterocytes, considering the excessive ROS production that would result from neutrophil activation. ROS have the ability to cause damage to all cellular components, including lipids, structural and regulatory proteins, carbohydrates and DNA. Excessive ROS production has been shown to play an important role in a number of diseases, including disorders of the gastrointestinal tract (Naito *et al.*, 1992). Mehta *et al.*, (1998) showed that *S. typhimurium*

infection led to the impairment of GSH levels and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity within enterocytes. This may render the enterocytes more susceptible to oxidative damage as a result of ROS production. It has been shown that live cultures of *S. typhimurium* cause the production of ROS by activated neutrophils as by-products of phagocytosis and these can induce severe tissue damage. The exposure of GSH to ROS causes it to be oxidised to form glutathione disulfide (GSSG) (Mehta *et al.*, 1998). GSSG can be reduced back to GSH by the enzyme glutathione reductase whose activity is controlled by the availability of NADPH (Wernerman and Hammarqvist, 1999). The NADPH that is utilised in the glutathione reductase catalysed step is provided by the oxidative branch of the pentose phosphate pathway. Hence, the synthesis of GSH is thought to be mainly regulated by the activity of the oxidative pentose pathway (OPP) (Mehta *et al.*, 1998).

1.5.1.2

Role of the Pentose Phosphate Pathway

The pentose phosphate pathway is an important biochemical pathway that is responsible for as much as 30% of glucose breakdown in the liver and even more than this in fat cells. It also provides the only *de novo* source of ribose-5-phosphate for synthesis of DNA and RNA (Butler and Arora, 1990). This pathway consists of two arms in most organisms, the oxidative arm (OPP) and the non-oxidative arm (NOPP) (Figure 1). The OPP is irreversible and, in addition to the production of ribose-5-phosphate, is the major provider of reducing equivalents (Williams, 1987). The NOPP is reversible providing C3-C8 glycolyl units which serve as cellular assembly units and as reserve energy metabolites.

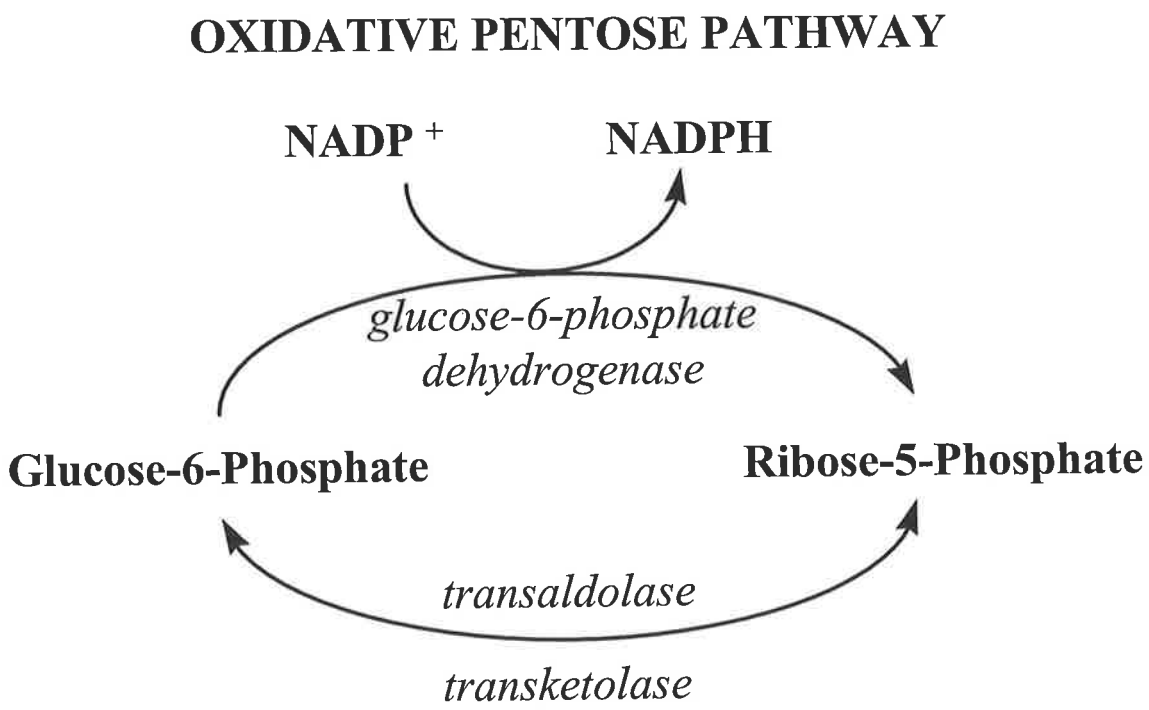

Figure 1: A schematic representation of the pentose phosphate pathways of glucose metabolism showing only enzymes of interest. Glucose-6-Phosphate is metabolised either through the irreversible oxidative pentose pathway or the reversible non-oxidative pentose pathway. The non-oxidative pathway can also recycle ribose-5-phosphate to glucose-6-phosphate. The rate-limiting enzyme of the oxidative pentose pathway is glucose-6-phosphate dehydrogenase (G6PDH), while both transaldolase and transketolase have been proposed as rate-limiting in the non-oxidative pathway.

It has been shown that the NOPP provides approximately 70 to 80% of ribose-5-phosphate in mammalian tissue with the remainder generated by the OPP (Rais *et al.*, 1999). The OPP is particularly important in that it provides the bulk of cytosolic NADPH which acts as a cofactor for a number of key antioxidant enzymes, such as superoxide dismutase and catalase (Tian *et al.*, 1999). Moreover, the provision of NADPH for GSH synthesis is extremely important in maintaining intracellular redox status and providing protection against oxidant induced injury (Tian *et al.*, 1999; Tian *et al.*, 1998; Izawa *et al.*, 1998).

Regulation of the OPP

G6PDH is the key regulatory enzyme of the oxidative arm of the pentose phosphate pathway and, therefore, controls the movement of carbon through this pathway (Figure 2) (Kletzien *et al.*, 1994). Its main role is in the production of NADPH to meet cellular needs for reductive biosynthesis and maintenance of cellular redox state. Therefore, G6PDH was initially considered to be an important "housekeeping" enzyme that was regulated solely by the ratio of NADPH to NADP (Kletzien *et al.*, 1994). However, it has since been shown to be highly regulated and to play major roles in a variety of cellular processes (Tian *et al.*, 1999). In this study G6PDH was observed to be under close transcriptional, translational and post-translational control and was seen to play a critical role in cell growth *via* its role in intracellular redox regulation.

OXIDATIVE PENTOSE PHOSPHATE PATHWAY

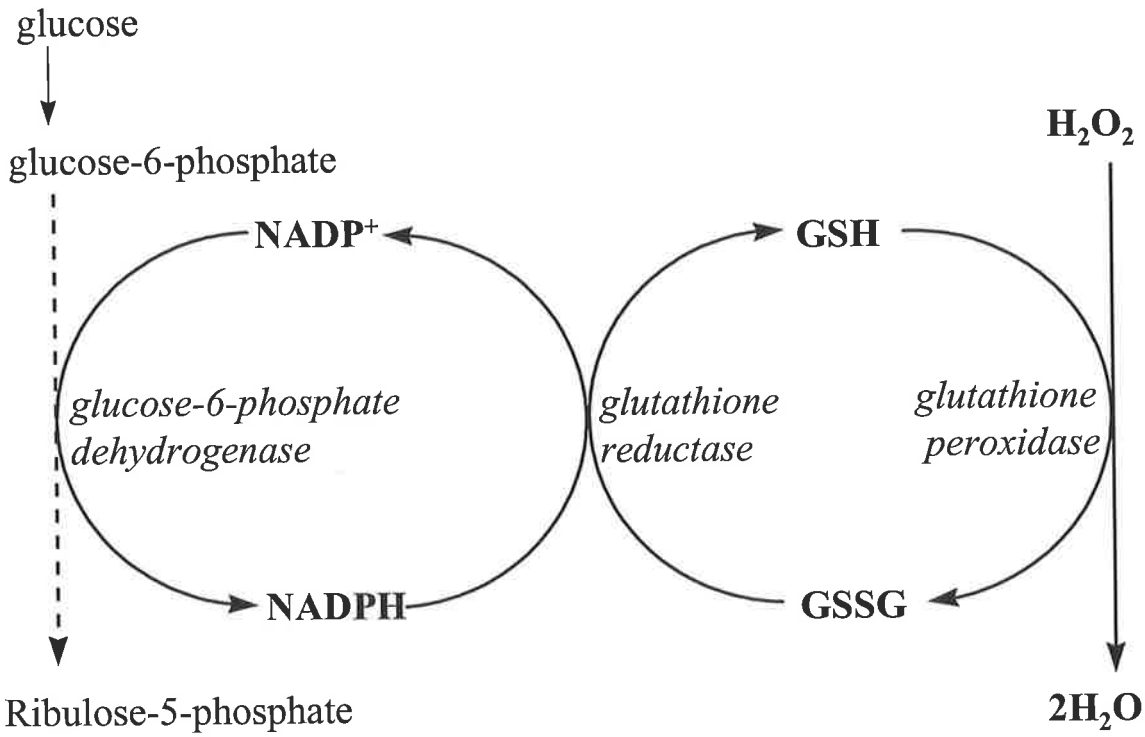

Figure 2: The oxidative pentose phosphate pathway (OPP) is the major provider of NADPH for synthesis and recycling of glutathione. An NADPH molecule is formed by G6PDH allowing the reduction of oxidised glutathione (GSSG) to glutathione (GSH). Glutathione is an important intracellular antioxidant that has the ability to detoxify reactive oxygen species (ROS), such as hydrogen peroxide (H_20_2).

1.6: *H. pylori infection and the pentose phosphate pathway*

The mechanisms of gastric mucosal injury by *H. pylori* infection are not fully understood. Yajima *et al.*, (1999) investigated the cytotoxic mechanism of NH_2Cl on the pathogenicity of *H. pylori* in relation to the intracellular levels of GSH. In this study, cultured rat gastric cells were incubated with NH_2Cl resulting in dose dependent increases in damage. However, pretreatment of the cultured cells with extracellular GSH, known to increase intracellular GSH levels (Hiraishi *et al.*, 1994), attenuated NH_2Cl-induced damage. Furthermore, it was observed that treatment of cells with diethyl maleate, a depletor of cellular stored GSH rendered the cells less resistant to NH_2Cl. This provides evidence that cellular GSH offers protection against NH_2Cl-mediated damage, potentially by the GSH antioxidant defence system in gastric mucosal cells, and is therefore likely to be important in the mucosal response to *H. pylori* infection.

Recently, Suzuki *et al.*, (1999) examined the levels of GSH in the gastric mucosa of the Mongolian gerbil model of *H. pylori* infection. It was observed that the content of total GSH within *H. pylori* infected mucosa was within the same range as the controls at six weeks, although the value had increased significantly by 12 weeks of infection. This result is again suggestive of a role for GSH as an important antioxidant in the host-response to *H. pylori* infection. The activity of G6PDH during *H. pylori* infection has not yet been investigated.

1.7: *Novel therapies for H. pylori-associated disease*

Increasing resistance to current antibiotics is beginning to highly impair our ability to treat *H. pylori* infection (Opekun *et al.*, 1999; Jorgensen *et al.*, 1996). Current treatment for *H. pylori* infection is moving from triple therapy (2 antibiotics and 1 acid suppressor) to quadruple therapy (3 antibiotics and 1 acid suppressor), further raising the possibility of increased antibiotic resistance in *H. pylori* and other bacteria not already resistant. Thus, there is a need for novel anti-*H. pylori* therapies that do not create antibiotic resistance.

It has been recently suggested that the regulation of GSH synthesis may provide a novel therapeutic regimen for *H. pylori* associated gastric mucosal damage (Wernerman and Hammarqvist, 1999). It is hypothesised that therapeutically increasing the intracellular levels of GSH will increase the ability of the antioxidant defence system to counteract the oxidant load produced during *H. pylori* infection. We aim to investigate the mechanistic aspects of this hypothesis by utilising two different methods aimed at increasing GSH synthesis by (a) the up-regulation of the OPP, or (b) the provision of a GSH precursor.

1.7.1 *Regulation of the OPP*

Regulating the availability of GSH by increasing the activity of the OPP was previously investigated by Banki *et al.*, (1996). Banki *et al.*, (1996) studied the role of transaldolase (EC 2.2.1.2), a key enzyme of the NOPP, in the protection of cellular integrity during oxidative stress in cells transfected with sense and anti-sense transaldolase. It was observed that over expression of transaldolase led to lowered GSH levels in transfected

Jurkat human Leukemic T cells. Moreover, it was seen that OPP activity in the same cell type was increased by suppression of transaldolase expression. These results suggest that the suppression of the NOPP leads to a compensatory up-regulation of the OPP. Hence, inhibition of the NOPP may provide a novel method of treating *H. pylori* associated disease.

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This thesis aims to evaluate the ability of an inhibitor of transketolase (EC 2.2.1.1), another key enzyme of the NOPP, in the treatment of *H. pylori* infection in the mouse. We hypothesise that the administration of the transketolase inhibitor (oxythiamine), an analogue of thiamine, to non-infected and *H. pylori* infected mice will induce a compensatory elevation of the OPP and hence lead to increased GSH levels in the gastric mucosa (Figure 3). Potentially, this will enhance the activity of the antioxidant defence system and prevent *H. pylori* associated gastric mucosal damage. The effects of oxythiamine on the gastric mucosa and tissues beyond the stomach would need to be investigated prior to investigations of its ability to eradicate *H. pylori* infection *in vivo*.

1.7.2

Provision of a GSH precursor

The possibility of influencing the availability of glutathione during disease states, such as *H. pylori* infection, has been suggested (Wernerman and Hammarqvist, 1999). However, a complete understanding of GSH regulation is not fully known. It is suggested that the provision of a GSH precursor may increase the rate of GSH synthesis and hence, increase GSH availability for use during oxidative stress.

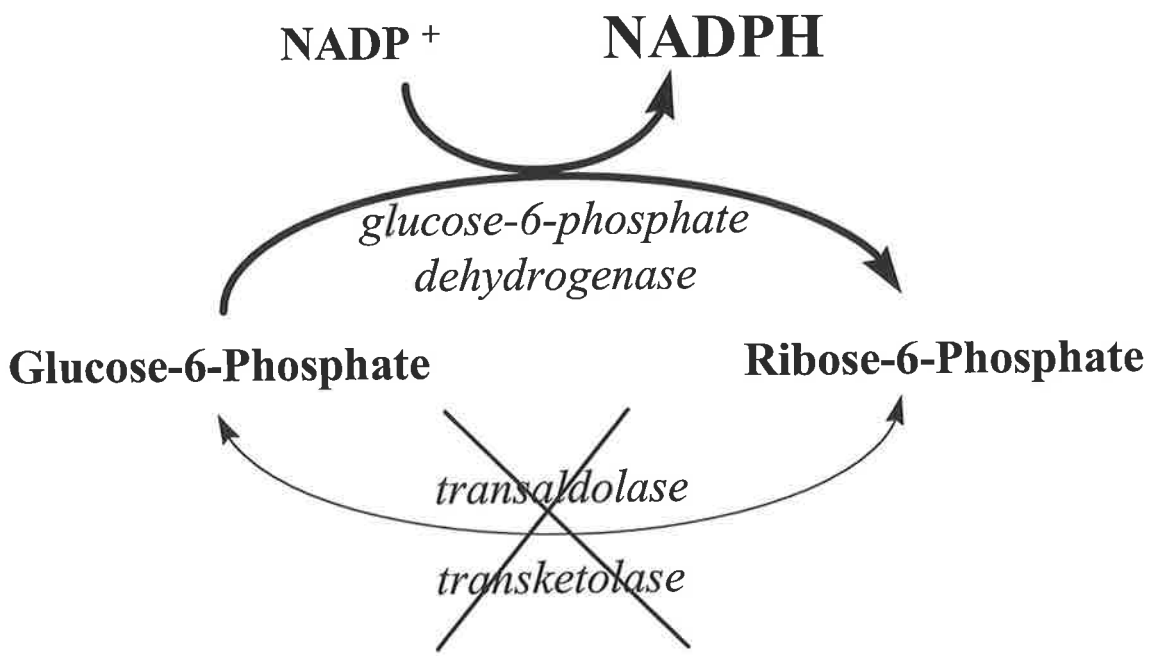

Figure 3: Proposed actions of oxythiamine administration on the pentose phosphate pathway. It is proposed that oxythiamine will inhibit the NOPP by inhibiting its key enzyme, transketolase. It is hypothesised that this will lead to a compensatory up-regulation of the OPP due to the need for ribose-5-phosphate. Moreover, it is proposed that a compensatory up-regulation of the OPP will increase the provision of NADPH for GSH synthesis and recycling, hence increasing GSH levels.

The initial step in GSH synthesis involves the combination of glutamate and cysteine which is catalysed by γ-glutamyl-cysteine synthetase (Wernerman and Hammarqvist, 1999). This is thought to be the rate limiting step in most cases. This is followed by the addition of glycine, catalysed by glutathione synthetase. Therefore, the synthesis of glutathione is dependent on the availability of cysteine and could be influenced by altering the levels of this compound. The administration of N-acetylcysteine (NAC) has been widely used for this purpose. N-acetylcysteine (NAC) has the ability to scavenge hydrogen peroxide, hydroxyl radicals and hypochlorous acid directly while also acting as a precursor for GSH synthesis. It is often used as an antidote in paracetamol intoxications and has been shown to improve the levels of GSH in HIV positive patients (Wernerman and Hammarqvist., 1999). It has not yet been utilised in the treatment of *H. pylori* associated disease. It is hypothesised that administration of NAC will lead to the increased availability of GSH within the gastric mucosa of both non-infected and *H. pylori* infected mice.

1.8: *Novel assessment of treatment efficacy in adult patients*

In *H. pylori* infected mice, the assessment of therapeutic efficacy usually involves the removal of the rodent stomach for bacterial culture and analysis of other investigated parameters. Obviously, this is not an option during therapeutic studies in patients although invasive procedures are often utilised, such as gastroduodenoscopy. Therefore, not only is there a need for a novel approach to the treatment of *H. pylori* associated gastric disease but there is also a requirement for a method of monitoring the efficacy of

these agents in patients without the need for invasive procedures. The ^{13}C-urea breath test (^{13}C-UBT) is a non-invasive tool that is currently used for the initial detection of *H. pylori* infection and for confirmation of *H. pylori* eradication following antibiotic therapy (Delvin *et al.*, 1999; Klein *et al.*, 1996; Logan *et al.*, 1991). With a sensitivity of 98% and a specificity 97% (Logan *et al.*, 1991) the ^{13}C-UBT has the potential to become the test of choice for determination of therapeutic efficacy and for utilisation during clinical trials.

Recent studies have indicated that results of the ^{13}C-UBT are correlated to a measure of intragastric bacterial load (Perri *et al.*, 1998; Ellenrieder *et al.*, 1997) and to the depth of *H. pylori* associated antral inflammation (Perri *et al.*, 1998). However, investigations have not yet evaluated the tests ability to detect changes to the gastric environment and/or the mucosal response following administration of novel agents against *H. pylori* infection and disease. This thesis has previously hypothesised that administration of NAC and/or oxythiamine to infected mice will affect the mucosal response to *H. pylori*. However, an alteration to the gastric mucosal response, such as increased G6PDH activity, after the administration of a therapeutic agent could be caused by a number of factors. These factors may include a change in bacterial load and/or a change in the immune and inflammatory status. Therefore, this thesis aims to determine the ability of the ^{13}C-UBT to assess bacterial load, the severity of mucosal inflammation and the urease activity of individual bacterial isolates in adult patients. It is hypothesised that the ^{13}C-UBT will non-invasively assess each of these parameters providing a useful tool for utilisation during therapeutic studies in patients.

1.9: *Summary*

H. pylori is the primary cause of gastritis and peptic ulcer disease and is known to infect greater than 50% of the world's population (Delvin *et al.*, 1999; Catherton, 1997). Also, infection with *H. pylori* is thought to lead to a predisposition to gastric cancer and, as such, it has been categorised as a group 1 carcinogen by the World Health Organisation (International Agency for Research on Cancer, 1994). Its ability to survive in the harsh conditions of the stomach and induce changes to the structure and function of the gastric mucosa has led to much research in the area of gastroenterology. The discovery of *H. pylori* by Warren (Warren, 1983) has revolutionised the way that peptic ulcers and gastritis are treated. However, the increasing resistance of *H. pylori* to current antibiotic treatment is impairing our ability to eradicate this organism. This has led *H. pylori* research towards the development of novel anti-*H. pylori* therapies (Opekun *et al.*, 1999).

Lee *et al.*, (1997) were the first to describe a strain of *H. pylori* (SS1) that was able to colonise the C57BL/6 mouse stomach for longer periods of time and in greater numbers than any previous strain reported. It has since facilitated investigations into the mechanisms underlying diseases associated with *H. pylori*, including gastritis and gastric cancer. However, although this strain of *H. pylori* is well characterised as readily colonising the mouse stomach, its pathology does not directly mimic that of the human response. The pathology of the mouse model is much slower to develop leading to an active chronic gastritis only after six months of infection. It was suggested by Lee (1998) that there may be a human-specific factor that induces an early active gastritis in humans not seen in the mouse model. However, we can utilise the mouse model to investigate the

events that occur in the host mucosal response to *H. pylori* infection. In particular, the events that occur in the early period post-colonisation.

It is proposed that the pentose phosphate pathway is of great importance in the host mucosal response to *H. pylori* infection and that differences in the activity of this pathway will relate to the different responses seen in humans and animals. It is hypothesised that an increase in G6PDH activity will act as an early event following *H. pylori* infection in humans that will facilitate an increase in GSH synthesis. An up-regulation of the OPP will be brought about by an increased need for antioxidant activity to offset the oxidant assault caused by the infection. It is also hypothesised that G6PDH activity and GSH availability will be increased in the gastric mucosa of the *H. pylori* infected mouse but decreased in gastric mucosa of the *H. pylori* infected adult patients. Increased OPP activity and concomitantly increased GSH levels may prevent the early active chronic gastritis in the mouse that is a characteristic of the human pathology to *H. pylori* infection. It is also hypothesised that the activity of the OPP will change over the duration of *H. pylori* infection.

It is the initial aim of this study to determine the activity of the OPP in the gastric mucosa of the *H. pylori* infected mouse model by measuring G6PDH activity and GSH levels. Assessment of MPO activity in the model will also be undertaken to investigate the role of neutrophil infiltration on the mucosal response to *H. pylori* infection. Also, two novel agents, NAC and oxythiamine, will be evaluated for their ability to alter the host gastric mucosal response during *H. pylori* infection. G6PDH activity, GSH levels and MPO

activity will then be measured in *H. pylori* infected human gastric mucosa and compared to the mouse model. Finally, the non-invasive ^{13}C-UBT will be evaluated for its ability to assess bacterial load, the severity of antral gastritis and urease activity in infected patients.

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Chapter 2

Gastric Mucosal Oxidative Pentose Phosphate Pathway Activity And Glutathione Levels Are Increased In The *Helicobacter pylori* Infected Mouse Model.

2.1 Introduction

Helicobacter pylori infection is the primary cause of gastritis and peptic ulcer disease and is now recognised as a group one carcinogen by the World Health Organisation (Suzuki and Ishii, et al., 2000; International Agency for Research on Cancer, 1994). In humans, infection with *H. pylori* is always associated with an inflammation of the gastric mucosa characterised by an infiltration of neutrophils (Yajima *et al.*, 1999; Sasayama *et al.*, 1997; Crabtree, 1996; Marshall *et al.*, 1985). The current *H. pylori* infected mouse model was developed by Lee *et al.*, (1997) and utilises the Sydney strain of *H. pylori* (SS1) shown to heavily colonise the mouse stomach for long periods. However, the pathology of the SS1 *H. pylori* infected mouse model lacks the early active chronic gastritis and fails to develop peptic ulcers (Lee *et al.*, 1997; Sakagami *et al.*, 1996) and hence the infection does not completely mimic the human disease.

Lee *et al.*, (1997) observed the pathology of the SS1 infected C57BL/6 mouse to be mild in both the antrum and the body after 3.5 months of infection. By six months the inflammatory score increased together with an increased polymorph infiltration. Following eight months of infection, a more severe body gastritis with atrophy was seen. This is unlike the human response to *H. pylori* infection where immediate inflammation

of the gastric mucosa is always seen (Marshall *et al.*, 1985). This highlights the importance of the host in the development of a response to *H. pylori* infection. However, the direct mechanisms mediating gastric mucosal damage by *H. pylori* infection are not yet known.

Recent studies have focussed on the importance of reactive oxygen species (ROS) in *H. pylori* mediated gastric mucosal damage (Suzuki and Ishii, 2000; Yajima *et al.*, 1999; Drake *et al.*, 1998; Farinati *et al.*, 1996). It is well documented that *H. pylori* infection is associated with increased production of ROS in the gastric mucosa by neutrophils (Suzuki and Ishii, 2000; Drake *et al.*, 1998; Moran, 1996) and are implicated as a major contributor to many other gastrointestinal diseases, including inflammatory bowel disease (Santra *et al.*, 2000; Antionetta *et al.*, 1999; Drake *et al.*, 1998; Sedghi *et al.*, 1994). Yajima *et al.*, (1999) investigated the role of the toxic monochloramine (NH_2Cl) in the mechanisms of *H. pylori* mediated mucosal injury. NH_2Cl is a product of the reaction between hypochlorous acid (HOCl), produced by the oxidation of Cl^- by H_2O_2, and NH3, produced by *H. pylori* urease. It was shown that NH_2Cl exposure to cultured rat gastric cells resulted in significant levels of cytolysis by one hour of incubation. Additionally, Yajima *et al.*, (1999) observed that the levels of NH_2Cl demonstrated to cause damage to cultured gastric cells were comparable to those known to be produced by activated neutrophils. Therefore, NH_2Cl, along with other ROS, seem to be very important mediators of mucosal damage in *H. pylori* infected humans.

The role of ROS in *H. pylori* mediated gastric damage highlights the potential importance of cellular antioxidant activity in the host response to the infection (Suzuki and Ishii, 2000; Yajima *et al.*, 1999; Drake *et al.*, 1998; Farinati *et al.*, 1996). Previous research has centred on the role of Glutathione (GSH), an important cellular antioxidant found in most tissues of the body. The levels of GSH increase under oxidative stress *in vitro* (Yajima *et al.*, 1999) and decrease during *H. pylori* infection *in vivo* (Farinati *et al.*, 1996). The antioxidant defence mechanism relies heavily on the production of NADPH for reducing equivalents during oxidative stress. However, little research has investigated the activity of the oxidative arm of the pentose phosphate pathway (OPP), a biochemical pathway that is the major provider of NADPH for the synthesis of GSH, during *H. pylori* infection (Tian *et al.*, 1999).

The present study is hypothesising that the activity of the host mucosal OPP is a very important factor in the host response to *H. pylori* infection. Moreover, this study hypothesises that the activity of the OPP will be increased following *H. pylori* infection in the mouse model resulting in increased levels of GSH. Potentially, this would prevent the mucosal damage that is routinely seen in the human disease.

Therefore, the present study aims to measure the activity of the OPP in the C57BL/6 mouse model of *H. pylori* infection (SS1) over a six month period by assessing the activity of its rate limiting enzyme, glucose-6-phosphate dehydrogenase (G6PDH). Mucosal levels of GSH will also be measured along with MPO activity, a marker of neutrophil activation (Krawisz *et al.*, 1984; Bradley *et al.*, 1982). G6PDH activity, GSH

levels and MPO activity will also be measured in non-infected mice. This study will provide important insights into the early events regulating the host response to *H. pylori* infection.

2.2 Methods

2.2.1: *Animals*

Sixty-nine mice were involved in this study. Forty-three of these mice were infected with *H. pylori* via an oro-gastric gavage of 0.1ml of a *H. pylori* suspension at an initial concentration equivalent to a 1 Mcfarland turbidity standard (approximately 1 x 10^9 cfu/ml). Mice were infected over a five day period on days one, three and five. Infected mice were sacrificed after one month of infection (n = 15), after four months of infection (n = 18) and after six months of infection (n = 10). The remaining 26 mice acted as non-infected controls. Non-infected control mice were sacrificed at the same time as the one month (n = 10), four month (n = 10) and six month (n = 6) infected mice. All mice were sacrificed by CO_2 asphyxiation and cervical dislocation. This study was approved by the Animal Ethics Committees of The University of Adelaide and the Women's and Children's Hospital.

2.2.2: *Tissue preparation*

Immediately following killing, the stomachs were excised and cut along the greater curvature prior to being washed in saline. Stomachs were cut in half from the oesophagus to the pylorus along the lesser curvature using a sterile scalpel. Half of the stomach was placed into preweighed vials containing two ml of sterile saline, weighed and placed on ice. Tissues were homogenised for ten seconds using an ultra turrex (Janke & Kunkel; 24,000 rpm). Eight-hundred µl of homogenate was aliquoted into screw cap eppendorf

tubes along with 10 µl of a 10 µM Pepstatin A solution (an inhibitor of Pepsin; Sigma-Aldrich Pty. Limited, St Louis, Missouri, USA). Two-hundred µl of this homogenate was assayed immediately for G6PDH activity while another 200 µl was plated onto *Helicobacter* agar (Columbia and 7.5% lysed horse blood). All plates were incubated at 37°C under microaerophilic conditions (10% CO_2, 7% O_2) for 4 days. *H. pylori* growth was confirmed by urease test, oxidase test and gram staining. Fifty µl of the homogenate was also placed into a urea broth containing a colour indicator (Hazell *et al.*, 1987) as a final determinate of *H. pylori* infection. The remaining homogenate was snap frozen in liquid N_2 and stored at -70°C until further analysis.

2.2.3: *G6PDH assay*

Extraction of cytosol

Cytosolic preparations were prepared using a revised method similar to Ikeda *et al.* (1998). Sixty µl of a 0.25 M sucrose solution (Sigma-Aldrich Pty. Ltd.) was added to 200 µl of the fresh homogenate and subsequently spun down in a microfuge for 30 mins at 4°C. The supernatant/cytosolic preparation was aliquoted into clean eppendorf tubes and immediately placed on ice until analysis.

G6PDH analysis

Cytosolic G6PDH activity was assayed using a commercially available Sigma Kit (Sigma-Aldrich Pty, Ltd, #345-A). Briefly, 50 µl of supernatant was added to a cuvette along with 400 µl of a G6PDH reagent mixture containing 1.5 mM NADP and 12 mM

maleimide. Eight-hundred µl of a G6PDH substrate solution containing 1.05 mM G6P, a buffer and magnesium salt was aliquoted into the cuvette and this was incubated at 30°C in a circulating water bath for three minutes. Following the incubation period, samples were placed into a spectrophotometer (Beckman, DU-65) and change in absorbance at 340 nm was read over a five minute period against water.

2.2.4: *Protein quantification assay*

Levels of protein were quantified using the method of Bradford (1976) but was modified to be carried out in microtitre plates. Briefly, protein standard solutions were prepared using bovine serum albumin (BSA; Sigma-Aldrich Pty. Ltd.) at concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.625 µg/ml, 7.8125 µg/ml and 3.9 µg/ml protein. Ten µl of each protein standard, in duplicate, was pipetted into the first 9 columns of a microtitre plate. Ten µl of sample cytosol diluted 1 in 10, prepared using the method 2.2.3, was plated out in all remaining wells in duplicate. One-hundred µl of a protein reagent containing 100 mg of Coomassie Brilliant Blue dissolved in 50 ml of 95% ethanol, 100 ml of 85% (w/v) phosphoric acid and 850 ml of water, was added to each well. The plate was mixed and the absorbance at 630 nm was measured after two mins. All absorbances were compared to a background of water. The weight of protein was plotted against the resulting absorbance to produce a standard curve and total protein (mg) was determined from this curve.

2.2.5: *GSH assay*

Preparation of tissue samples

Glutathione levels were assayed using a similar method to Griffith, (1980), however, revised to allow many samples to be undertaken at the same time using a microtitre plate technique. Briefly, 200 µl of homogenate was thawed in a circulating water bath at 37°C for five minutes. The homogenate was precipitated with 100 µl of a 10% sulfosalacylic acid solution and spun down for 4 min at 4°C using a microfuge.

GSH standard preparation

GSH standards were initially prepared by making up a 20mM GSH standard solution with water (Roche Diagnostics, Manheim, Germany) and 0.5 ml of this standard solution was then aliquoted into a separate vessel and diluted with water to 10 ml. 2.5 µl (standard 1), 5.0 µl (standard 2), 7.5 µl (standard 3) and 10 µl (standard 4) of the latter solution was then added to approximately 400 µl of water. Hence, 40 µl of standards one, two, three and four would contain 0.25 nmol, 0.5 nmol, 0.75 nmol and 1.0 nmol of GSH, respectively.

GSH assay

One-hundred and forty µl of an NADPH buffer solution containing 0.125 M sodium phosphate, 6.3 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and 0.3 mM NADPH tetra sodium salt was added to wells of a microtitre plate. Twenty µl of a solution containing 0.125 mM sodium phosphate, 6.3 mM EDTA, and 6 mM 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) was added to each well. Forty µl of each

standard solution was added in duplicate to wells of the microtitre plate. One-hundred µl of sample supernatant was added to the wells not containing standards for analysis and all steps were carried out at 4°C. The plate was allowed to sit at room temperature for five minutes prior to analysis. 210 µl of water (milliQ) was added to 2 wells to act as background.

2.2.5.4

Analysis of GSH levels

Following stabilisation with room temperature, 10 µl of a GSH reductase solution (Sigma-Aldrich, Pty. Ltd; 100 µl of 120 U/mg in 500 µl of a buffer solution containing 0.125 M sodium phosphate and 6.3 mM EDTA, finally diluted to 1 in 10 with water) was added to each well except for those containing water. Plates were immediately mixed for three seconds in a plate reader (Dynatech MR 7000) and read at 410 nm every minute for four to five minutes. The slope of change in absorbance was determined by taking the slope over the first 180 seconds.

2.2.6: *MPO assay*

2.2.6.1 *Preparation of tissue samples*

After killing, animal tissues were prepared using method 2.2.2. Two-hundred µl of homogenate was aliquoted into an eppendorf tube to be analysed for MPO activity by the method of Sekizuka *et al.*, (1988). Briefly, eppendorf tubes were centrifuged in a microfuge at 4°C for 15 min to pellet insoluble cellular debris. The supernatant, which contains less than 5% of total myeloperoxidase activity, was discarded and was

resuspended in 200 µl of a 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB), a detergent that releases MPO from the primary granules of the neutrophil. Samples were vortexed for one min and then centrifuged at 4°C for two mins. Samples were then placed on ice.

MPO assay reaction

Sample supernatant was assayed for MPO activity using a similar method to Sekizuka *et al.*, (1988). For this assay 50 µl of sample supernatant was plated out, in duplicate, in microtitre plates. Two-hundred µl of an MPO reaction mixture, containing 16.7 mg of O-dianisidine dihydrochloride, 50 µl of H_2O_2, 10 mls of stock potassium phosphate buffer (pH 6.0) and 90 mls of H_2O, was added to each well. 250 µl of water was also added to two wells as background. Absorbances were read at 450 nm using a Dynatech MR 7000 plate reader over a ten minute period and rates of MPO activity were determined from readings at six, seven and eight mins. These time points had been previously determined to be at the most linear part of the absorbance trace.

2.2.6: *Statistics*

G6PDH activity, GSH levels and MPO activity in all groups of mice were compared using a one-way analysis of variance (ANOVA) followed by individual students two-tailed t-tests between infected and non-infected groups. Statistical significance was assumed with P value < 0.05.

2.3: Results

2.3.1: G6PDH activity in the gastric mucosa of C57BL/6 mice over a 6 month period

G6PDH activity in non-infected control mice

G6PDH activity was assessed in the gastric mucosa of non-infected mice age matched to the one, four and six month infected mice. No significant variation in G6PDH activity was observed in these mice ($P > 0.05$; Figure 4).

G6PDH activity in H. pylori infected mice

G6PDH activity was observed to significantly change over the period of *H. pylori* infection ($P < 0.0001$; Figure 4). Analysis of G6PDH activity between non-infected and infected animals demonstrated a significant increase in activity at one month of infection ($P < 0.001$), with a further increase at four months of infection ($P < 0.0001$) and remaining significantly elevated six months after infection ($P < 0.001$) when compared to mice of the same age (Figure 4). There was a downward trend in G6PDH activity from the four month to the 6 month infected animals; however, this was not significant ($P > 0.05$).

2.3.2: GSH levels in the gastric mucosa of C57BL/6 mice over a 6 month period

GSH levels in non-infected control mice

The levels of GSH in the gastric mucosa of non-infected control mice were investigated in mice that were age matched to the one month, four month and six month infected mice.

GSH levels in the gastric mucosa of non-infected mice significantly decreased with increasing age ($P < 0.01$; Figure 5).

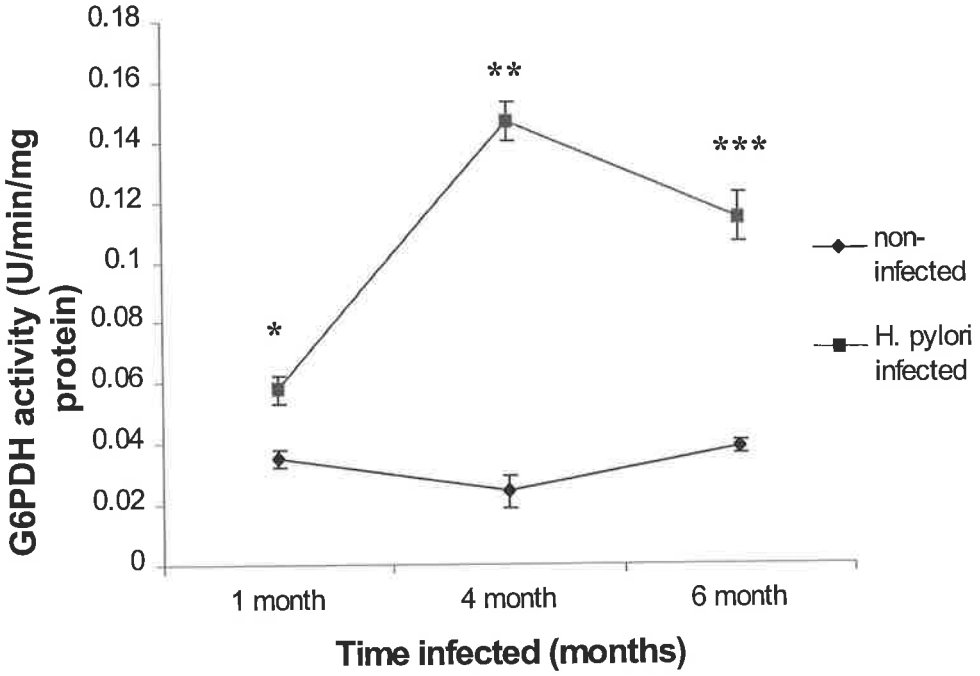

Figure 4: Gastric mucosal G6PDH activity in non-infected vs. *H. pylori* infected mice (mean ± SEM). G6PDH activity did not significantly vary with the age of the animal in non-infected mice ($P > 0.05$). However, G6PDH activity was significantly increased by 1 month of infection (*$P < 0.001$; $n = 15$) with a further increase at 4 months (**$P < 0.0001$; $n = 18$) and remaining significantly elevated after 6 months of infection (***$P < 0.001$; $n = 10$). Means were compared using a one-way ANOVA with Tukey's post-hoc test.

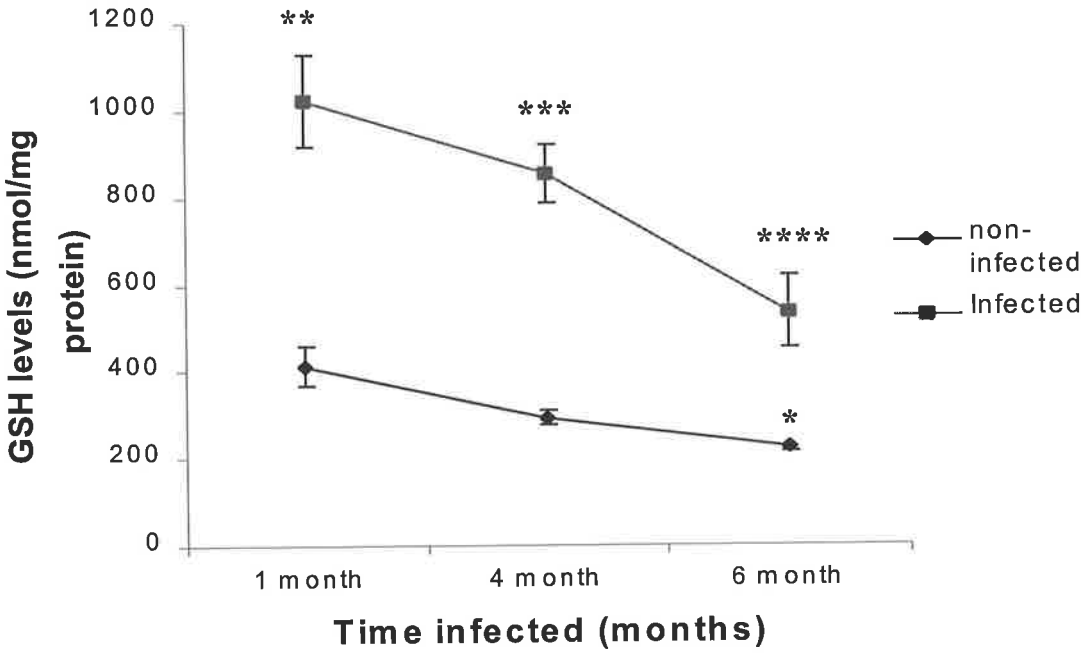

Figure 5: Gastric mucosal GSH levels in non-infected vs. *H. pylori* infected mice over time (mean ± SEM). GSH levels significantly decreased in non-infected mice over the 6 months (*P < 0.01). GSH levels were significantly increased by 1 month of infection (**P < 0.001; n = 15) remaining raised after 4 months of infection (***P < 0.001; n =18). GSH levels had decreased significantly by 6 months of infection (P < 0.001; n =10) but were still significantly higher than non-infected levels (****P < 0.001). Means were compared using a one-way ANOVA with Tukey's post-hoc test.

GSH levels in H. pylori infected mice

GSH levels varied significantly over the 6 month period of *H. pylori* infection ($P < 0.001$; Figure 5). Statistical analysis indicated that GSH levels were significantly increased by one month of infection ($P < 0.001$) and were still raised after four months of infection ($P < 0.001$) when compared to non-infected mice of the same age. After six months of infection, GSH levels were observed to be significantly lower than that at one month ($P < 0.001$) but were still significantly higher than the age matched non-infected mice ($P < 0.001$; Figure 5).

2.3.3: MPO activity in the gastric mucosa of H. pylori infected mice over a 6 month period

MPO activity in non-infected control mice

MPO activity was assessed in the gastric mucosa of non-infected mice that were age matched to the one month, four month and six month infected mice. No significant variation in MPO activity was observed over this time period ($P > 0.05$; Figure 6).

MPO activity in H. pylori infected mice

Analysis of MPO results revealed a small but significant increase in MPO activity ($P = 0.046$) after one month of infection, however by four months of infection a large variation in MPO activity was seen showing no significant difference in MPO activity when compared to control animals ($P > 0.05$; Figure 6). Variation in MPO activity had decreased by six months of infection; however, there was no significant difference

between MPO activity in infected animals compared to age matched non-infected animals ($P > 0.05$; Figure 6).

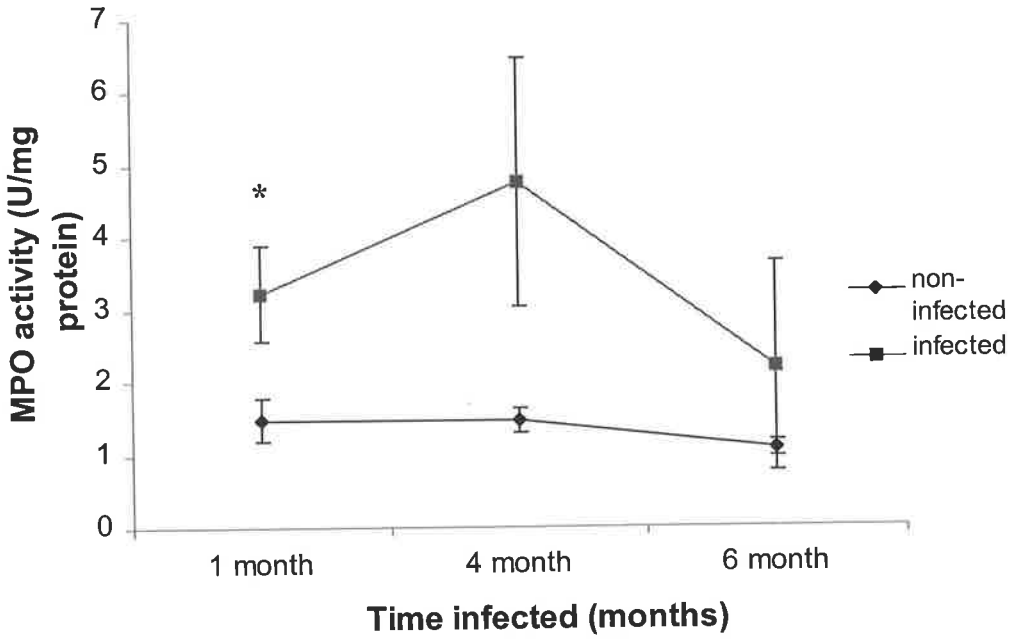

Figure 6: Gastric mucosal MPO activity in non-infected vs. *H. pylori* infected mice over time (mean ± SEM). No significant variation in MPO activity was observed in non-infected mice over the 6 months ($P > 0.05$). However, MPO activity was significantly increased after 1 month of infection (*$P = 0.46$; $n = 15$). MPO activity was not significantly different to normal levels after 4 months of infection ($P > 0.05$; $n = 18$) remaining at these levels after 6 months ($P > 0.05$; $n = 10$). Means were compared using a one-way ANOVA with Tukey's post-hoc test.

2.4: Discussion

The present study investigated the importance of the OPP in the host mucosal response to *H. pylori* infection in a mouse model of the disease. Mice were initially infected with the SS1 strain of *H. pylori* and then sacrificed either at one month, four months or six months following infection. The activity of the OPP and the levels of GSH were both significantly increased following infection with *H. pylori*. Interestingly, the activity of the OPP was seen to increase with the duration of infection whereas the levels of GSH diminished with time. GSH levels were also observed to decrease in age matched controls whereas G6PDH did not appear to.

The present study observed GSH levels to be significantly elevated by one month of infection in the mouse model together with a significant increase in G6PDH activity. It was also shown that mucosal GSH levels in the mouse decreased with increasing length of the infection, although at six months of infection the levels were still higher than age matched, non-infected animals. This study observed that G6PDH activity continued to increase by four months of infection and remained virtually unchanged at this level after six months. This data contrasts that of Santra *et al.*, (2000) who investigated the role of GSH in the mucosal response to *H. pylori* in patients. It was demonstrated that the availability of GSH was significantly decreased during infection in humans. Furthermore, Mehta *et al.*, (1998) observed a decrease in both G6PDH activity and GSH levels in the small intestine of mice infected with *Salmonella typhimurium*. It was proposed that the depleted levels of gastric mucosal GSH in the patients was the result of a failure of the

antioxidant defence mechanism due to high levels of oxidative stress caused by the infections. The increased levels of GSH in the gastric mucosa of *H. pylori* infected mice observed in the present study suggests that an induction of the cellular antioxidant defence system in the mouse occurs in the early stages of infection. It is hypothesised that an induction of the antioxidant defence mechanism in the gastric mucosa of the mouse model immediately following *H. pylori* colonisation enables it to effectively defend itself against the oxidant load produced by the infection.

The OPP, controlled by the rate limiting enzyme, G6PDH, is the major provider of NADPH for the synthesis of GSH (Tian *et al.*, 1999). Hence, G6PDH activity would be important in the ability of the mucosa to defend itself against the oxidant stress produced by *H. pylori* infection. Therefore, the initial increase in G6PDH activity seen by the present study after one month of infection would be indicative of an oxidant load being placed upon the mucosa by the infection. The up-regulated activity of the OPP would be consistent with the increased synthesis of GSH to counteract the oxidant load which was also observed by this study. Yajima *et al.*, (1999) showed that extracellularly administered GSH *in vitro* leads to the attenuation of cellular damage caused by *H. pylori* by increasing intracellular levels of GSH. This highlights the importance of the cellular antioxidant defence system in *H. pylori* infection and the role of the OPP in this mechanism.

MPO activity was used to investigate the degree of neutrophil infiltration and activation resulting from *H. pylori* infection. A significant increase in MPO activity was seen at one

month of infection suggesting an immediate infiltration of neutrophils to the infected mucosa that may be associated with a mild gastritis. Lee *et al.*, (1990) observed the development of a mild inflammation of the gastric mucosa in the C57BL/6 mouse model, 3.5 months after infection with *H. pylori*. However, no observations were made earlier in the infection. By four months of infection, the present study observed that MPO activity was no longer increased although its activity was seen to very variable at this time. Normal MPO activity was also seen after six months of infection. This suggests that an early neutrophil infiltration is decreased to normal levels by four months of infection and remains at this level by six months. Potentially, a small but significant neutrophil infiltration after one month of *H. pylori* colonisation may reflect a mild mucosal inflammation that has diminished by four months of infection. These observations may indicate an initial host immune response to *H. pylori* infection in the mouse that is attenuated by increasing GSH levels, and hence induction of the antioxidant defence system. Increased mucosal GSH levels would act to decrease the oxidant load produced during the infection leading to a normalisation of the redox status of the mucosal cells. This may result in an inhibition of any further neutrophil infiltration and activation and may involve the inhibition of Nuclear Factor-κB, a transcription factor known to be involved in neutrophil activation and that has been shown to be expressed during *H. pylori* infection (Isomoto *et al.*, 2000). Normal levels of MPO activity by four months and also at six months of infection is suggestive of this process.

Highly increased G6PDH activity by four and six months of infection, together with decreased levels of GSH by six months of infection compared to those after one month, suggests an increased utilisation of GSH by the constant oxidant load. Additionally, there is a parallel decrease in gastric mucosal GSH levels with age although GSH levels in *H. pylori* infected animals were always significantly higher than non-infected animals. Therefore, if this model of *H. pylori* infection develops its pathology in a similar, but slower, way to the human infection, unchanged (Farinati *et al.*, 1996) or decreased (Santra *et al.*, 2000) levels of mucosal GSH seen during patient studies may indicate a failure of the antioxidant defence mechanism to offset the oxidant load. Potentially, this would result in the accumulation of ROS leading to the mucosal damage seen in humans with *H. pylori* infection. Perhaps the C57BL/6 mouse model is able to prevent the accumulation of these ROS until later in the infection unlike adults who develop pathological changes immediately following *H. pylori* colonisation (Marshall *et al.*, 1985). The development of mucosal pathology following *H. pylori* infection in children has not been investigated. However, from the results of the present study, whereby GSH levels appeared to decrease with age, it may be suggested that it would be more similar to the mouse than the adult. The mouse model only develops substantial pathological changes after eight months of infection (Lee *et al.*, 1990). This suggests that mucosal GSH levels may be diminished to a degree where its utilisation overcomes recycling and its rate of synthesis leading to failure of the antioxidant defence system after eight months. The present study only measured GSH levels up to six months following infection and hence this suggestion remains speculative.

In conclusion, the present study has shown that the activity of the OPP and the levels of mucosal GSH are significantly increased after one month of infection in the C57BL/6 mouse model of *H. pylori* infection. This was also associated with a small, but significant, increase in mucosal MPO activity that diminishes by four months of infection presumably resulting from the upregulated antioxidant defence mechanism. It appears that the antioxidant defence mechanism slowly becomes overcome by the constant oxidant load placed on the mucosa by *H. pylori*. This may result in the onset of significant pathological changes seen after eight months of infection by previous studies. Potentially, these mucosal changes, indicative of the early host mucosal response to *H. pylori* infection, may relate to early events that occur upon *H. pylori* colonisation in humans which are believed to occur early in life.

Chapter 3

N-Acetylcysteine And Oxythiamine Increase Gastric Mucosal Glutathione Levels In Non-*Helicobacter pylori* Infected Mice.

3.1: Introduction

The eradication of *Helicobacter pylori* using triple therapy is the current regimen for the treatment of *H. pylori* associated gastric disease (Bazzoli *et al.*, 2000; Bazolli, 2000). Recent studies have shown that eradication of *H. pylori* leads to the complete disappearance of peptic ulcer disease and the resolution of gastritis (Bell and Powell, 1993; Rauws *et al.*, 1988). Moreover, the recurrence of peptic ulcer disease following adequate *H. pylori* eradication is virtually nil, compared to 65% in patients who have not had eradication therapy (De Boer and Tytgat, 2000; Bell and Powell, 1993). Increasing resistance to the current antibiotics is beginning to highly impair the ability to eradicate this organism (Opekun *et al.*, 1999; Jorgensen *et al.*, 1996). Hence, novel therapies for the treatment of *H. pylori* infection and disease are needed.

Currently, the most common treatment for *H. pylori* eradication is triple therapy and involves the administration of a proton pump inhibitor (PPI) together with two antibiotics, comprising either amoxycillin, and metronidazole or amoxycillin and clarithromycin (Bazzoli, 2000). A large multi-centre study in 1997, the MACH1 study, found high eradication rates in the order of 90% using triple therapy (The European *Helicobacter pylori* study group, 1997). However, since the MACH1 study, resistance to metronidazole and clarithromycin has risen to 56% and 6%, respectively, in Australia

(Katelaris *et al.*, 2000) and to 25% and 5%, respectively, in Northern Europe (Megraud, 1999). This has resulted in decreased eradication rates, in the order of 15%, using the current treatment regimens compared to the eradication rates seen in the MACH1 study (Megraud, 1999).

Recent studies have demonstrated the role of ROS on the host mucosal response to *H. pylori* infection (Santra *et al.*, 2000; Yajima *et al.*, 1999; Suzuki *et al.*, 1999). These ROS have been shown to play major roles in many gastrointestinal diseases including inflammatory bowel disease (Santra *et al.*, 2000; Antionetta *et al.*, 1999; Drake *et al.*, 1998; Sedghi *et al.*, 1994). This suggests that cellular antioxidants would be beneficial in preventing, or minimising, the gastric mucosal damage caused by *H. pylori* infection.

Reduced glutathione (GSH) is the most important intracellular antioxidant and its levels have been observed to be increased under oxidative stress *in vitro* (Yajima *et al.*, 1999) and decreased during *H. pylori* infection *in vivo* (Farinati *et al.*, 1996). The availability of GSH is thought to be mainly regulated by the activity of the OPP, a major pathway for the provision of NADPH needed to reduce oxidised glutathione to GSH (Tian *et al.*, 1999; Mehta *et al.*, 1998). Therefore, the possibility of influencing the availability of GSH during *H. pylori* infection has been suggested as a potential therapy for *H. pylori* associated gastric disease (Wernerman and Hammarqvist, 1999).

It is proposed that a potential therapy for *H. pylori* associated gastric disease could involve the administration of oxythiamine, an inhibitor of a key enzyme of the non-

oxidative pentose pathway (NOPP), transketolase (Rais *et al.*, 1999; Boros *et al.*, 1997; Gubler and Murdock, 1982). The NOPP provides C3-C8 glycolyl units which serve as cellular assembly units and as reserve energy metabolites (Butler *et al.*, 1990; Williams, 1987). It has been shown that the NOPP provides approximately 70 to 80% of ribose-5-phosphate in mammalian tissues with the remainder being generated by the OPP (Rais *et al.*, 1999). G6PDH activity can be induced by administration of various growth factors, such as platelet derived growth factor (Rais *et al.*, 1999), and has been shown to be inhibited following administration of somatostatin, an agent that suppresses the release of growth factors (Boros *et al.*, 1998). Banki *et al.*, (1996) showed that suppressing the expression of transaldolase, a key regulatory enzyme of the NOPP, resulted in an increased OPP activity. Concomitantly, GSH levels were significantly increased following suppression of transaldolase. The current study is hypothesising that inhibition of another key enzyme of the NOPP, transketolase, by administration of oxythiamine, will also lead to a compensatory up-regulation of the OPP. Furthermore, considering the results of Banki *et al.*, (1996), it is also hypothesised that administration of oxythiamine will result in increased GSH synthesis following an increased provision of NADPH by an induced OPP. The effects of oxythiamine on the gastrointestinal mucosa would need to be determined prior to its utilisation as a therapy for *H. pylori* disease.

Another potential therapy for *H. pylori* associated mucosal damage could involve directly regulating the synthesis of GSH. GSH synthesis is dependent on the availability of cysteine suggesting that its provision could influence the levels of GSH within the mucosa (Guevara *et al.*, 2000; Wernerman and Hammarqvist, 1999). A widely used

approach for influencing GSH availability has been through the provision of N-acetylcysteine (NAC). NAC has the ability to scavenge ROS directly (Aruome *et al.*, 1989) while also acting as a GSH precursor (Guevara *et al.*, 2000; Herzenberg *et al.*, 1997) and is used clinically as an antidote for oxidant drug intoxications. Recent studies have shown that provision of NAC to individuals with human immuno-deficiency virus results in the replenishment of their GSH levels (Herzenberg *et al.*, 1997). Furthermore, administration of NAC to healthy individuals was observed to lead to an improvement in their GSH status. The present study hypothesises that administration of NAC to *H. pylori* infected mice will lead to an increase in gastric mucosal GSH levels potentially contributing to the prevention of the disease caused by the bacterial infection.

In summary, the increasing antibiotic resistance of *H. pylori* has led this study to investigate the potential utilisation of two novel therapies, NAC and oxythiamine. It is hypothesised that NAC will act as a precursor for GSH synthesis leading to increased gastric mucosal levels of GSH. It is also hypothesised that inhibition of the NOPP by oxythiamine will lead to a compensatory elevation of the OPP resulting in an increased synthesis of GSH. Therefore, it is the primary aim of this study to administer both agents, individually, to *H. pylori* infected and non-infected mice and to measure the activity of the OPP and the levels of GSH following treatment. Neutrophil infiltration and activation will also be measured by assessing myeloperoxidase (MPO) activity as a marker of the gastric mucosal inflammatory response.

3.2: Methods

3.2.1: *Animals*

Sixty-eight mice were included in the treatment study. Thirty-eight of these were infected with *H. pylori* while the remaining 30 mice remained uninfected. Mice were infected via an oro-gastric gavage of 0.1ml of a *H. pylori* suspension at an initial concentration equivalent to a 1 Mcfarland turbidity standard (approximately 1 x 10^9 cfu/ml). Mice were infected over a five day period on days one, three and five. Mice were housed for a total period of two and a half weeks from the initial day of infection prior to treatment with N-acetylcysteine or oxythiamine. Thirteen of these mice acted as *H. pylori* positive controls and were gavaged sterile water at the same time as the treatment groups. This study was approved by the Animal Ethics Committees of The University of Adelaide and the Women's and Children's Hospital.

3.2.2: *Treatment protocols*

3.2.2.1 *Oxythiamine treatment (n = 28)*

Thirteen *H. pylori* infected mice and 15 non-infected mice underwent treatment with oxythiamine. All mice received a 0.1 ml oro-gastric gavage of oxythiamine at an initial concentration of 20 mg/ml two times daily resulting in a total daily dose of 4 mg per mouse. This dosage was selected due to previous investigations into the bactericidal concentrations of oxythiamine on *H. pylori in vitro* (2.5 mg/ml) (Butler and Tan, 2000). Oxythiamine treatment was continued for 14 days. All mice were killed via CO_2

asphyxiation and cervical dislocation. Gastric tissues were excised and prepared for analysis of G6PDH activity, GSH synthesis and MPO activity.

N-acetylcysteine treatment (n = 29)

Fourteen *H. pylori* infected mice and 15 non-infected mice underwent treatment with NAC. Treatment with NAC consisted of a 0.1 ml orogastric gavage of an NAC solution with an initial concentration of 200 mg/ml. This was carried out twice daily resulting in a total daily dose of 120 mg per mouse. This dosage of NAC was selected due to studies within our laboratory that had shown it to be bactericidal at an *in vitro* concentration of 15 mg/ml (personal communications, Hien Huynh). Treatment with NAC was undertaken for a total of 14 days. Mice were sacrificed via CO_2 asphyxiation and cervical dislocation. Gastric tissues were excised and prepared for analysis of G6PDH activity, GSH levels and MPO activity using the techniques outlined in chapter 2 (2.2).

3.2.3: *Analysis of samples*

G6PDH activity assay

G6PDH activity in gastric samples from mice of both treatment groups were assayed using the method described in section 2.2.3.

Protein quantification assay

Total protein was quantified using the protocol described in section 2.2.4.

3.2.3.3 *GSH synthesis assay*

GSH synthesis in gastric tissue resulting from treatment with N-acetylcysteine or oxythiamine was determined using the method described in section 2.2.5.

3.2.3.4 *MPO activity assay*

Gastric MPO activity was assayed using the method described in section 2.2.6.

3.2.4: *Statistics*

Changes in G6PDH activity, GSH levels and MPO activity over the period of infection were compared to the same parameters in non-infected control mice from the previous chapter using Student's t-tests. Statistical significance was assumed with P value < 0.05.

3.3: Results

3.3.1: *Oxythiamine treatment*

G6PDH activity

Treatment with oxythiamine did not alter G6PDH activity in non-infected animals when compared to non-infected control animals ($P > 0.05$; Figure 7). However, treatment with oxythiamine led to a significant decrease in G6PDH activity in *H. pylori* infected animals when compared to *H. pylori* infected control animals ($P < 0.01$; Figure 7). No significant variation in G6PDH activity was observed following oxythiamine treatment between *H. pylori* infected and non-infected animals ($P > 0.05$; Figure 7).

GSH levels

Treatment with oxythiamine significantly increased gastric mucosal levels of GSH in non-infected animals when compared to non-infected control animals ($P < 0.001$; Figure 8). However, administration of oxythiamine to *H. pylori* infected animals led to a significant decrease in GSH levels when compared to *H. pylori* infected control animals ($P < 0.001$; Figure 8). GSH levels in *H. pylori* infected mice following oxythiamine treatment were significantly lower than in non-infected animals following treatment with oxythiamine ($P < 0.05$; Figure 8).

MPO activity

An increase in MPO activity was observed in non-infected animals following oxythiamine treatment when compared to non-infected control animals ($P < 0.05$; Figure

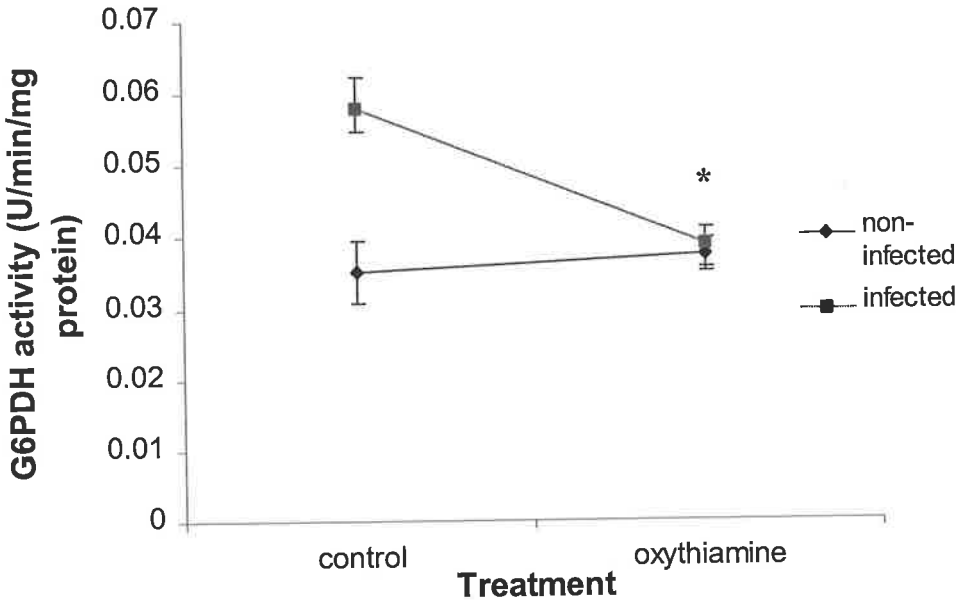

Figure 7: Gastric mucosal G6PDH activity in non-infected vs. *H. pylori* infected mice following oxythiamine administration (mean ± SEM). No change in G6PDH activity was observed in non-infected mice following oxythiamine administration ($P > 0.05$; $n = 12$). However, oxythiamine administration led to a significant decrease in G6PDH activity in *H. pylori* infected mice to normal levels ($^*P < 0.01$; $n = 13$). Means were compared using a two-way Student's t-test.

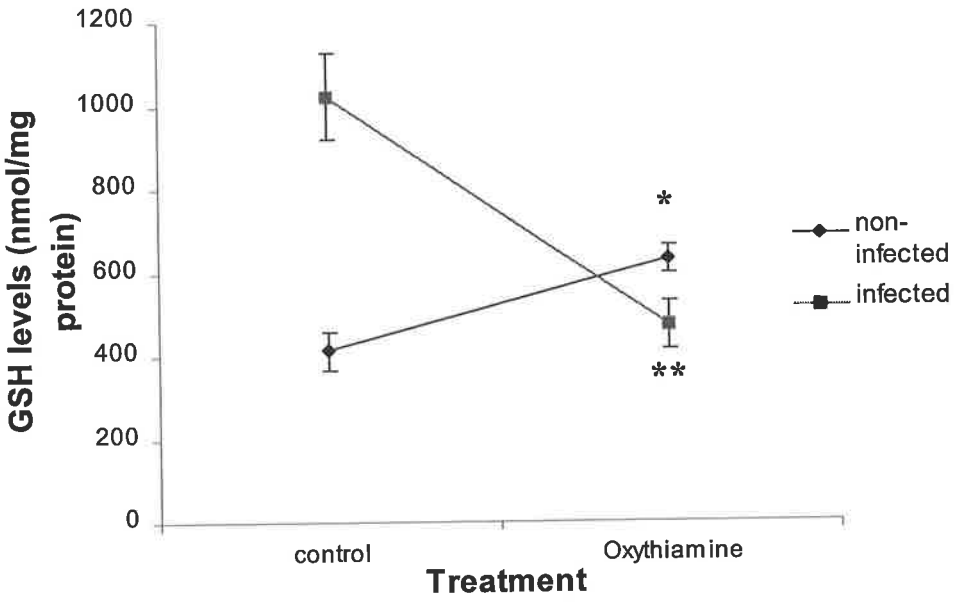

Figure 8: Gastric mucosal GSH levels in non-infected vs. *H. pylori* infected mice following oxythiamine administration. GSH levels were significantly increased in non-infected mice following oxythiamine administration (*P < 0.001; n = 12). In contrast, GSH levels were significantly decreased in *H. pylori* infected mice following oxythiamine treatment (*P < 0.001; n = 13). Means were compared using a two-way student's t-test.

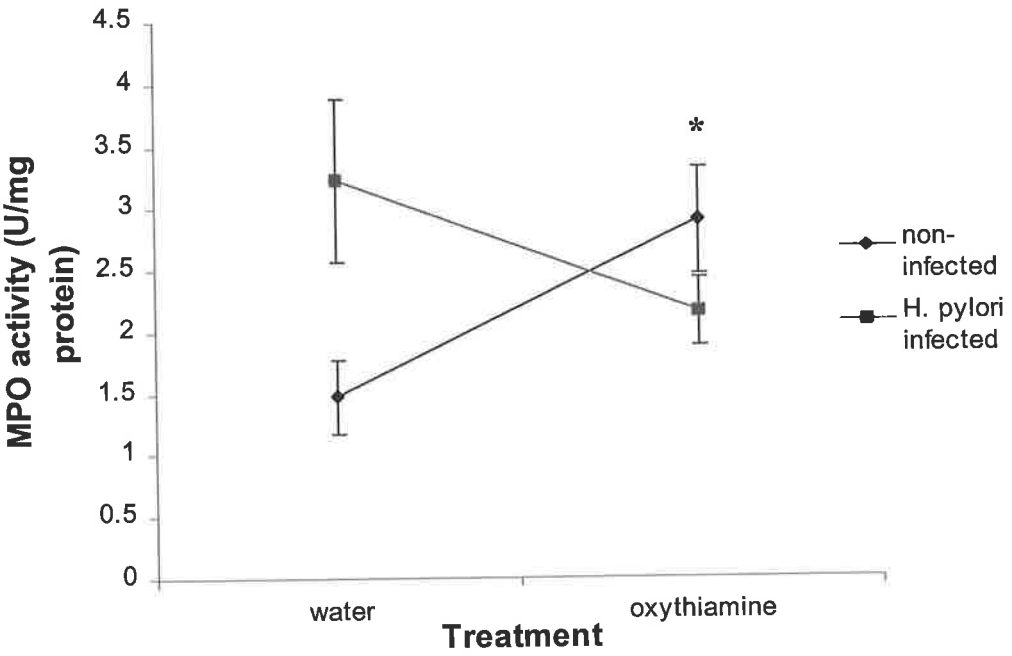

Figure 9: Gastric mucosal MPO activity in non-infected vs. *H. pylori* infected mice following oxythiamine administration. MPO activity was significantly increased in non-infected mice after treatment with oxythiamine (*$P < 0.05$; $n = 12$). No change in MPO activity was observed following oxythiamine administration in *H. pylori* infected mice ($P > 0.05$; $n = 13$). Means were compared using a two-way Student's t-test.

9). There was no significant difference between the MPO activity of non-infected and *H. pylori* infected mice following oxythiamine treatment ($P > 0.05$; Figure 9). Finally, there was no significant difference between the MPO activity of oxythiamine treated *H. pylori* infected mice and *H. pylori* infected control mice ($P > 0.05$; Figure 9).

3.3.2: *N-acetylcysteine treatment*

G6PDH activity

G6PDH activity did not significantly change from normal levels when non-infected mice were treated with NAC ($P > 0.05$; Figure 10). Following NAC treatment in *H. pylori* infected animals, G6PDH activity was not significantly different to non-infected, NAC treated animals ($P > 0.05$; Figure 10). However, following NAC treatment, G6PDH activity in *H. pylori* infected animals was significantly decreased when compared to non-treated (control), *H. pylori* infected animals ($P < 0.001$; Figure 10).

GSH levels

Gastric mucosal levels of GSH were significantly increased in non-infected animals when treated with NAC compared to non-treated (control), non-infected animals ($P < 0.05$; Figure 11). Treatment with NAC led to a significant decrease in GSH levels in *H. pylori* infected animals when compared to *H. pylori* infected, non-treated animals ($P < 0.001$; Figure 11). GSH levels in *H. pylori* infected animals following NAC treatment were significantly lower than NAC treated, non-infected animals ($P < 0.05$; Figure 11).

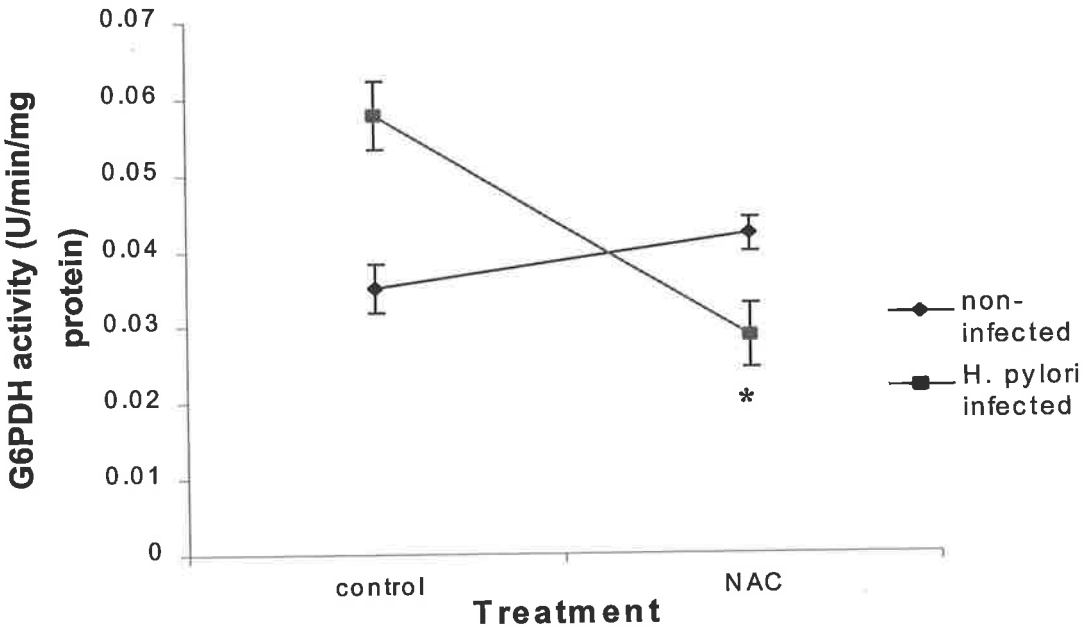

Figure 10: Gastric mucosal G6PDH activity in non-infected vs. *H. pylori* infected mice following NAC administration. No change in G6PDH activity was observed in non-infected mice post-NAC treatment ($P > 0.05$; $n = 11$). However, G6PDH activity was significantly lower in *H. pylori* infected mice following NAC treatment (*$P < 0.001$; $n = 14$). Means were compared using a two-way Student's t-test.

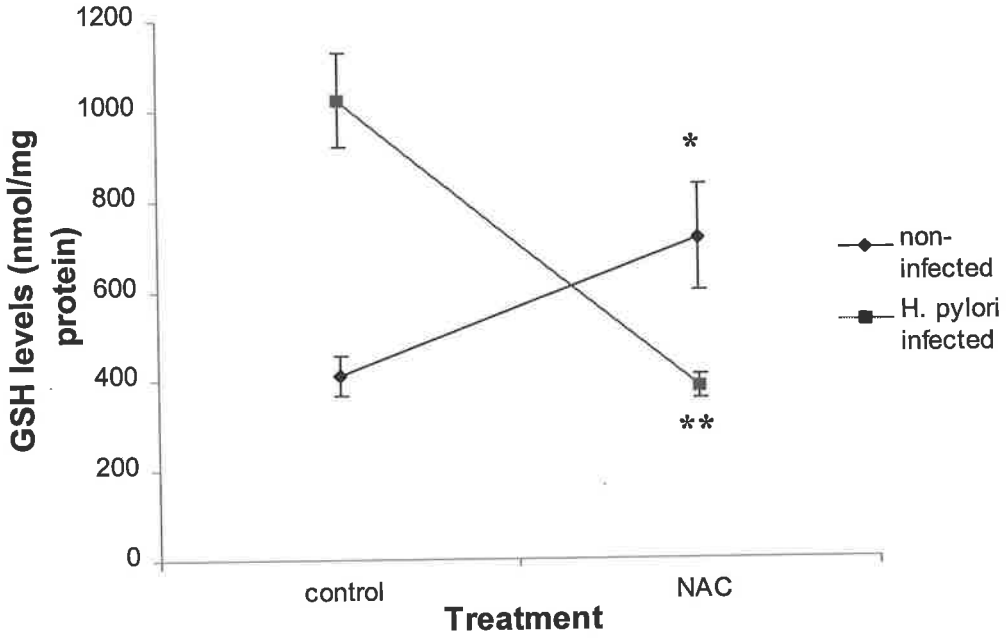

Figure 11: Gastric mucosal GSH levels in non-infected vs. *H. pylori* infected mice following NAC treatment (mean ± SEM). GSH levels were significantly increased in non-infected mice after NAC treatment (*$P < 0.05$; $n = 11$). In contrast, GSH levels were significantly decreased following NAC administration in *H. pylori* infected mice (**$P < 0.001$; $n = 14$). Means were compared using a two-way Student's t-test.

MPO activity

Treatment with NAC significantly increased MPO activity in non-infected animals ($P < 0.01$; Figure 12). In contrast, NAC treatment led to a significant decrease in MPO activity in *H. pylori* infected animals when compared to non-treated, *H. pylori* infected animals ($P < 0.01$; Figure 12). Additionally, MPO activity was significantly lower in NAC treated, *H. pylori* infected animals following treatment when compared to NAC treated, non-infected animals ($P < 0.05$; Figure 12).

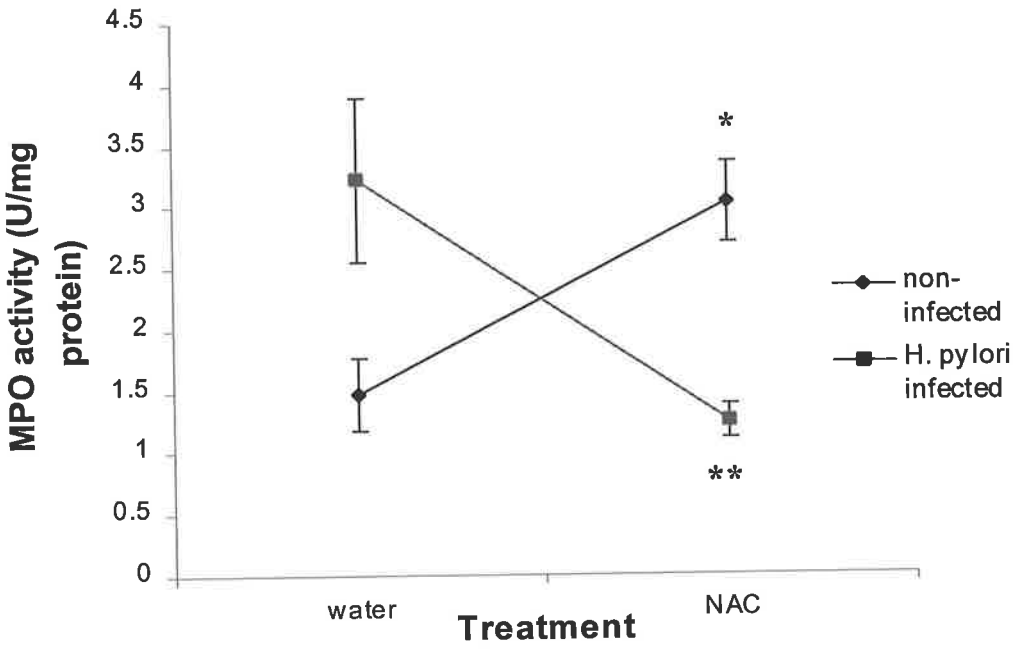

Figure 12: Gastric mucosal MPO activity in non-infected vs. *H. pylori* infected mice following NAC treatment (mean ± SEM). NAC treatment significantly increased MPO activity in non-infected mice (*$P < 0.05$; $n = 11$). In contrast, NAC treatment led to a significant decrease in MPO activity in *H. pylori* infected mice (**$P < 0.01$; $n = 14$). Means were compared using a two-way Student's t-test.

3.4: Discussion

The present study investigated the potential utilisation of two novel orally administered agents, oxythiamine and NAC, in the treatment of *H. pylori* associated disease. It was hypothesised that administration of oxythiamine, an inhibitor of the NOPP, would result in a compensatory up-regulation of the OPP leading to the increased provision of NADPH for the recycling of GSH and its synthesis. It was also hypothesised that NAC treatment would increase gastric mucosal levels of GSH by acting as a precursor for its synthesis.

It was observed that administration of oxythiamine to *H. pylori* infected and non-infected mice did not alter the activity of the OPP as measured by G6PDH activity, the rate limiting enzyme of the OPP. This infers that inhibition of the NOPP by oxythiamine does not cause a compensatory up-regulation of the OPP as was originally hypothesised. However, this study also observed a decrease in G6PDH activity post-oxythiamine treatment when *H. pylori* infected mice were compared to *H. pylori* infected control mice.

Recent studies have shown that the activity of the pentose phosphate pathway is significantly increased within cancer cells *in vitro* (Rais *et al.*, 1999; Boros *et al.*, 1997). Moreover, these studies have shown that inhibition of the NOPP within cancer cells by oxythiamine, as a novel anti-cancer therapy, also resulted in a significant inhibition of the OPP. This suggests that inhibition of the NOPP also leads to an inhibition of the OPP, but only under conditions where its activity is up-regulated, such as during *H. pylori* infection

and/or in cancer cells. Alternatively, it may be due to a reduction in *H. pylori* load and a concomitant decreased response of the gastric mucosa.

An interesting finding in the current study was that oxythiamine administration to non-infected mice appeared to significantly increase the mucosal levels of GSH which was not paralleled by an increase in G6PDH activity. Potentially, this may indicate that there are sufficient stores of NADPH within the cell for GSH recycling and synthesis. This suggests that the OPP may only be induced to provide reducing equivalents for GSH synthesis when the NADPH stores have been utilised. Indeed, previous studies have shown that the activity of the OPP is regulated by the ratio of NADPH to NADP (Kletzien *et al.*, 1994). It could also be hypothesised that inhibiting the up-regulation of the OPP, the main provider of NADPH for GSH synthesis, may induce an alternative biochemical pathway to provide NADPH for GSH synthesis. However, the present study did not investigate these hypotheses.

Gastric mucosal MPO activity was seen to significantly increase in non-infected animals post-oxythiamine treatment. However, MPO activity was not significantly different from control levels in oxythiamine treated, *H. pylori* infected mice. This suggests that oxythiamine may be damaging the gastric mucosa in non-infected animals but not affecting it in *H. pylori* infected animals. In infected animals, it is possible that *H. pylori* may utilise the oxythiamine before it is able to have any effect on the gastric mucosa. This is another interesting finding as it was recently suggested that oxythiamine could be utilised as an anti-*H. pylori* agent (Butler and Tan, 2000). Butler and Tan, (2000)

hypothesised that inhibition of the bacterial NOPP would prevent the synthesis of ribose-5-phosphate leading to cell death and eventually eradication of the infection. The present study did not utilise any quantitative measure of bacterial load and can, therefore, only hypothesise that this may have had some effect within this study. However, if oxythiamine is to be utilised in the future as a treatment for *H. pylori* infection, this study has shown that it has no effect on *H. pylori* infected mucosa.

The present study also investigated that utilisation of NAC as a novel treatment for *H. pylori* associated disease. It was demonstrated that treatment with NAC did not significantly alter the activity of the OPP in non-infected animals. However, G6PDH activity was significantly decreased following NAC treatment in *H. pylori* infected mice compared to *H. pylori* infected, non-treated mice. This suggests, that whereas NAC has no effect on the activity of the OPP in the gastric mucosa of non-infected mice it acts in a way that lowers the activity during *H. pylori* infection. NAC is a GSH precursor and was seen to increase the availability of GSH in non-infected mice within this study. Previous studies have observed the protective effects of increased GSH availability following NAC administration, for example in Trypanosoma cruzi infection and HIV patients (Guevara *et al.*, 2000; Wernerman and Hammarqvist, 1999).

Following NAC treatment, the levels of GSH were observed to be significantly decreased during *H. pylori* infection, returning to non-infected control levels. This is in contrast to GSH levels in *H. pylori* infected control mice which were observed to be significantly increased. However, this is possibly not a surprising result as NAC is not only a GSH

precursor but is also able to act as a scavenger of ROS, and is therefore an antioxidant in itself (Tredger, 2000; Wernerman and Hammarqvist, 1999). This suggests, that although in the non-infected animal NAC acts as a GSH precursor, its properties as an antioxidant may dominate in the infected animal. Potentially, the antioxidant nature of NAC allows it to scavenge the ROS produced during *H. pylori* infection preventing the need for induction of the cellular antioxidant defence mechanism. Thus, the normal GSH levels seen in *H. pylori* infected mice following NAC treatment may indicate an inhibition of the ROS mediated gastric mucosal response to *H. pylori* as a result of the antioxidant properties of NAC and not because of it being a GSH precursor. Alternatively, NAC has been reported to kill *H. pylori in vitro* and reduce bacterial load *in vivo* (Huynh *et al.*, 2000).

MPO activity following NAC treatment was observed to be significantly increased in non-infected mice but significantly lower in *H. pylori* infected animals. This suggests that NAC may exhibit some toxicity to the gastric mucosa in non-infected animals. It is possible that an increase in GSH availability following NAC treatment in non-infected mice may result from any mucosal damage leading to an induction of the antioxidant defence system. However, the increase in gastric mucosal MPO activity was only small suggesting that the increase in GSH levels following NAC treatment was due to its actions as a GSH precursor as previously discussed. It also appears that when *H. pylori* is present NAC does not affect the gastric mucosa and this may be a result of its actions as an antioxidant.

In summary, the present study has shown that treatment with oxythiamine has no effect on OPP activity in non-infected animals but inhibits an up-regulation of the OPP in *H. pylori* infected mice. Interestingly, an increase in gastric GSH levels were observed following oxythiamine treatment in non-infected mice. NAC administration was seen to increase the GSH availability in non-infected mice. However, its levels in *H. pylori* infected mice following treatment were not significantly different to that of non-infected mice. It was hypothesised that this result was indicative of the antioxidant properties of NAC being dominant during *H. pylori* infection rather than it acting as a GSH precursor. Therefore, this study has shown that oral administration of NAC and oxythiamine may prove to be novel therapeutic agents for *H. pylori* associated disease in the future. It is suggested that future studies investigate an interaction between these two agents both with respect to the host gastric mucosa and the viability of the bacteria.

Chapter 4

Gastric Mucosal G6PDH Activity And GSH Levels Are Not Increased In *Helicobacter pylori* Infected Adult Patients.

4.1: Introduction

Warren (1983) was the first to report the presence of *Helicobacter pylori* in the gastric mucosa of patients with gastritis and this has since led to much research into the organisms role in gastroduodenal disease. It has been demonstrated that *H. pylori* infection causes immunological and inflammatory responses in host gastric mucosa resulting in mucosal cell damage (Yajima *et al.*, 1999; Sasayama *et al.*, 1997; Smoot, 1997; Crabtree, 1996). The exact mechanisms that cause *H. pylori* associated gastric mucosal injury are not fully understood. However, recent studies have focussed on the role of reactive oxygen species (ROS) in the process of mucosal cell damage (Shimada *et al.*, 1999; Yajima *et al.*, 1999).

A large infiltration of neutrophils into the gastric mucosa of *H. pylori* infected individuals is observed pathologically (Yajima *et al.*, 1999; Sasayama *et al.*, 1997; Zhang *et al.*, 1997). It is known that *H. pylori* activates neutrophils *in vitro* and *in vivo* via epithelial production of IL-8 (Shimada *et al.*, 1999; Sasayama *et al.*, 1997). Activated neutrophils generate ROS, and excessive ROS production is known to play a part in many diseases of the gastrointestinal tract, including peptic ulcer disease (Naito *et al.*, 1992). Neutrophils also release myeloperoxidase (MPO), an enzyme that catalyses the oxidation of electron donors by hydrogen peroxide (Krawisz *et al.*, 1984). MPO catalyses the oxidation of

chloride to hypochlorous acid (HOCl) by H_2O_2. It has been proposed by recent studies that luminal NH_3, produced by *H. pylori* urease, reacts with the HOCl to form the highly reactive and toxic monochloramine (NH_2Cl) ([b]Suzuki *et al.*, 1998). NH_2Cl is able to penetrate biological membranes leading to the oxidation of intracellular components. Chemiluminescence studies have shown HOCl content in *H. pylori* infected mucosa to be increased. Furthermore, *H. pylori* was shown to directly evoke the infiltration of neutrophils leading to gastric mucosal injury mediated by NH_2Cl ([b]Suzuki *et al.*, 1998). This suggests that ROS play a major role in the gastric mucosal damage caused by *H. pylori* infection. Therefore, cellular antioxidants would be very important in the host response to this bacterium.

Most mammalian cells are equipped with an antioxidant defence system enabling them to combat free-radical-mediated damage (Mehta *et al.*, 1998). The gastrointestinal system has been shown to be particularly rich in antioxidant activity. Indeed, the glandular mucosa of the stomach is known to have one of the highest glutathione (GSH) levels in the body (Ovrebo *et al.*, 1999). Intracellular GSH is possibly the most important antioxidant factor preventing oxidative damage (Yajima *et al.*, 1999; Mehta *et al.*, 1998). Exposure of GSH to ROS causes it to be oxidised to form glutathione disulfide (GSSG) which can then be reduced back to GSH by glutathione reductase. The reducing equivalents (NADPH) utilised in the glutathione reductase catalysed step are principally produced by glucose-6-phosphate dehydrogenase (G6PDH), the rate limiting enzyme of the oxidative pentose phosphate pathway (Tian *et al.*, 1999). Hence, the levels of GSH in gastric mucosa are mainly regulated by the activity of G6PDH.

Yajima *et al.*, (1999) characterised the importance of GSH in the redox regulated response to NH_2Cl mediated damage. Cells were treated with either extracellular GSH, which increases intracellular GSH, or diethylmaleate (DEM), which reduces GSH levels. Pretreatment with extracellular GSH was found to attenuate NH_2Cl mediated damage while pretreatment with DEM led to cells being less resistant to NH_2Cl. These results provide evidence for the protective role of cellular GSH against NH_2Cl mediated damage which may play a major role in *H. pylori* associated gastric mucosal damage.

Farinati *et al.*, (1996) investigated the levels of GSH within gastric mucosal tissue of *H. pylori* infected patients. This study observed a trend towards higher mucosal levels of GSH in patients with chronic gastritis, atrophy and *H. pylori* infection although this was not statistically significant. However, GSH turnover was seen to be significantly higher in patients with *H. pylori* associated gastritis when compared with normals, resulting in higher levels of GSSG. In contrast, Santra *et al.*, (2000) observed a significant decrease in gastric mucosal GSH levels during *H. pylori* infection in another group of symptomatic patients. It was suggested that this decrease was a result of a failure of the gastric mucosal antioxidant defence system to counteract the oxidant load produced during *H. pylori* infection. However, the role of the OPP in the host mucosal response to *H. pylori* infection in patients has not been assessed.

Therefore, the present study aims to evaluate the activity of the OPP and the levels of GSH within the gastric mucosa of *H. pylori* infected symptomatic individuals and compare this to non-infected individuals. It is hypothesised that, during *H. pylori*

infection, the activity of the OPP and the mucosal levels of GSH will be significantly decreased in symptomatic patients as a result of the increased levels of ROS produced during the infection. Furthermore, it is hypothesised that myeloperoxidase activity, a measure of neutrophil infiltration and activation, will be significantly increased in *H. pylori* infected patients when compared to non-infected individuals. This will be investigated by measuring the activity of the rate limiting enzyme of the OPP, G6PDH, along with gastric mucosal levels of GSH using biochemical methodologies. Histological assessment of gastric biopsy samples will also be undertaken.

4.2: Methods

4.2.1: *Symptomatic patients*

Symptomatic patients (n = 48) previously referred for gastroduodenoscopy were recruited from the Gastroenterology Unit of The Queen Elizabeth Hospital. All patients had fasted overnight prior to endoscopy (no food and only small amounts of water). Inclusion criteria were chronic abdominal pain and/or other gastroesophageal symptoms (reflux, dysphagia, dyspepsia, pyrosis or odynophagia). Patients were excluded if they had received antibiotics in the preceding four weeks. Informed consent was obtained from all subjects and the study was approved by the Human Ethics Committees of the Women's and Children's Hospital and of The Queen Elizabeth Hospital.

4.2.2: *Endoscopy*

Patients were intravenously sedated followed immediately by gastroduodenoscopy. During endoscopy, multiple biopsies (≥ 5) of gastric mucosa one to three millimetres in area were obtained from the gastric antrum using flexible forceps and placed in pre-weighed tubes containing 900 µl of phosphate buffered saline (PBS) and maintained at 4°C until tissue preparation. Antral biopsies were also obtained for histopathological assessment (Institute of Medical and Veterinary Science, The Queen Elizabeth Hospital), culture (Microbiology Department, The Queen Elizabeth Hospital) and for CLO test (Delta West, Bentley, Western Australia), a commercially available rapid urease test.

4.2.3: *^{13}C-urea breath test*

Methodology

In the days immediately following the endoscopy, all patients were required to undertake a ^{13}C-urea breath test for the non-invasive assessment of *H. pylori* infection. After an overnight fast, patients were required to ingest 100 mL of Vitafresh® lemon-barley drink containing citric acid before exhaling completely into duplicate baseline breath collection tubes (Exetainer®, Labco, High Wycombe, England). One-hundred mg of ^{13}C labelled urea (Cambridge Isotope Laboratories Inc, Massachusetts, USA) was dissolved in two millilitres of warm water before being added to a further 100 mL of the citric acid-containing drink which was then ingested. Subjects were required to refrain from any activity for 30 minutes before exhaling into duplicate breath collection tubes.

Analysis of ^{13}C-urea breath test

Exhaled $^{13}CO_2$ was detected by isotope ratio mass spectrometry (ABCA20/20 Europa Scientific equipped with a V410 data collection system) thirty minutes following ingestion of the ^{13}C-urea. $^{13}CO_2/^{12}CO_2$ ratios were expressed as δ ^{13}C values (permil, ‰) relative to the PeeDee Belemnite Limestone (PBD) standard, and changes in the δ ^{13}C level compared with baseline are expressed as δ over baseline ^{13}C ‰ (DOB ^{13}C) (Perri *et al.*, 1998). A positive test was defined as a breath sample with a DOB ^{13}C of 5 and above (Kindermann *et al.*, 2000).

4.2.4: *Tissue preparation*

Biopsies in PBS were immediately analysed for G6PDH activity. Tissues were initially weighed and then homogenised using an ultra turrex (Janke & Kunkel; 24,000rpm) for 4 seconds. Ten µl of a 10 µM solution of Pepstatin A was added to the homogenate. Two-hundred µl of homogenate was immediately aliquoted into a clean eppendorf tube for analysis of G6PDH activity. The remaining homogenate was snap frozen in liquid N_2 and stored at -70°C until analysis of GSH levels and MPO activity.

4.2.5: *G6PDH assay*

Cytosolic preparation

A cytosolic preparation of the two-hundred µl of homogenate was prepared using the method described in section 2.2.3. G6PDH activity was assayed by the method of Dror *et al.*, (1970) as described in the section 2.2.3, however 100 µl of the cytosolic preparation was used in this study. G6PDH activity was measured spectrophotometrically for change in absorbance at 340 nm over a five minute period using a Beckman, DU–65 spectrophotometer.

4.2.6: *Protein quantification assay*

Total protein was measured by the method of Bradford (1970) described in section 2.2.4.

4.2.7: *GSH assay*

Two-hundred µl of homogenate was assayed for GSH synthesis using the protocol of Griffith (1980) previously described in section 2.2.5.

4.2.8: *MPO assay*

Gastric MPO activity was assessed using the method of Sekizuka *et al.*, (1988) previously described in section 2.2.6.

Not listed

4.2.9: *Statistics*

Changes in G6PDH activity, GSH synthesis and/or MPO activity in *H. pylori* positive patients were compared to non-infected patients using unpaired Student's t-tests. MPO activity was compared to a histological score of the severity of gastric inflammation using regression analysis. All values are reported as mean ± SEM. Statistical significance is assumed with a P value < 0.05.

4.3 Results

4.3.1: *Patients*

A total of 48 patients with mean age of 51.15 ± 2.51 years (range 20 to 80 yrs; 27M: 21F) were recruited by this study. Analysis of G6PDH activity was undertaken in 39 of these patients of whom 11 (28%) were determined to be *H. pylori* positive by ^{13}C-urea breath test, culture and/or CLO test. Analysis of GSH levels and MPO activity was undertaken in 44 patients and 16 (36%) of these were found to be *H. pylori* positive by the same parameters.

4.3.2: *G6PDH activity*

G6PDH activity was measured in antral mucosal samples from both *H. pylori* infected and non-infected patients. No significant difference was observed between the G6PDH activity of *H. pylori* infected and non-infected patients ($P > 0.05$; Figure 13). G6PDH activity was not correlated to the DOB ^{13}C vales ($r = 0.103$; $P > 0.05$; Figure 14).

4.3.3: *GSH levels*

Gastric antral mucosal samples were assessed for GSH levels in both *H. pylori* infected and non-infected patients. No significant difference was observed between *H. pylori* infected and non-infected patients ($P > 0.05$; Figure 15). Gastric mucosal GSH levels were not correlated to DOB ^{13}C values ($r = 0.3$; $P > 0.05$; Figure 16).

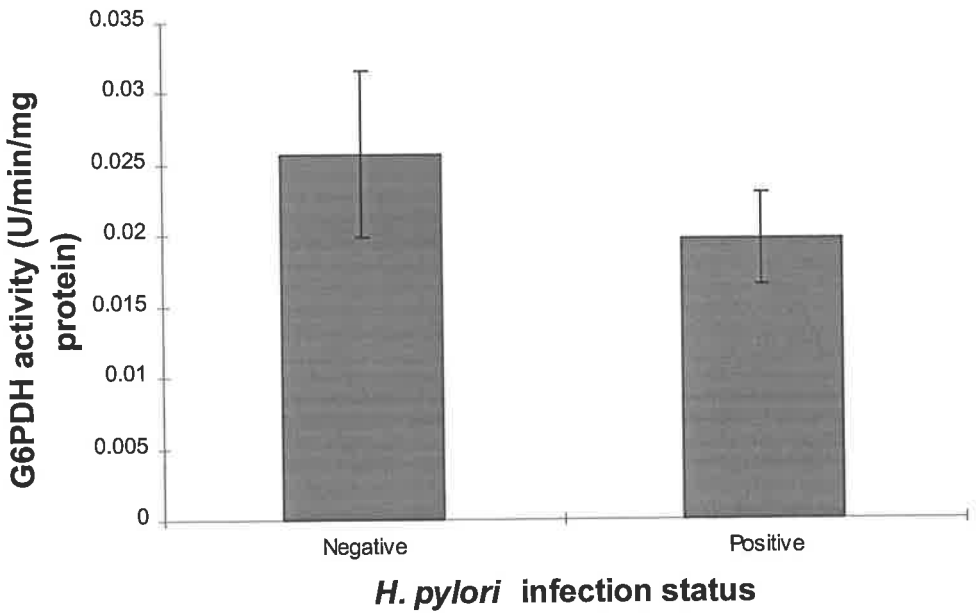

Figure 13: Gastric mucosal G6PDH activity in non-infected (n = 28) vs. *H. pylori* infected (n = 11) adult patients (mean ± SEM). G6PDH activity was not significantly different between the two groups ($P > 0.05$). Means were compared using a two-way Student's t-test.

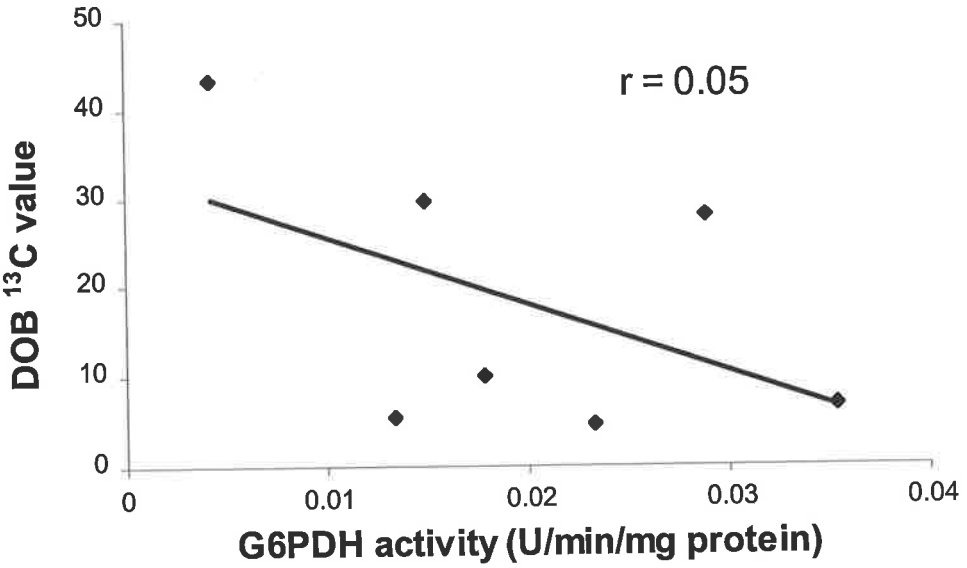

Figure 14: A correlation between DOB ^{13}C values from the ^{13}C-urea breath test and the gastric mucosal G6PDH activity in *H. pylori* infected adult patients (n = 7). A correlation coefficient of r = 0.5 was obtained, however this was not significantly different from zero (P > 0.05).

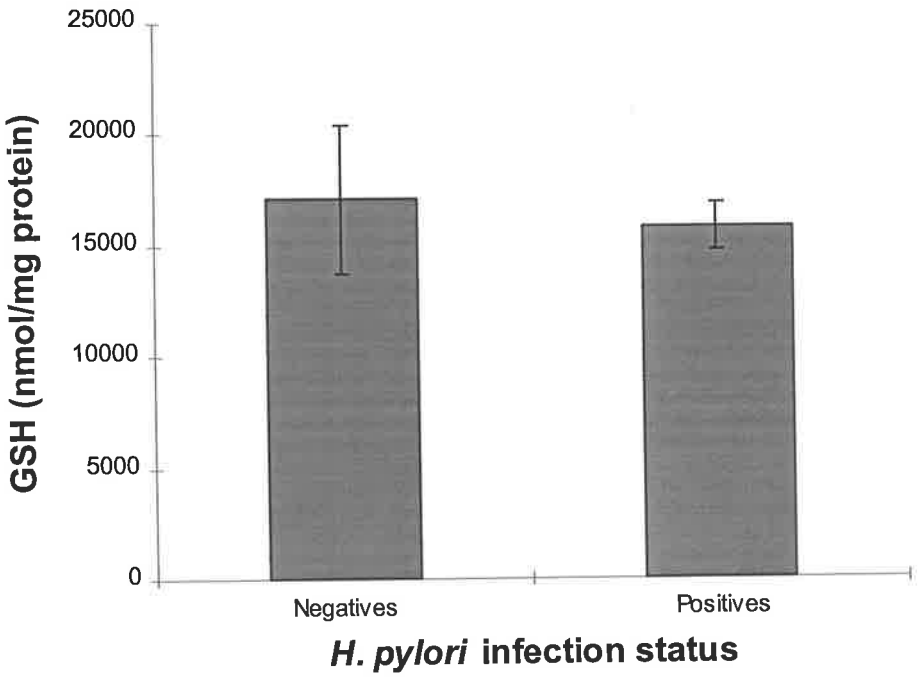

Figure 15: Gastric mucosal GSH levels in non-infected (n = 28) vs. *H. pylori* infected (n = 16) adult patients (mean ± SEM). No significant difference in GSH levels were observed ($P > 0.05$). Means were compared using a two-way Student's t-test.

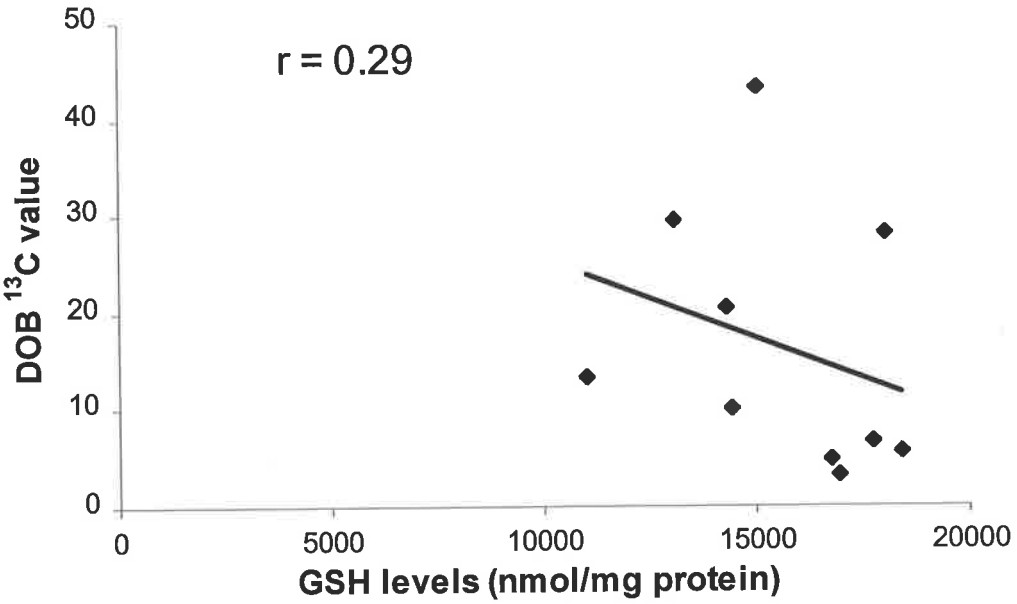

Figure 16: A correlation between DOB ^{13}C values from the ^{13}C-urea breath test and gastric mucosal GSH levels in *H. pylori* infected adult patients (n = 10). No significant correlation was obtained (r = 0.29; P > 0.05).

4.3.4: *MPO activity*

MPO activity within antral mucosal specimens was assessed in both *H. pylori* infected patients and non-infected individuals. A significant increase in MPO activity, of greater than 4 times, was observed in *H. pylori* infected patients compared to non-infected patients ($P = 0.001$; Figure 17). MPO activity was also compared between individuals with normal and inflamed gastric mucosa. Patients with histologically demonstrated gastric inflammation had significantly higher MPO activity than those with normal pathology ($P = 0.014$; Figure 18). Additionally, MPO activity was compared to a histological score of gastric inflammation in both *H. pylori* infected and non-infected patients. A significant correlation was found between the two parameters ($r = 0.65$; $P < 0.0001$; Figure 19). Also, a significant correlation was found between MPO activity and DOB ^{13}C values ($r = 0.82$; $P < 0.01$; Figure 20).

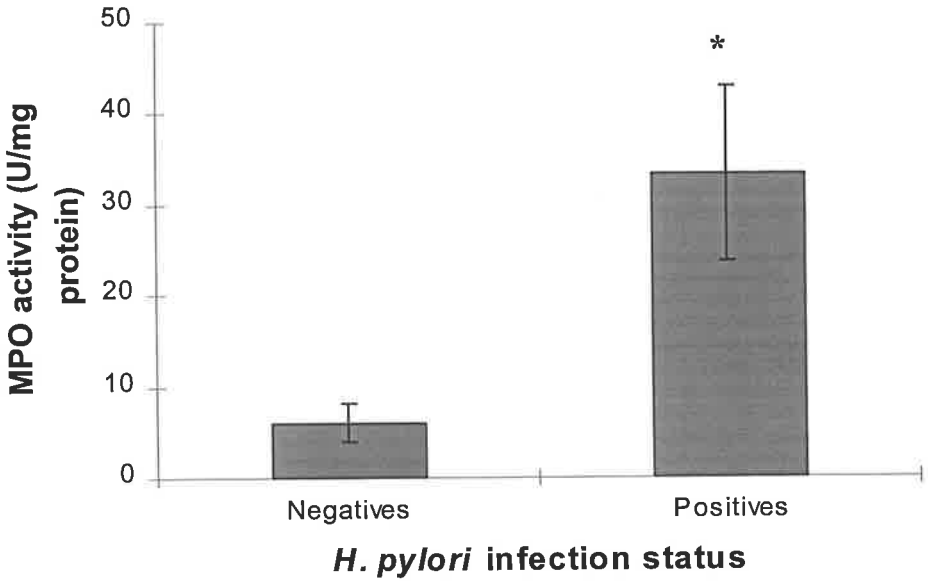

Figure 17: Gastric mucosal MPO activity in non-infected (n = 28) vs. *H. pylori* infected (n = 16) adult patients (mean ± SEM). MPO activity was significantly higher in *H. pylori* infected patients than non-infected patients (*P = 0.001). Means were compared using a two-way Student's t-test.

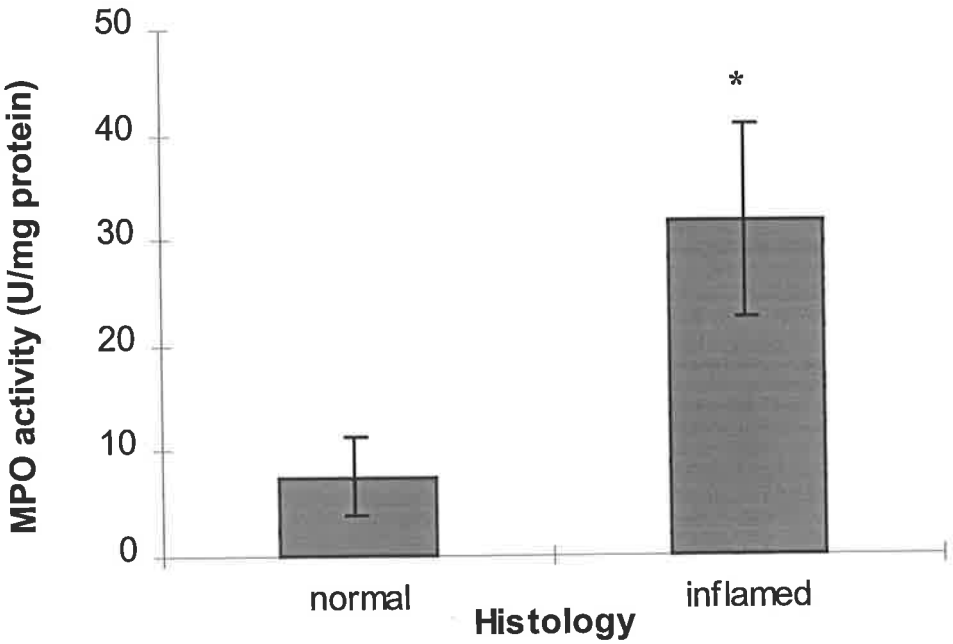

Figure 18: Gastric mucosal MPO activity compared to a histological measure of gastric pathology (normal and inflamed) in adult patients (mean ± SEM). Patients with gastric inflammation (n = 13) also had significantly higher MPO activity than non-infected patients (n = 30; *P = 0.014). Means were compared using a two-way Student's t-test.

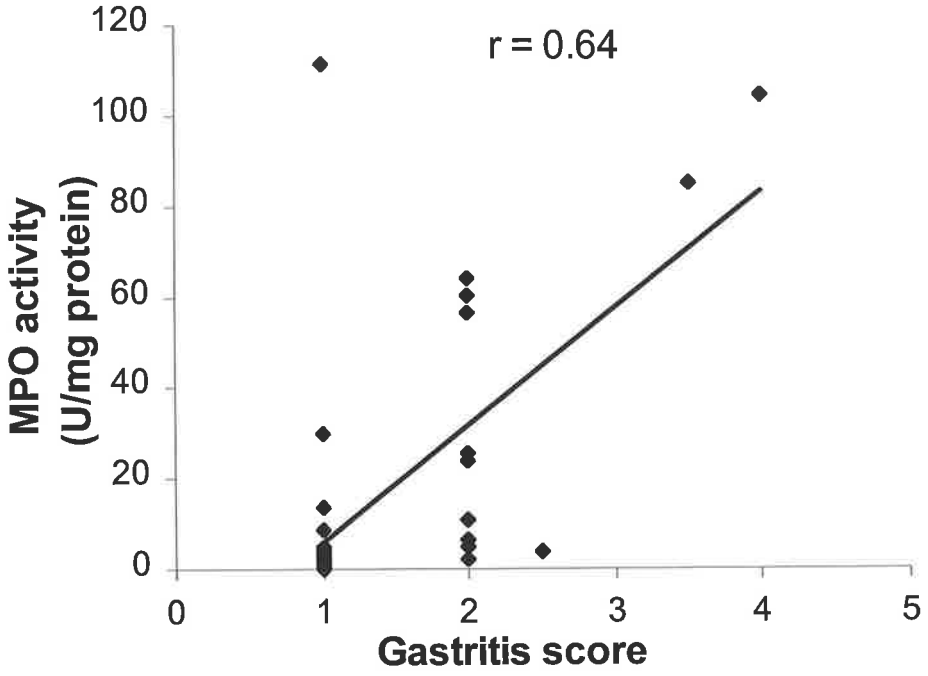

Figure 19: A correlation between gastric mucosal MPO activity and a histologically determined gastritis score in both non-infected and *H. pylori* infected adult patients. Gastric pathology was scored as; normal (1; n = 31), mild gastritis (2; n = 10), mil to moderate gastritis (2.5; n = 1), moderate gastritis (3; n = 0), moderate to severe gastritis (3.5; n = 1) and severe gastritis (4; n = 1). A significant correlation coefficient of r = 0.64 was obtained between these parameters ($P < 0.0001$).

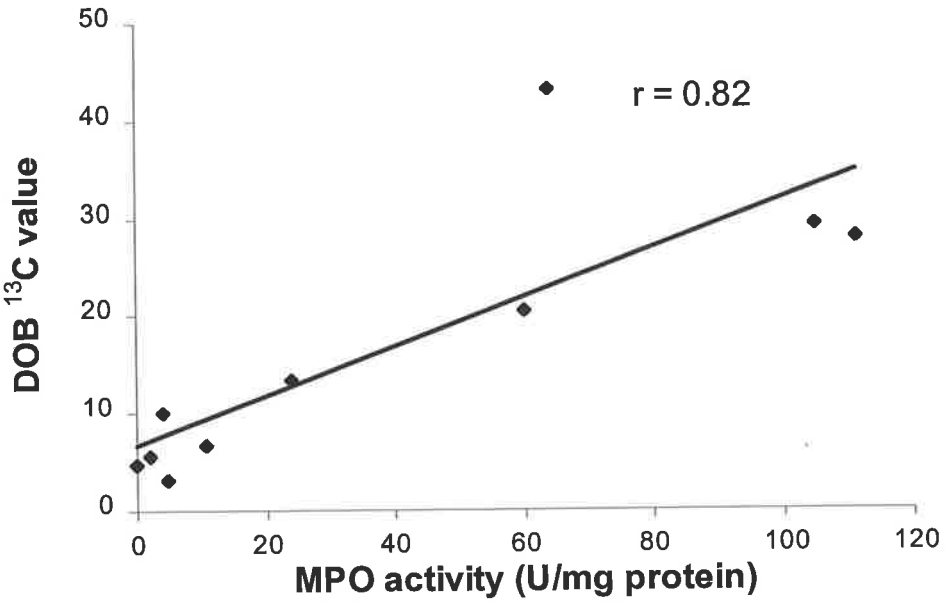

Figure 20: A correlation between DOB ^{13}C values from the ^{13}C-urea breath test and gastric mucosal MPO activity in *H. pylori* infected adult patients (n = 10). A significant correlation coefficient of r = 0.82 was obtained indicating a relationship between DOB ^{13}C and MPO activity ($P < 0.01$).

4.4 Discussion

The present study demonstrated that the activity of the OPP in the gastric mucosa of *H. pylori* infected patients was not different to that of non-infected patients. Furthermore, the mucosal levels of the antioxidant, GSH, were also not different in *H. pylori* infected patients when compared to non-infected patients. It was also observed that *H. pylori* infection in symptomatic patients was associated with a significant increase in mucosal MPO activity.

The antioxidant defence mechanism relies heavily on the adequate production of reducing equivalents in the form of NADPH during oxidant stress (Tian *et al.*, 1999). G6PDH, the rate limiting enzyme of the OPP, provides the major source of NADPH for the synthesis of GSH from its oxidised form, GSSG. Previous studies have shown G6PDH activity to be increased under oxidant stress (Pandolfi *et al.*, (1995). Hence, the levels of GSH are thought to be mainly regulated by the activity of G6PDH (Tian *et al.*, 1999). The present study measured the levels of GSH in human *H. pylori* infected gastric mucosal samples together with the activity of G6PDH.

It was observed that both the activity of G6PDH and GSH levels in *H. pylori* infected mucosa were not significantly different to non-infected mucosa in the present study. A similar study (Farinati *et al.*, 1996) demonstrated a trend towards increased GSH levels in the gastric mucosa of *H. pylori* infected subjects. However, in agreement with the present study, this was not statistically different to non-infected patients ($P = 0.08$).

Unfortunately, Farinati *et al.*, (1996) did not measure the activity of the G6PDH in their study. However, their investigations assessed the levels of oxidised glutathione (GSSG) in the tissue samples and found that both GSSG levels and the %GSSG/total glutathione were significantly higher in the presence of chronic atrophic gastritis. This possibly indicates an overall increase in the rate of GSH recycling by the glutathione redox cycle (Suttorp *et al.*, 1986) and/or increased utilisation of GSH by oxidant stress. Potentially, an increased glutathione recycling rate would act to supply continuing amounts of GSH at a level able to maintain antioxidant activity against the ROS. The levels of gastric mucosal GSSG were not measured within the current study. However, as G6PDH is the major source of NADPH for GSH synthesis (Tian *et al.*, 1999), the lack of an increase in G6PDH activity in *H. pylori* infected patients suggests that GSH cycling, or turnover, was not significantly increased in these patients. Nonetheless, it could be suggested that GSH turnover could occur if another source of NADPH, or NADPH stores, were able to provide for GSH synthesis.

Indeed, it could be also suggested by the present study that the lack of any difference in gastric GSH levels between *H. pylori* infected and non-infected patients may be due to the chronicity of the infection. Earlier studies with *H. pylori* infected mice within our laboratory have suggested that the activity of the antioxidant defence system does change over the duration of the infection (refer chapter 2.0). The mean age of patients recruited by this study was 50 years (range 20 to 80 years). This was very similar to the age range in the study by Farinati *et al.*, (1996). The high mean age is highly suggestive of long term infection in many of the patients and it is not yet known whether the role of GSH as

an antioxidant in *H. pylori* infection changes over the duration of the infection in humans (≥ 50 years). It is possible that the antioxidant defense mechanism that is provided by GSH may act as an early event during the initial period of *H. pylori* infection and may be overwhelmed, or down regulated, by an ever increasing oxidant load as the chronicity of the infection increases. This could be investigated by assessing the activity of the OPP and GSH levels in the gastric mucosa of children found to be infected with *H. pylori*. Gastric mucosal GSH levels and OPP activity would be expected to be increased in *H. pylori* infected children indicating the importance of the antioxidant defense mechanism provided by the OPP and GSH in the initial period of *H. pylori* infection.

The present study also observed a significant increase in MPO activity in *H. pylori* infected mucosa compared with normal mucosa. This indicates a significant infiltration of neutrophils to the gastric mucosa of *H. pylori* infected individuals. Neutrophil infiltration is always observed pathologically in *H. pylori* infected human mucosa and has been documented in many studies (Suzuki *et al.*, 2000; Sasayama *et al.*, 1997; Crabtree, 1996; Marshall *et al.*, 1985). MPO activity was also shown to correlate with an inflammatory score determined from histological parameters and the DOB ^{13}C values from the ^{13}C-urea breath test (^{13}C-UBT). This suggests that the measurement of MPO activity in tissue homogenate is a quantitative marker of the severity of *H. pylori* associated gastric inflammation and this in agreement with similar studies (Sasayama *et al.*, 1997; Krawisz *et al.*, 1984). Moreover, these results suggest that the ^{13}C-UBT is able to non-invasivley

assess the the severity of *H. pylori* associated mucosal inflammation. However, larger group sizes are needed to confim these results.

The data from this study suggests that the mucosal response to *H. pylori* infection in humans later in a chronic infection is not regulated by the antioxidant defence system. However, it could be hypothesised that the sequence of events that regulate the response early in the infection may be begin with *H. pylori* infection causing ROS production, either directly and/or indirectly, which is closely followed by an increase in mucosal G6PDH activity and concomitant GSH levels to protect the mucosa from the ROS. However, as the duration of the infection proceeds, the GSH antioxidant defence system becomes overwhelmed by the continued oxidant load and this allows the recruitment of neutrophils by the production of IL-8 by epithelial cells. It is suggested that this may involve the oxidant induced activation of Nuclear Factor-κB that is known to be involved in the expression of IL-8 (Kim *et al.*, 2000). Activated neutrophils produce further ROS and act to promote mucosal inflammation in order to eradicate the organism. However, the inflammatory response fails to eradicate the infection and concomitantly, the antioxidant defence system fails to counteract the continued oxidant load. Potentially, this would lead to mucosal damage, such as peptic ulcers, that is characteristic of *H. pylori* infection in humans. It is suggested that certain hosts may have a more effective antioxidant defence system allowing it to escape ulcer formation. Alternatively, differing strains of *H. pylori* might elicit different mucosal responses, and in some patients (symptomatics) these strains may overcome the cellular defence mechanisms.

In conclusion, the present study has shown that although gastric mucosal MPO activity is significantly increased in *H. pylori* infected patients compared to normals, no change in the activity of G6PDH or the level of GSH is seen. These results agree with previous studies evaluating GSH levels and suggest an overwhelmed antioxidant mechanism that may play more of a role in the host response to *H. pylori* infection in the early period post-colonisation. The present study suggests that GSH levels and OPP activity should be assessed, if possible, in *H. pylori* infected children during the initial period of infection and in asymptomatic *H. pylori* infected adults. Finally, further studies are required to indicate a definitive relationship between DOB ^{13}C values and the severity of *H. pylori* associated inflammation.

Chapter 5

The ^{13}C-Urea Breath Test: Reproducibility And Association With The Severity Of *Helicobacter pylori* Associated Antral Gastritis.

5.1: Introduction

Helicobacter pylori is the primary cause of gastritis and peptic ulcer disease and has been linked to the onset of gastric cancer (McColl and El-Omar, 1996; Marshall *et al.*, 1985; International Agency for Research on Cancer, 1994). This organism has been shown to infect more than 50% of the world's population with a prevalence of up to 80% in developing countries (Delvin *et al.*, 1999; Catherton, 1997). The need for a simple, non-invasive test for the detection of *H. pylori* infection was met by the development of the urea breath test (Logan *et al.*, 1991). This test allowed infection status to be known without the need for costly and invasive gastroduodenoscopy. The urea breath tests have been refined with the introduction of test meals to delay gastric emptying (Perri et al., 1996), shortened sampling periods (Klein *et al.*, 1996) and with the introduction of a stable isotope (^{13}C) alternative to radioactive ^{14}C-urea (Logan *et al.*, 1991). With a sensitivity of 98% and a specificity of 97% (Logan, 1993), the ^{13}C-UBT is rapidly becoming the test of choice in determining the efficacy of *H. pylori* eradication regimens following antibiotic therapy.

At present, many strategies exist for the treatment of *H. pylori* infection, however, increased resistance to current antibiotics is beginning to highly impair our ability to eradicate this organism (Opekun *et al.*, 1999). There is a need for the introduction of

novel therapies against *H. pylori* that are without the bacterial resistance experienced with current antibiotics. Furthermore, there is a requirement for a non-invasive method of assessing the efficacy of anti-*H. pylori* therapies during trials without the need for endoscopy. Hence, this study has investigated the ability of the ^{13}C-UBT to assess *H. pylori* infection and associated disease in both asymptomatic and symptomatic individuals.

Subjects undergoing a ^{13}C-UBT swallow an isotopically labelled (^{13}C) urea tablet that is hydrolysed to ammonia and labelled CO_2 by *H. pylori* urease activity (Klein *et al.*, 1996; Cutler *et al.*, 1995; Logan *et al.*, 1991). Exhaled labelled CO_2 is then detected in the breath of patients, by isotope ratio mass spectrometry. Recently it has been shown that the ^{13}C-UBT can be used as a non-invasive tool to predict *H. pylori* load *in vivo* (Perri *et al.*, 1998; Ellenrieder *et al.*, 1997). Excess exhaled $^{13}CO_2$ was found to correlate significantly with a measurement of intragastric bacterial load. This suggests that the ^{13}C-UBT has the potential to provide a non-invasive index of bacterial growth in addition to assessing the efficacy of therapeutic agents in infected populations.

Quantitative assessment of the ^{13}C-UBT requires both the inter and intra-subject variability of the test to be assessed. We have aimed to assess the intraindividual variation of the ^{13}C-UBT in a group of asymptomatic *H. pylori* positive individuals. Additionally, we compared the level of excess exhaled $^{13}CO_2$, 30 minutes following the ingestion of ^{13}C-urea, to a measure of *H. pylori* load, the severity of *H. pylori* associated gastritis and *H. pylori* urease activity in a cohort of symptomatic patients.

5.2: Methods

5.2.1: *Subjects*

5.2.1.1 *Asymptomatic subjects*

Sixty-five healthy, asymptomatic volunteers randomly selected from the population aged 54.2 ± 2.0 years (mean ± SEM; range 20 to 86; 24 males: 41 females) were involved in this study. All subjects were initially screened for *H. pylori* infection by ^{13}C-UBT after an overnight fast. Those subjects determined to be *H. pylori* positive were requested to undertake the ^{13}C-UBT on 5 further occasions over a 2 week period.

5.2.1.2 *Symptomatic Subjects*

This study also recruited 119 symptomatic patients aged 57.2 ± 1.8 years (mean ± SEM; range 16 to 91; 61 males: 58 females) referred to The Queen Elizabeth Hospital gastroenterology unit for diagnostic upper gastrointestinal endoscopy. All patients had fasted overnight (no food and only small amounts of water). Inclusion criteria were chronic abdominal pain and/or other gastroesophageal symptoms (reflux, dysphagia, dyspepsia, pyrosis or odynophagia). Patients were excluded if having received antibiotics in the preceding four weeks. Informed consent was obtained from all subjects and the study was approved by the Ethics Committees of the Women's and Children's Hospital and The Queen Elizabeth Hospital.

5.2.2: *Endoscopy*

Patients were intravenously sedated followed immediately by gastroduodenoscopy. During endoscopy, multiple biopsies (≥ 4) of gastric mucosa one to three millimetres in area were obtained from the gastric antrum using flexible forceps. Following endoscopy, patients were required to fast for at least a further 60 minutes before undergoing a ^{13}C-UBT carried out by the investigator.

5.2.3: *^{13}C-urea breath test*

The HELICO-BT™, a ^{13}C-UBT devised by the Gastroenterology Unit of the Women's and Children's Hospital, was used to assess *H. pylori* infection in this study. This test was undertaken using the same protocol as previously outlined in section 4.2.3. Briefly, the breath test involved the ingestion of 100 mg of ^{13}C-labelled urea (Cambridge Isotope Laboratories Inc, Massachusetts, USA) dissolved in warm water and ingested with 200 mL of a Vitafresh® lemon-barley drink containing citric acid. A baseline breath sample was collected and all subjects were then required to refrain from any activity for 30 minutes before exhaling into duplicate breath collection tubes (Exetainer, Labco, High Wycombe, England). These were then stored at room temperature until analysis. Breath samples were analysed as outlined in section 4.2.3. A positive test was defined as a breath sample with a DOB ^{13}C of 5 and above (Kindermann *et al.*, 2000).

5.2.4: *Histology*

Antral biopsy specimens were assessed for inflammation and *H. pylori* infection by

experienced histopathologists using the Sydney System for the grading of gastritis (Dixon *et al.*, 1996). Gastric inflammation was classified as normal, mild, moderate or severe. Histopathology results were then subdivided into three major groupings i.) *H. pylori* negative individuals with normal histological appearance, ii.) *H. pylori* positive individuals with a mild inflammation, and iii.) *H. pylori* positive individuals with a moderate to severe gastric inflammation.

5.2.5: *Bacterial culture*

One biopsy was placed in 100 µl of saline and transported to the Microbiology department of the Women's and Children's Hospital to be cultured for *H. pylori*. All biopsies were initially weighed and then macerated using a sterile scalpel. A 1 in 10 dilution of the specimen was made using phosphate buffered saline (PBS). Ten µl of the PBS dilution was inoculated onto chocolate agar and *Helicobacter* agar (Columbia and 7.5% lysed horse blood) plates. A second 1 in 10 dilution was made from the previous dilution and plated onto agar plates as described. All plates were then incubated at 37°C under microaerophilic conditions (10% CO_2, 7% O_2) for 4 days. *H. pylori* growth was confirmed by urease test, oxidase test and Gram staining. *H. pylori* colony forming units were also counted.

5.2.6: *Urease activity assay*

Bacterial isolates from *H. pylori* positive individuals were subcultured on chocolate agar and incubated as per the method described previously. Cultures were then diluted in PBS

to obtain a *H. pylori* suspension at an initial concentration equivalent to a 1 McFarland turbidity standard (10^9 cfu/ml). Twenty µl of the *H. pylori* suspension was inoculated, in quadruplicate, into wells of an ELISA plate containing 80 µl of a urea reagent (Hazell *et al.*, 1987). Urease activity was then measured spectrophotometrically at 630nm using a plate reader (Dynatech MR 7000) half hourly for 300 minutes.

5.2.7: *Statistics*

Coefficients of variation (CV) were calculated from standard deviations of the mean from the 6 breath tests undertaken by asymptomatic subjects. Also, asymptomatic patient data were analysed using sample size determination statistics to determine the ability of the ^{13}C-urea breath test to detect a change in the level of *H. pylori* infection. Data from symptomatic patients were analysed using regression analysis and/or Students t-tests. All values were reported as mean ± SEM. Statistical significance was assumed with a P value < 0.05.

5.3: Results

5.3.1: *Subjects*

5.3.1.1 *Asymptomatic Subjects*

Twenty-one of the 65 (32%) asymptomatic subjects of mean age 59.7 ± 3.4 years (mean ± SEM; range 20 to 86; 10 men and 11 women) were determined to be *H. pylori* positive by the ^{13}C-UBT. Subjects classified as *H. pylori* positive had mean DOB ^{13}C of 23.6 ± 1.1 (mean ± SEM; range 5 to 60.58). Subjects classified as *H. pylori* negative had mean DOB ^{13}C of 1.06 ± 0.1 (mean ± SEM; range -0.16 to 2.9).

5.3.2: *Variability of DOB ^{13}C*

DOB ^{13}C did not significantly vary over the 2 weeks of testing (P = 0.07; Table 1). The intraindividual CV of the ^{13}C-UBT was calculated to be 26.8 ± 1.6% (mean ± SEM). Variation in the ^{13}C-UBT results were observed to be marginally greater in those individuals with a mean DOB ^{13}C of between 5 and 15 (CV = 34.8 ± 2.3%) compared with DOB ^{13}C ≥ 15 (CV = 24.9 ± 1.5%). Sample size determination statistics were only carried out on data from those individuals with a mean DOB ^{13}C of greater than 15. Statistics (from a mean DOB ^{13}C of six ^{13}C-UBT's) indicated that groups of 12 or more individuals with a DOB ^{13}C ≥ 15 would be sufficient to detect a 33% change in DOB ^{13}C at a significance of 0.05 and a power of 0.9. It was also determined that mean DOB ^{13}C values from two breath tests were highly correlated to mean DOB ^{13}C values from four separate breath tests (P < 0.0001; r = 0.94; Figure 21).

Table 1. Mean group DOB ^{13}C over the two week period of testing in asymptomatic *H. pylori* positive subjects.

	Breath test number over two week period of testing						
	1	2	3	4	5	6	P value
Mean DOB ^{13}C	22.936	22.322	23.313	21.266	27.024	24.508	**P > 0.05**
SEM	2.5753	2.4259	2.8874	2.6529	2.9604	3.0555	

Data are shown as mean ± SEM. No significant variation in mean DOB ^{13}C was observed over the two week period of testing in asymptomatic individuals ($P > 0.05$; $n = 21$). Means were compared using an analysis of variance (ANOVA).

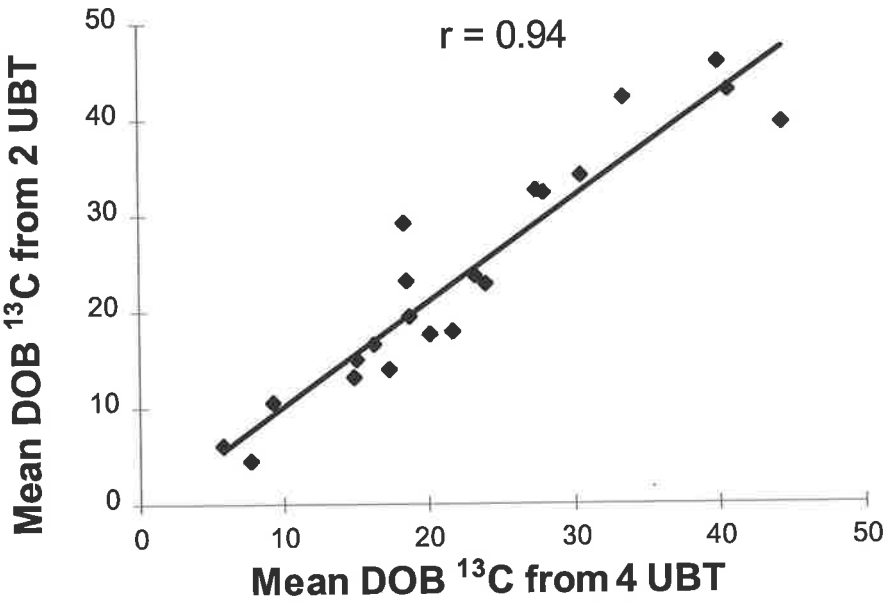

Figure 21: A correlation between mean DOB ^{13}C value from four ^{13}C-urea breath tests and mean DOB ^{13}C value from a separate two ^{13}C-urea breath tests (n = 21). A correlation coefficient of r = 0.94 was obtained ($P < 0.0001$).

5.3.3: *Symptomatic Subjects*

One hundred and nineteen patients with mean age 57.2 ± 1.8 years (mean ± SEM; range 16 to 91 years) attending the gastroenterology unit of The Queen Elizabeth Hospital were included in this study.

5.3.4: *^{13}C-urea breath test*

One hundred and six patients (89%) were ^{13}C-urea breath tested. Thirty-nine (36.8%) patients, of mean age 57.9 ± 3.0 years (mean ± SEM; range 16 to 91; 20 men and 19 women), were determined to be *H. pylori* positive by ^{13}C-UBT. Patients classified as *H. pylori* positive had mean DOB ^{13}C of 25.4 ± 3.2 (mean ± SEM; range 5.35 to 106.7). Patients classified as *H. pylori* negative had a mean DOB ^{13}C of 1.16 ± 0.1 (mean ± SEM; range -0.07 to 3.58). One patient was falsely negative on ^{13}C-UBT but positive on all other tests (DOB ^{13}C = 2.41).

5.3.5: *Histology*

Antral biopsies were obtained from 110 (92%) patients. Twenty-eight patients (25.5%) were determined to be *H. pylori* positive by histological analysis (Institute of Medical and Veterinary Science, The Queen Elizabeth Hospital, Woodville, S.A.). Mean DOB ^{13}C was significantly higher in individuals with a mild gastritis (17.7 ± 2.8; n = 14) compared to individuals with no evident inflammation (1.77 ± 0.13; n = 44; $P < 0.001$; Figure 22). DOB ^{13}C in those individuals with a moderate to severe gastritis (34.5 ± 4.4; n = 13) was significantly higher than those individuals with a mild gastritis (17.7 ± 2.8; n = 14; P =

.002; Figure 22). One patient was not included in this analysis as the person could not be placed into a specific gastritis severity group due to an ill-defined histopathology report.

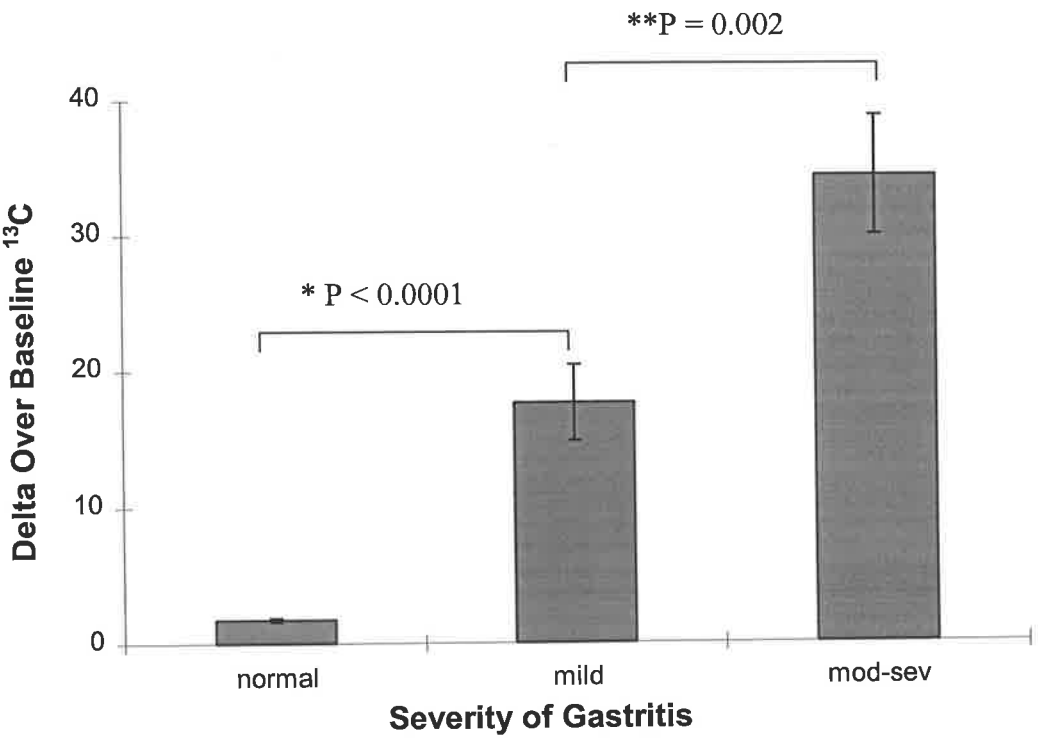

Figure 22: Severity of *H. pylori* associated antral gastritis compared to mean DOB ^{13}C value. Mean DOB ^{13}C was significantly higher in individuals with a mild gastritis (17.7 ± 2.8; n = 14) compared to the *H. pylori* negative individuals with no evident inflammation (1.77 ± 0.13; n = 44; *P < 0.0001). DOB ^{13}C in those individuals with a moderate to severe gastritis (34.5 ± 4.4; n = 13) was significantly higher than those individuals with a mild gastritis (17.7 ± 2.8; n = 14; **P = 0.002). Means were compared using a two-way Student's t-test.

5.3.6: *Bacterial culture*

Antral biopsies were taken from 105 (88%) patients to be cultured at the Microbiology Department of the Women's and Children's Hospital. Thirty-one (29.5%) were determined to be *H. pylori* positive. No significant correlation was found between bacterial load, measured by colony forming unit per gram (cfu/g), and DOB ^{13}C ($r = 0.04$; $P > 0.05$; Figure 23).

5.3.7: Urease activity

A total of 25 antral biopsy isolates were subcultured for the urease activity assay. However, *H. pylori* growth was only observed on 12 of these plates. The lack of *H. pylori* growth is assumed to have been a result of freezing the biopsies in the period prior to the urease activity assay. Urease activities of the remaining tissue samples ($n = 12$) showed a moderate correlation with DOB ^{13}C ($r = 0.55$; $P = 0.6$; Figure 24).

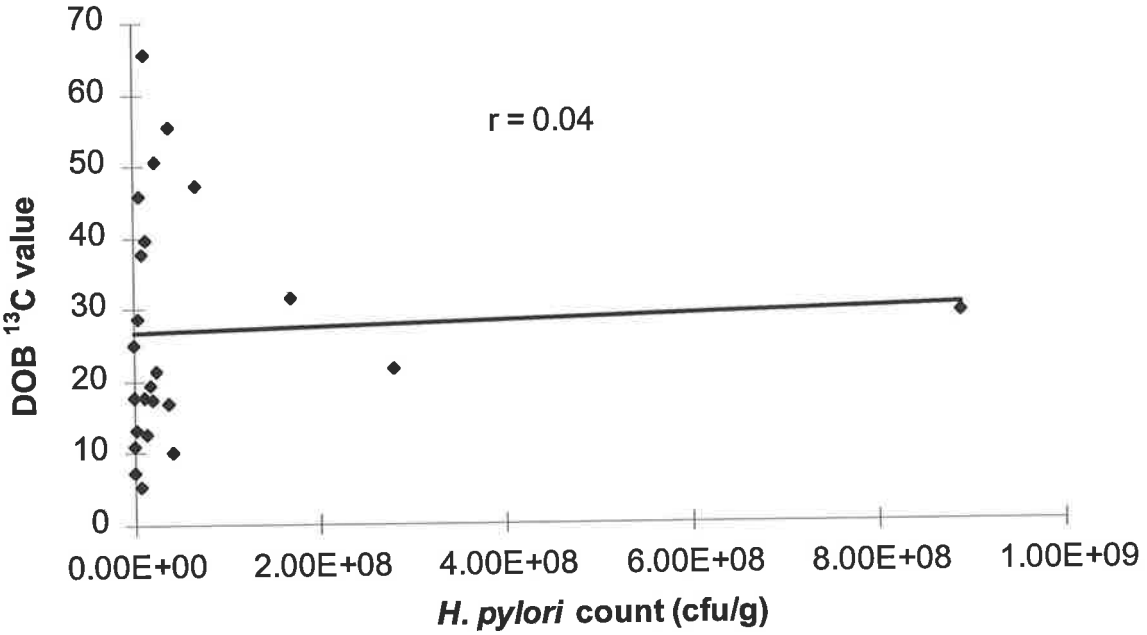

Figure 23: A correlation between DOB ^{13}C value from the ^{13}C-urea breath test and *H. pylori* count (cfu/g) (n = 31). No significant correlation was obtained (r = 0.04; P > 0.05).

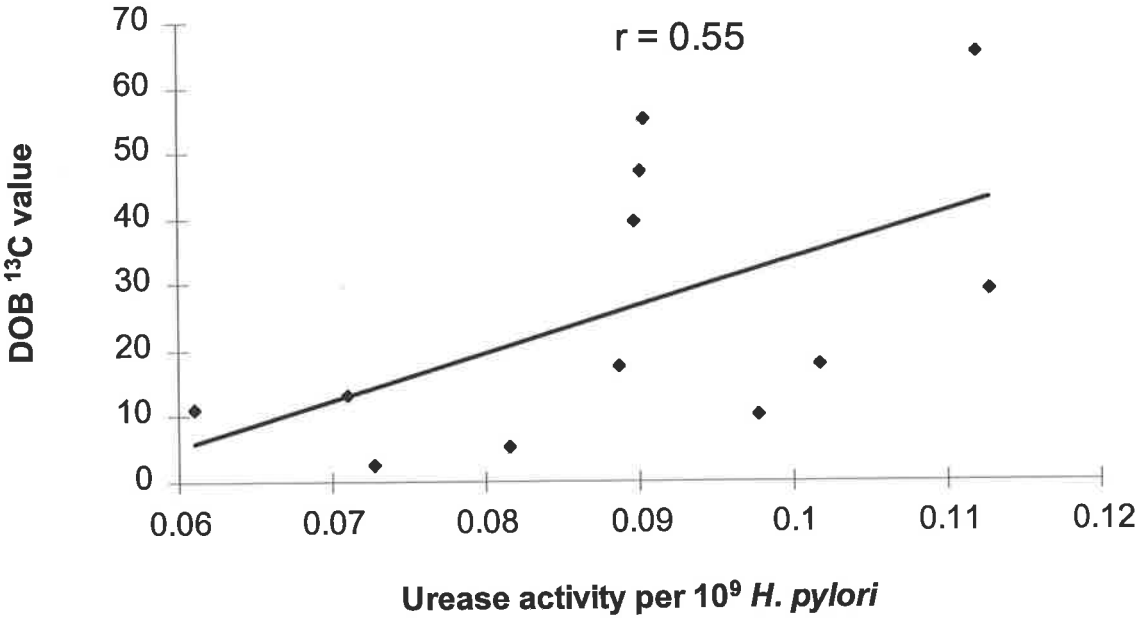

Figure 24: A correlation between DOB ^{13}C value and the urease activity of isolated *H. pylori* from symptomatic patients (n = 12). A trend towards a significant moderate correlation of r = 0.55 was obtained (P = 0.06).

5.4: Discussion

The results of this study demonstrate that the ^{13}C-UBT is a reproducible test that can reliably detect *H. pylori* infection. Mean levels of DOB ^{13}C did not significantly vary over the two week period of testing. Furthermore, it was shown that the mean DOB ^{13}C from six repeat breath tests was significantly correlated with the mean from two breath tests. This indicates that a mean DOB ^{13}C from two tests is sufficient to accurately, precisely and reliably indicate this parameter.

The low variation of the ^{13}C-UBT would permit it to be utilised as a non-invasive index of the level of *H. pylori* infection. This would allow the long term monitoring of *H. pylori* infection non-invasively in asymptomatic individuals who may develop *H. pylori* associated disease later in life. Also, the ^{13}C-UBT could provide a non-invasive index of *H. pylori* suppression and/or eradication during anti-*H. pylori* therapeutic studies where no other non-invasive measure exists. Results of this study indicate that for future therapeutic studies, groups of 12 to 15 individuals would need to be recruited in order to detect a change in the level of DOB ^{13}C of at least 33%. This would be at a significance of 0.05 and with a confidence of 0.9. During anti-*H. pylori* studies the ^{13}C-UBT would potentially indicate the efficacy of therapeutic agents where complete eradication may not be achieved.

Interestingly, it was noted that the level of DOB ^{13}C was more variable in individuals

with a DOB ^{13}C of between 5 and 15 (CV = 34.8 ± 2.3%) than those with a level greater than 15 (CV = 24.9 ± 1.5%). This possibly indicates that when utilising the ^{13}C-UBT as an index of the efficacy of anti-*H. pylori* agents, only individuals with a DOB ^{13}C of greater than 15 should be recruited. Also, a change in the level of *H. pylori* infection following therapy would be more easily detected when the pre-treatment level of DOB ^{13}C is high.

Recent studies have suggested that the ^{13}C-UBT can non-invasively measure intragastric *H. pylori* load (Perri *et al.*, 1998; Ellenrieder *et al.*, 1997). Perri *et al.*, (1998) observed that the level of DOB ^{13}C correlated significantly with bacterial load measured histologically, although this was only moderate (r = 0.32). Moreover, Ellenreider *et al.*, (1997) found a higher correlation between a semi-quantitative count of *H. pylori* density and the level of DOB ^{13}C (r = 0.56). In contrast, the current study found no correlation between *H. pylori* load, measured by bacterial culture, and the level of DOB ^{13}C (r = 0.04). It is well known that *H. pylori* colonises the antral glands (Scott *et al.*, 1998) which suggests that bacteria within the glands may not be detected by the urea breath test. Potentially, this would result in an insignificant measure of the number of bacteria by DOB ^{13}C value, and hence an incorrect prediction of the total number of intragastric bacteria.

These results may also be explained by findings of a similar study which determined that the amount of growth seen by bacterial culture did not correlate with the number of

organisms seen by light microscopy of histological sections (Logan *et al.*, 1991). Furthermore, Logan *et al.*, (1991) could not find a relationship between the level of DOB ^{13}C and growth seen by bacterial culture. It could, therefore, be suggested that the lack of a correlation observed by the current study was a result of bacterial growth on culture not being indicative of actual mucosal *H. pylori* load. However, more recently Atherton *et al.*, (1996) compared a quantitative measure of antral *H. pylori* density, by culture, to a histological count of *H. pylori* density and found a significant positive correlation ($r = 0.44$, $P = 0.027$). This is still a small correlation and cannot, therefore, negate our finding. Without a histological measure of bacterial density the current study can simply conclude that no relationship exists between the value of DOB ^{13}C and the *H. pylori* density measured by the method of bacterial culture utilised within this study.

In contrast, a pertinent finding by the current study was the obvious relationship between the DOB ^{13}C value and the severity of antral gastritis. Perri *et al.*, (1998) observed a relationship between DOB ^{13}C and the depth of antral gastritis ($P = 0.036$), but not the severity of gastritis in *H. pylori* positive individuals. The depth of gastritis is more a measure of the chronicity of the infection rather than its severity. Hence, the current study has furthered these findings by indicating that the ^{13}C-UBT is able to measure the severity of the antral inflammation caused by *H. pylori* infection. These findings suggest that the ^{13}C-UBT has the potential to act as an index of *H. pylori* associated disease. This may allow the non-invasive assessment of the efficacy of therapeutic agents against *H. pylori* which may lead to the resolution of gastritis within human studies. However, further

studies are indicated to determine the ability of the ^{13}C-UBT to assess the resolution of gastritis during therapeutic studies.

The relationship between the DOB ^{13}C value and the severity of gastritis is interesting due to the lack of a relationship between bacterial load and the DOB ^{13}C value discussed above. It could be hypothesised that the severity of the inflammation is a more reliable measure of the virulence of the organism than a bacterial count. Furthermore, it is possible that another factor, such as the urease activity (Mirshahi *et al.*, 1998; Ellenrieder *et al.*, 1997), may be more closely related to the virulence of the organism, and hence the DOB ^{13}C value, than the total bacterial count.

The present study investigated this hypothesis by assessing the urease activity of the bacteria isolated from different individuals within the study. When the urease activities were compared to the corresponding DOB ^{13}C value of each individual, a moderate correlation was observed. The level of urease activity measured *in vitro* may not be indicative of total intragastric *H. pylori* urease activity due to focal sampling from biopsies that does not represent the entire gastric surface area, unlike the ^{13}C-UBT. However, this correlation is promising and, with further studies, may eventually provide a non-invasive determinant of the relative level of urease activity *in vitro* that can then be related to the total intragastric activity. Therefore, this is the first study to investigate the correlation between a direct measure of urease activity of individual isolates to the results of the DOB ^{13}C value.

A recent study suggested that the urease activity of *H. pylori*, measured by ^{13}C-UBT, is inversely associated with the success of treatment regimens ([a]Suzuki *et al.*, 1998). Hence, the current study indicates that the level of DOB ^{13}C is moderately related to the level of urease activity *in vitro* and, therefore, has the potential to be utilised in a clinical setting for efficacy of therapies along with determination of the likelihood of success of therapy prior to treatment.

In conclusion, the present study has shown that the ^{13}C-urea breath test is a reliable and reproducible determinant of *H. pylori* infection and has the potential to provide a non-invasive alternative to endoscopy during human therapeutic studies. Further work is needed to determine a conclusive relationship between intragastric *H. pylori* urease activity and the DOB ^{13}C value. However, the high correlation between DOB ^{13}C levels and the severity of antral inflammation suggests that the ^{13}C-urea breath test is already a very powerful test that is simple, accurate and non-invasive and able to determine the severity of *H. pylori* associated disease without the need for more invasive procedures.

6.0 DISCUSSION/ CONCLUSIONS

6.1: *Background*

This thesis has investigated the role of the oxidative pentose phosphate pathway (OPP) in the host mucosal response to *Helicobacter pylori* infection in a mouse model and in symptomatic patients. The animal model of *H. pylori* infection, the SS1 infected C57BL/6 strain of mouse, was originally developed by Lee *et al.*, (1997). This model does not develop early active chronic gastritis that is characteristic of the human mucosal response to this organism. A significant mucosal response to *H. pylori* does not develop in the *H. pylori* infected mouse until approximately six to eight months following infection. This suggests that there may be an early change, or event, that occurs within the gastric mucosa following *H. pylori* infection that defends the mucosa for a long period in the mouse. However, it is proposed that this event fails to counteract the oxidant stress in the adult patient. It was hypothesised that the OPP would play an important role in this early event in relation to its role as a major provider of NADPH for the synthesis of GSH (Mehta *et al.*, 1998), an important antioxidant, that has been recently shown to be involved in the mucosal response to *H. pylori* (Santra *et al.*, 2000; Suzuki *et al.*, 1999; Yajima *et al.*, 1999; Farinati *et al.*, 1996). It was also hypothesised that the late inflammatory response of the mouse model to *H. pylori* infection would allow us to compare the early mucosal events of the mouse with that of the human response of which, in the adult patients studied, was almost certainly of long standing.

A marked infiltration of neutrophils to the gastric mucosa is characteristic of the human pathological response to *H. pylori* infection (Yajima *et al.*, 1999; Sasayama *et al.*, 1997; Crabtree, 1996). Once activated, neutrophils generate ROS and it is these damaging agents that have been proposed to play a major role in the mediation of mucosal damage during *H. pylori* infection. ROS have been implicated in the process of cellular damage in other gastrointestinal diseases, such as inflammatory bowel disease, where they cause damage by membrane lipid peroxidation (Santra *et al.*, 2000; Antionetta *et al.*, 1999; Drake *et al.*, 1998; Sedghi *et al.*, 1994).

Yajima *et al.*, (1999) proposed that the gastric mucosal damage caused by *H. pylori* was mainly mediated by the production of the toxic monochloramine (NH_2Cl), a highly reactive, low molecular weight and lipophilic molecule. The production of NH_2Cl results from the reaction between NH_3, produced by *H. pylori* urease, and hypochlorous acid (HOCl), produced by the oxidation of Cl^- by H_2O_2, a ROS. NH_2Cl was shown to cause cellular damage to cultured gastric cells at concentrations that are similar to those produced by activated neutrophils *in vivo*. Yajima *et al.*, (1999) also investigated the ability of the cellular antioxidant defence mechanism to prevent NH_2Cl mediated damage. It was shown that treatment of cultured cells with extracellular GSH, that has been shown to increase intracellular GSH levels (Hiraishi *et al.*, 1994), caused the attenuation of damage mediated by NH_2Cl. Furthermore, when intracellular GSH levels were depleted, the cells were less resistant to NH_2Cl mediated damage. This study highlights the role of the cellular antioxidant defense system in the mucosal response to *H. pylori* infection.

The present study followed the gastric mucosal response to *H. pylori* infection in the *H. pylori* infected mouse model for six months to investigate changes to three main parameters; namely, the activity of the OPP, GSH availability and MPO activity. A concurrent study of humans investigated the same three parameters in *H. pylori* infected patients. These studies were carried out to determine differences between the host mucosal response to *H. pylori* infection of the mouse and human patients. Results show that in the *H. pylori* infected mouse, G6PDH activity and GSH levels are increased whereas they are unchanged in *H. pylori* infected patients. In contrast, MPO activity was highly increased in the mucosa of *H. pylori* infected patients but is only slightly increased in the gastric mucosa of the *H. pylori* mouse model and this returns to normal levels after four months of infection.

6.2: *Host response studies*

Activity of the OPP was measured within these studies by the assessment of G6PDH activity, the rate limiting enzyme of the OPP (Mehta *et al.*, 1998; Kletzien ~~and Harris~~ et al., 1994). It was observed in the *H. pylori* infected mouse that G6PDH activity was significantly increased after one month of infection and further increased at four and six months when compared to non-infected animals of the same age. G6PDH activity showed a downward trend from four to six months of infection, however this was not significant. These observations indicate an increased activity of the OPP in response to *H. pylori* infection in the mouse. In contrast, G6PDH activity in *H. pylori* infected and non-infected patients was not significantly different. This suggests that the activity of the OPP is possibly increased early during *H. pylori* infection but is down-regulated as the infection

continues. If the activity of the OPP had been followed for a longer period in the mouse, the observed trend downwards may have possibly become significant. Therefore, it is possible that G6PDH activity may eventually reach normal levels in the mouse, paralleling the activity seen in samples from adult patients.

The unchanged OPP activity in *H. pylori* infected patients was associated with normal levels of GSH. These results differ from a recent study which investigated the role of oxidative stress during *H. pylori* infection and showed GSH levels were depleted (Santra *et al.*, 2000). It was suggested that a depletion in GSH availability was indicative of a failure of the mucosal antioxidant system to counteract the oxidant stress produced during *H. pylori* infection. It is possible that initial *H. pylori* infection in humans is associated with an initial increase in OPP activity and GSH levels similar to the mouse, but this fails due to increased GSH utilisation by ROS. Potentially, this could be investigated by assessing the mucosal response to *H. pylori* infection early in the period following infection in humans, for example in children. It is currently accepted that *H. pylori* infection is acquired during childhood and that primary infections in adults are rare (Rowland *et al.*, 1999). However, the mean age of patients within this study was 57 years inferring that many may have been infected for over 50 years. Therefore, an investigation of the early mucosal events that occur in *H. pylori* infected humans could only be evaluated in young subjects.

The increased OPP activity and concomitant GSH levels in the *H. pylori* infected mouse appears to prevent the infiltration and activation of neutrophils to the gastric mucosa. A

small significant increase in MPO activity was observed one month post-infection, however, this had decreased to normal levels by four and six months of infection. This may be explained by the continued increase in G6PDH activity by four and six months of infection providing a continued supply of NADPH to GSH reductase for GSH synthesis and recycling. The availability of GSH at one month of infection was at its highest levels and slowly decreased by six months of infection, although not to normal, non-infected levels. It is suggested that the decrease in GSH levels is associated with an increased utilisation of the intracellular antioxidant by the continued oxidant load placed on the mucosa during *H. pylori* infection. The small initial increase in G6PDH activity observed by one month of infection may indicate the increased need to up-regulate the synthesis of GSH to the degree seen in this study. Hence, the continued increase in G6PDH activity may infer the continued need to counteract the oxidant stress at increasing levels as the duration of infection continues. Alternatively, the decreased GSH availability by six months of infection may be a result of a decrease in the rate of its synthesis.

Although up-regulated G6PDH activity is potentially able to provide NADPH for increased GSH synthesis, this study did not investigate the rate of GSH synthesis. An investigation of glutathione reductase (GR), the rate limiting enzyme for GSH synthesis, would assess the rate of GSH synthesis (Mehta *et al.*, 1998). This would indicate whether the GSH levels were decreased due to increased utilisation or due to decreased synthesis. For example, Mehta *et al.* (1998) demonstrated decreased GSH levels following *Salmonella typhimurium* infection but showed that this was associated with a decrease in the activity of GR. Measurement of oxidised glutathione (GSSG) would also be useful in

determining the levels of GSH utilisation during *H. pylori* infection. Farinati *et al.*, (1996) demonstrated unchanged GSH levels but an increased rate of glutathione turnover in patients infected with *H. pylori*. This suggests a failure of the GSH mediated mucosal antioxidant defence system to counteract the oxidant stress caused by the organism. Potentially, a failure of the mucosal antioxidant defence mechanism would allow further infiltration and activation of neutrophils to the gastric mucosa resulting in *H. pylori* associated mucosal damage and disease.

Studies ~~within this thesis~~ suggest that an increased OPP activity and concomitantly increased GSH levels are important factors mediating the mucosal response to *H. pylori* infection. In the mouse, these factors appear to act as an effective first line defence mechanism preventing, or lowering, the level of neutrophil infiltration to the gastric mucosa. Potentially, this could prevent ROS mediated damage. However, it appears that this defence mechanism begins to fail after approximately 8 months of infection after which a significant mucosal pathology is observed (Lee *et al.*, 1997). In the adult human infection, the mucosal defence mechanism appears less effective. It is suggested that a failure of the human mucosal OPP and GSH defence system to effectively defend the mucosa against ROS results in the mucosal inflammation and damage that is associated with *H. pylori* infection. Recent studies have focussed on the role of Nuclear Factor-κB (NF-κB) in the mucosal inflammatory response to *H. pylori* infection (Isomoto *et al.*, 2000; Kim *et al.*, 2000).

It is suggested that a mucosal pathology develops during *H. pylori* infection through

activation of NF-κB, an important transcription factor that controls the expression of many genes involved in immune and inflammatory responses, including IL-8 (Isomoto *et al.*, 2000; Kim *et al.*, 2000). IL-8 is a potent chemokine that is involved in the recruitment of neutrophils and its levels in the gastric mucosa are seen to be significantly increased during *H. pylori* infection (Yamaoka *et al.*, 1999; Yamaoka *et al.*, 1998; Crowe *et al.*, 1995). Moreover, it has been shown that *H. pylori* is able to activate NF-κB suggesting that it is through this pathway that a mucosal immune and inflammatory response to *H. pylori* infection is mediated.

NF-κB activation is primarily induced by oxidative stress (Isomoto *et al.*, 2000; Kim *et al.*, 2000) suggesting that antioxidant activity may be beneficial in the treatment of *H. pylori* associated gastric mucosal damage. Kim *et al.*, (2000) investigated the effects of rebamipide, an anti-ulcer agent that also has antioxidant properties, on gastric mucosal NF-κB activation and IL-8 production. As hypothesised, rebamipide inhibited NF-κB activation, thereby decreasing IL-8 production during *H. pylori* infection. Therefore, it is suggested by the present study that gastric mucosal GSH acts to prevent an inflammatory response of the mucosa in the early stages of *H. pylori* infection by preventing the oxidant induced activation of NF-κB (Figure 25 & 26).

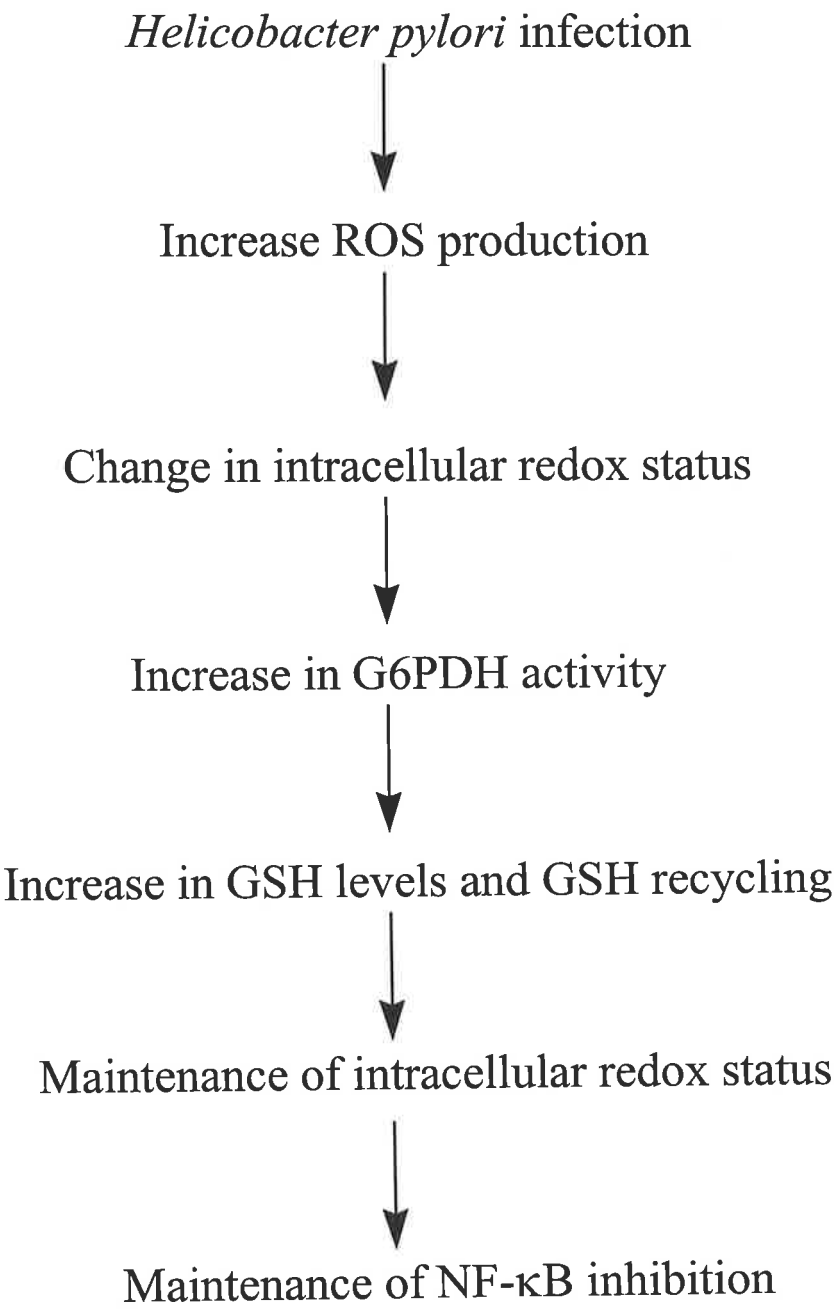

Figure 25: Proposed early events in the gastric mucosa of *Helicobacter pylori* infected mice in regard to OPP activity, GSH levels and NF-κB regulation.

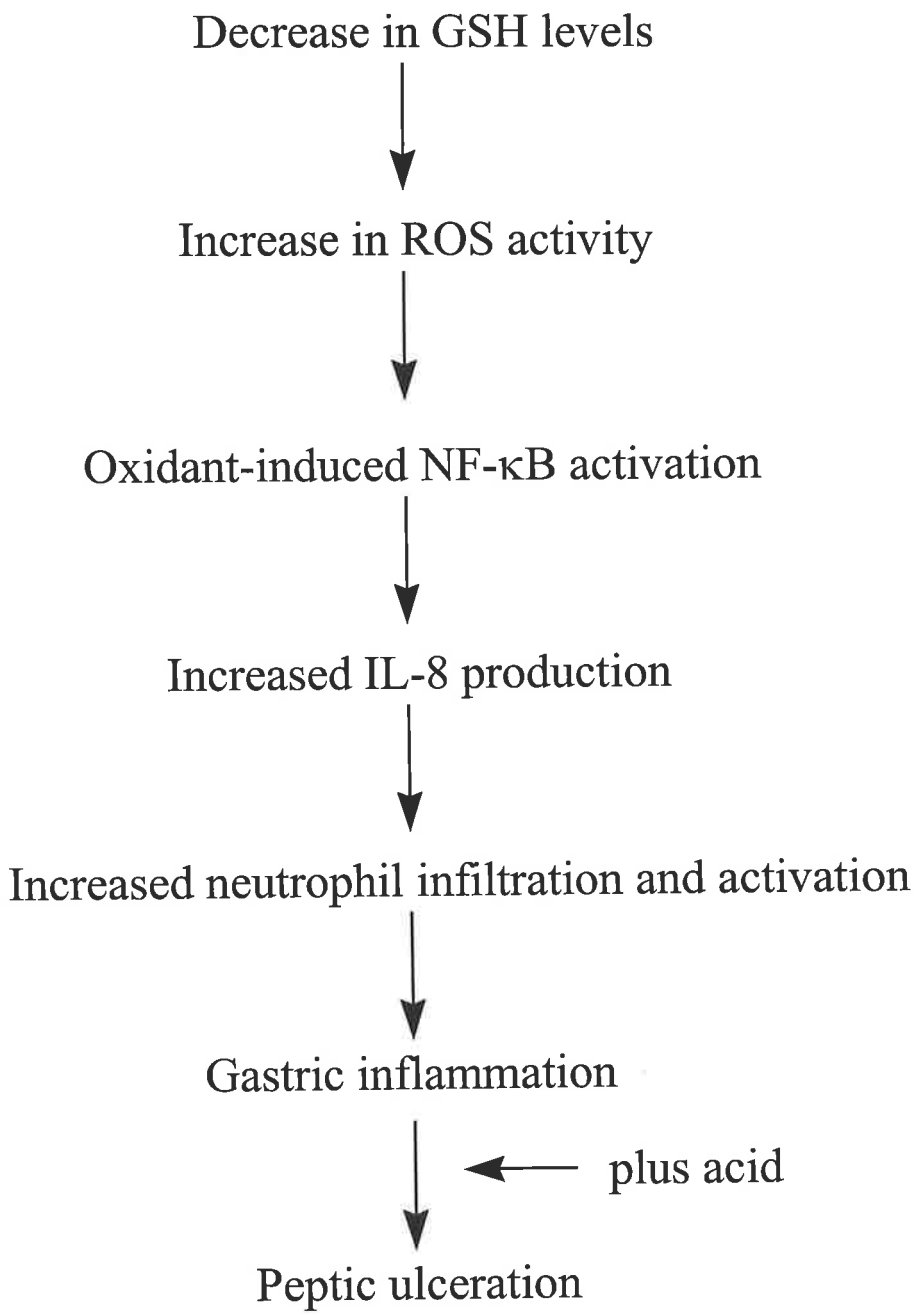

Figure 26: Proposed gastric mucosal events following decreased GSH levels during chronic *H. pylori* infection in regard to ROS activity, NF-κB activation and IL-8 secretion.

6.3: *Treatment studies*

Given the results of the present study, it was hypothesised that regulating the activity of the OPP and/or the levels of GSH would prove to be clinically useful therapy for treating *H. pylori* associated disease. Potentially, increasing the levels of gastric mucosal GSH may prevent an inflammatory response during *H. pylori* infection. This hypothesis was investigated by evaluating the ability of two novel agents to alter the activity of the OPP and/or GSH availability in both non-infected and *H. pylori* infected mice.

Oxythiamine treatment

Butler *et al.*, (1993) showed that inhibition of the OPP by dehydroepiandrosterone (DHEA) led to an up-regulation of the NOPP in normal rat colonic epithelium. Furthermore, Banki *et al.*, (1996) showed that suppressing the expression of a key enzyme of the NOPP, transaldolase, led to the increased expression of G6PDH and a concomitant increase in GSH levels. Therefore, it was hypothesised by the present study that inhibition of another key enzyme of the NOPP would lead to an increase in GSH synthesis via a compensatory up-regulation of the OPP in gastric mucosal cells. This would possibly result in an increased ability of the antioxidant defence system to counteract the oxidant load produced during *H. pylori* infection.

Oxythiamine, an inhibitor of a key enzyme of the NOPP, transketolase, was administered orally to a group of both non-infected and *H. pylori* infected mice. It was observed that oxythiamine did not alter gastric mucosal G6PDH activity in non-infected mice but significantly reduced this parameter in *H. pylori* infected mice. Recent studies

investigating the role of the pentose phosphate pathway in tumour proliferation have observed that the OPP is significantly increased within cancer cells *in vitro* (Rais *et al.*, 1999, Boros *et al.*, 1997). More importantly, these studies have shown that inhibition of the NOPP within cancer cells by oxythiamine led to a significant inhibition of the OPP. Therefore, the results of this thesis and those of other studies suggest that in cells where OPP activity is otherwise increased, such as during *H. pylori* infection and in tumour cells, oxythiamine will act to inhibit the NOPP leading to the inhibition of the OPP.

Interestingly, oxythiamine administration was noted to significantly increase both GSH levels and MPO activity in non-infected mice, but had no effect in *H. pylori* infected mice. It was suggested that the increase in GSH levels may have resulted from the utilisation of stored NADPH, or NADPH from another source, rather than being provided by the OPP. It is possible that in non-infected animals, oxythiamine causes mucosal damage and this may be supported by the significant increase in mucosal MPO activity seen in non-infected mice after its administration. This suggests that the increase in GSH levels may have been a mucosal response to the damage caused by oxythiamine. However, without the provision of NADPH by the OPP, another source of reducing equivalents must have been utilised.

A recent study suggested that oxythiamine could be utilised as an anti-*H. pylori* agent by inhibiting the bacterial pentose phosphate pathway (Butler and Tan, 2000). It was observed that administration of oxythiamine *in vitro* led to a significant suppression of the organism possibly by preventing the synthesis of bacterial ribose-5-phosphate (refer

Figure 1). Therefore, it may be suggested that oxythiamine is utilised by *H. pylori* before it is able to have any damaging effects on the gastric mucosa. Potentially, this would prevent an increase in G6PDH activity and any change to GSH levels as were seen in the present study. Therefore, it is suggested that future investigations focus on the potential ability of oxythiamine to inhibit the activity of the pentose phosphate pathway of *H. pylori*.

6.3.2

N-acetylcysteine treatment

A second novel therapy for *H. pylori* associated disease investigated in this thesis was the oral administration of NAC, a therapeutic agent currently used for the treatment of oxidant drug ingestion (Geuvara *et al.*, 2000; Wernerman and Hammarqvist, 1999). NAC is an antioxidant due to its capacity to scavenge ROS. It is also a precursor of GSH because of its ability to provide cysteine for the initial step in GSH synthesis. NAC administration has been shown to replenish GSH availability in HIV patients and improve GSH status in healthy individuals (Wernerman and Hammarqvist, 1999). Therefore, it was hypothesised that NAC would provide a novel treatment for *H. pylori* associated disease by acting as a precursor for GSH synthesis during infection. Potentially, increasing gastric mucosal GSH levels will prevent, or lessen, the mucosal damage seen during *H. pylori* infection.

NAC administration did not affect G6PDH activity in non-infected mice although it was significantly decreased post-treatment in *H. pylori* infected mice. Treatment with NAC led to a significant increase in GSH availability in non-infected animals but led to lower

GSH levels in *H. pylori* infected mice. MPO activity was significantly lower in *H. pylori* infected animals when compared to *H. pylori* infected control animals. These results suggest that NAC acted as a precursor of GSH in non-infected mice resulting in increased GSH levels. However, during *H. pylori* infection, NAC treatment was not effective in maintaining GSH levels perhaps because of its ability to scavenge oxidants produced during *H. pylori* infection, thereby preventing them from causing the mucosal events seen during untreated *H. pylori* infection. Moreover, it appears that NAC diminishes the requirement of the mucosa to mount the first line antioxidant defence mechanism which was observed during untreated *H. pylori* infection within this study.

It is also suggested that NAC may act via inhibition of the transcription factor, NF-κB, which is involved in the expression of many immune and inflammatory genes (Isomoto *et al.*, 2000; Kim *et al.*, 2000). This has been shown by Tanaka *et al.*, (1997). Potentially, NAC would inhibit *H. pylori* induced cytokine production, such as IL-8, by preventing the oxidant mediated activation of NF-κB. It could be proposed that if this was to occur then GSH levels would still be increased due to the oxidant load produced during *H. pylori* infection. However, the lack of an increase in GSH levels observed within this study suggests that NAC acts as both an antioxidant and an inhibitor of NF-κB. That is, NAC does not only act to inhibit NF-κB in this model of *H. pylori* infection.

Decreased G6PDH activity and GSH availability seen in *H. pylori* infected mice following NAC treatment may also have occurred as a result of NAC decreasing the

bacterial load within the mucosa. Recently, Huynh *et al.*, (2000) showed that NAC treatment in mice led to a significant reduction in bacterial load. Potentially, a reduction in bacterial load would lead to a decrease in the levels of oxidant stress placed on the gastric mucosa during *H. pylori* infection. Bacterial counts were not made during the present study and it can, therefore, only be suggested that a change in bacterial load may partly explain the results seen. However, it appears that NAC administration could provide a novel therapy for *H. pylori* associated disease without yet knowing which property of NAC dominates during *H. pylori* infection. It is suggested that future investigations should investigate whether continued NAC treatment is able to prevent the gastritis seen by approximately eight months in the mouse model.

Bacterial enumeration could have been carried out!

6.4: *Other agents that modulate the OPP and/or GSH levels*

The results of the treatment studies indicate that future studies trialing novel therapies for *H. pylori* associated disease should focus on the utilisation of other antioxidants and also agents that stimulate the synthesis of GSH. Two such agents are dehydroscorbate (Puskas *et al.*, 2000) and DHEA (Boros *et al.*, 1997) A recent study investigated the antioxidant properties of the dehydroscorbate, the oxidised form of ascorbic acid (vitamin C), and its ability to prevent ascorbic acid mediated apoptosis (Puskas *et al.*, 2000). It was shown that dehydroscorbate not only acted as an antioxidant but also stimulated the antioxidant defence system of cells through activation of G6PDH. The increased GSH levels inhibited ascorbic acid induced apoptosis of cells. Therefore, induction of the OPP using novel agents, such as dehydroscorbate, may prove useful in the treatment for *H. pylori* associated disease.

It appears that the pentose phosphate pathway is important in modulating the gastric mucosal host response to *H. pylori* during the initial stages of infection in the mouse. It is hypothesised that inhibition of the OPP using agents such as DHEA, may prevent the induction of the first line mucosal defence mechanism. Potentially, this would allow the activation of NF-κB leading to the stimulation of IL-8 production and subsequent infiltration of neutrophils to the gastric mucosa. This may lead to the onset of pathological changes, such as gastritis and peptic ulcers. Therefore, it is suggested that the pathology of the SS1 infected mouse may mimic that of humans following inhibition of the OPP. Furthermore, prevention of GSH synthesis, by agents such as diethyl maleate, could also allow the induction of pathological changes otherwise prevented by the antioxidant defence system. This may provide a better mouse model whose response to *H. pylori* infection closely resembles that of the human response. Future studies could investigate this hypothesis.

6.5: *Future studies: The Helicobacter felis model*

It is important to consider that the infected humans within these studies were almost certainly infected with a variety of different strains of *H. pylori* which may elicit a spectrum of responses. It is known that the SS1 strain of *H. pylori* used within the mouse studies causes a different first line mucosal response to that of *Helicobacter felis* in the same strain of mouse (C57Bl/6) (Lee *et al.*, 1998; Lee *et al.*, 1999). This highlights the potential importance of virulence properties of the infecting organism.

The *H. felis* model has been shown to develop histopathological changes that closely resemble that of the human response to *H. pylori* infection (Lee *et al.*, 1990). Therefore, future studies evaluating the mucosal response to *Helicobacter* infection could also utilise the *H. felis* model (Lee, 2000; Dick-Hegedus and Lee, 1991; Lee *et al.*, 1990) in place of the *H. pylori* (SS1) model. *H. felis* was cultured from the feline stomach and has a wider host range than *H. pylori* (Lee *et al.*, 1990). Moreover, it was observed that the *H. felis* infected mouse developed an active gastritis after two weeks of infection. This later developed into active chronic gastritis by eight weeks of infection. In contrast, the *H. pylori* infected mouse model does not develop significant histopathological changes until at least six to eight months post-infection. Hence, the pathology of the *H. felis* model may reflect the pathology of the *H. pylori* infected adult and could, therefore, be utilised as a model for chronic *H. pylori* infection. As such, it is proposed that mucosal G6PDH activity, GSH levels and MPO activity in the *H. felis* model would be very similar to the *H. pylori* infected adult patient.

As a small pilot study, G6PDH activity, GSH levels and MPO activity were investigated in four *H. felis* infected mice. Mice were inoculated with *H. felis* via oro-gastric gavage and were killed after six weeks of infection to allow a significant pathology to develop. Following six weeks of *H. felis* infection, G6PDH activity was significantly increased compared to non-infected mice and this was similar to the four week *H. pylori* infected mice. Also, GSH levels were not significantly different to non-infected mice but were significantly lower than mucosal levels in the *H. pylori* infected mice. Finally, MPO activity was significantly higher in the *H. felis* mouse when compared to both the non-

infected and the four week *H. pylori* infected mouse. These results are similar to those seen in the human response to infection where there is no change, or a decrease, in GSH levels (Santra *et al.*, 2000; Farinati *et al.*, 1996). G6PDH activity was not observed to be significantly changed within the patient study of chapter 4 which is in contrast to the increased activity seen in the *H. felis* infected mucosa. It could be suggested that this may be a result of the chronicity of the infection in the patients tested (mean age of 57 years) as opposed to the relatively short (six week) length of infection in the *H. felis* infected mouse. The increased MPO activity in the *H. felis* model is similar to the results seen in the *H. pylori* infected patients.

The *H. felis* mouse model has been used to evaluate the efficacy of antibiotic drugs on *Helicobacter* eradication (Dick-Hegedus and Lee, 1991). Future studies utilising the *H. felis* mouse model could also investigate a number of factors relating to the role of the antioxidant defence system in chronic *Helicobacter* infection and the utilisation of novel treatments. Agents, such as those evaluated within this thesis, that alter the activity of the OPP and/or the levels of GSH, may provide important information into the defence properties of the OPP and GSH in adult patients. Potential studies could involve agents that cause the up-regulation of G6PDH and/ or GSH levels within the gastric mucosa. It is hypothesised that an up-regulation of both G6PDH activity and GSH levels may prevent the pathology of the *H. felis* mouse by enhancing the activity of the first line defence mechanism. Future studies could investigate this hypothesis.

6.6: *Non-invasive assessment of treatment efficacy*

The assessment of novel therapies for the treatment of *H. pylori* infection and associated disease can be easily undertaken in the mouse utilising methods such as bacterial culture and histological investigation of entire stomachs. However, during human studies only small biopsy samples of gastric mucosa can be assessed and this is not an ethically viable option for investigations in asymptomatic individuals. Previous studies have investigated the ability of the ^{13}C-urea breath test (^{13}C-UBT) to non-invasively assess *H. pylori* load (Ellenrieder *et al.*, 1997; Perri *et al.*, 1998). Perri *et al.*, (1998) observed a correlation between the results of the ^{13}C-UBT and *H. pylori* load, however, this was only small (r = 0.32). Hence, this thesis assessed the ability of the ^{13}C-UBT to assess *H. pylori* infection and associated disease in both asymptomatic and symptomatic individuals.

In order to utilise the ^{13}C-UBT in any quantitative manner the intraindividual variation of the test needed to be assessed. This was carried out in a cohort of randomly selected asymptomatic subjects with *H. pylori* infection initially determined using this test. Each subject undertook the ^{13}C-UBT six times over a two week period and results were described as change in $^{13}CO_2/^{12}CO_2$ ratio 30 minutes following ingestion of ^{13}C-labelled urea (DOB ^{13}C).

6.6.1 *Variation in DOB ^{13}C value over time*

Mean DOB ^{13}C did not significantly vary over the six tests with an intraindividual coefficient of variation (CV) of approximately 26%. The moderately high CV of 26% could be due to variation in the level of *H. pylori* infection over the two weeks of testing,

although it is unknown whether the bacterial load and/or the severity of *H. pylori* associated disease changes over time.

DOB ^{13}C value vs. severity of antral gastritis

The most interesting result from this study was the significant correlation between DOB ^{13}C and the severity of *H. pylori* associated antral gastritis. Perri *et al.*, (1998) showed that DOB ^{13}C could significantly indicate the depth of *H. pylori* associated gastritis. However, the assessment of the depth of inflammation is more a measurement of the chronicity of the infection rather then the severity of inflammation. Therefore, this work extends previous studies by showing that the DOB ^{13}C value significantly reflected the severity of antral inflammation. This may allow the non-invasive assessment of *H. pylori* associated disease over time in patients where *H. pylori* eradication is not undertaken. Moreover, it may enable the evaluation of novel therapies against *H. pylori* infection and associated disease in asymptomatic patients without the need for expensive and invasive endoscopies.

DOB ^{13}C value vs. H. pylori load

In contrast to Perri *et al.*, (1998), this study did not find a correlation between DOB ^{13}C and *H. pylori* load. It is well known that *H. pylori* colonises the antral glands (Scott *et al.*, 1998) which may prevent the ^{13}C-labelled urea from reaching all of the organisms. This may account for the small number of false negatives (approximately 1%) that are associated with the test. However, it may also explain the lack of a correlation between DOB ^{13}C and intragastric bacterial load observed by this study. Bacterial load was

measured in this study by counting the number of colony forming units growing on *H. pylori* agar which would allow the growth of all bacteria within the sample taken, including those within the gastric glands and crypts. It is suggested that a possible inability of ^{13}C-urea to reach bacteria that are within these gastric glands may result in a lower and/or incorrect indication of bacterial load. Also, the number of bacteria that are within the glands may not be constant between different individuals.

6.6.4

DOB ^{13}C value vs. H. pylori urease activity

A moderate correlation was observed between DOB ^{13}C and *H. pylori* urease activity. A recent study showed that the urease activity of *H. pylori* is inversely associated with the success of eradication regimens (Suzuki *et al.*, 1998a or b). Suzuki *et al.*, (a1998) showed that individuals with low DOB ^{13}C values had higher cure rates than those with high values. However, the results of the ^{13}C-UBT in this study were not directly compared to the actual activity from isolated bacteria as was undertaken in this thesis. Therefore, the results of the current study again indicate the potential utilisation of the ^{13}C-UBT as a non-invasive index of *H. pylori* infection during therapeutic studies.

6.7: *Summary*

Helicobacter pylori is the primary cause of gastritis and peptic ulcer disease and has been classified as a group 1 carcinogen by the World Health Organisation (Suzuki and Ishii, 2000; International Agency for Research on Cancer, 1994). Recent studies have suggested that ROS play a major role in the pathogenesis of *H. pylori* associated disease (Suzuki and Ishii, 2000; Yajima *et al.*, 1999; Drake *et al.*, 1998; Farinati *et al.*, 1996).

Therefore the role of an important intracellular antioxidant and its primary source of reducing equivalents, the OPP, was investigated in this thesis in the mouse model and in symptomatic patients. Additionally, due to the increasing antibiotic resistance of *H. pylori*, two novel agents were evaluated in the mouse for the treatment of *H. pylori* associated disease. There is also a need for an accurate method to non-invasively detect and follow *H. pylori* infection both in asymptomatic individuals and during therapeutic trials. Hence, the reproducibility of the ^{13}C-urea breath test and its ability to reflect the severity of associated antral gastritis, bacterial load and urease activity of isolated organisms was also evaluated.

The gastric mucosal pathology of the mouse model of *H. pylori* infection does not completely mimic the mucosal response seen in *H. pylori* infected patients (Lee *et al.*, 1997; Lee *et al.*, 1990). Infection with *H. pylori* in humans leads to an early active chronic gastritis. However, significant pathology does not develop in the gastric mucosa of the *H. pylori* infected mouse until approximately six to eight months post-colonisation. It was hypothesised that the mouse model could be utilised to investigate the early events that occur in the gastric mucosa following *H. pylori* infection, particularly the levels of the antioxidant GSH and the OPP activity. In patients, the availability of GSH has been shown to be significantly depleted during *H. pylori* infection (Santra *et al.*, 2000). It was suggested that the GSH depletion was indicative of a failure of the antioxidant defence system to counteract the continued ROS onslaught in *H. pylori* infected patients. Thus, it was hypothesised that differences in the activity of the OPP and GSH availability would be related to the different host responses seen in the human and the mouse.

Results from these studies have demonstrated large differences between the host mucosal response of humans and the mouse to *H. pylori* infection. The hypothesis was that an increased OPP activity and a concomitant increase in GSH availability are important factors mediating the mucosal response to *H. pylori* infection. In the *H. pylori* infected mouse it appears that these factors are able to act as an effective first line defence mechanism that counteracts the oxidant stress placed on the mucosa until at least six months post-infection. In contrast, it appears that the human mucosal antioxidant defence mechanism fails to effectively defend the mucosa against the increased ROS activity early following infection. It is suggested that the activity of the intracellular antioxidant defence system acts to prevent the oxidant induced activation of NF-κB by ROS. However, when GSH levels become limited this mechanism fails to counteract the ROS activity. Potentially, this would lead to the onset of an immune and inflammatory response, characterised by the infiltration of neutrophils to the gastric mucosa, through the oxidant induced activation of NF-κB.

This thesis also investigated the utilisation of two novel therapies for the treatment of *H. pylori* associated disease, NAC and oxythiamine. It was hypothesised that NAC would act as a precursor of GSH, due to its cysteine moiety, leading to an increase in GSH synthesis. It was also hypothesised that administration of oxythiamine, an inhibitor of the NOPP, would result in a compensatory up-regulation of the OPP. Potentially, this would allow the increased provision of NADPH for GSH synthesis. It was observed that NAC increased GSH levels without altering OPP activity in the non-infected mouse and appeared to act as an antioxidant in the *H. pylori* infected mouse. Interestingly,

oxythiamine had no effect on OPP activity, but was seen to increase the availability of GSH in non-infected animals. Finally, oxythiamine was observed to prevent the up-regulation of the OPP in *H. pylori* infected mice. It is suggested that future studies should evaluate the utilisation of therapeutic agents that are able to regulate the activity of the OPP and/or the availability of GSH.

This thesis also evaluated the ability of the ^{13}C-UBT to assess the level of *H. pylori* infection in adult patients. It was observed that results of the breath test significantly indicated the severity of *H. pylori* associated antral gastritis and were also related to the urease activity of the organism. However, the ^{13}C-UBT did not indicate the intragastric bacterial load. It is suggested that the ^{13}C-UBT has the ability to be used as a non-invasive index of *H. pylori* associated disease during therapeutic studies and also to follow asymptomatic infections over time.

6.8: *Conclusion*

In conclusion, this thesis has shown that the activity of the OPP and the levels of GSH in the gastric mucosa play an important role in regulating the host mucosal response to *H. pylori* infection in both the human and the mouse. It appears that the antioxidant defence system of the mouse is able to effectively defend the mucosa against the oxidant load produced during *H. pylori* infection. However, the antioxidant defence system of the adult patients studied does not appear as effective. It is suggested that this may be due to a failure of the antioxidant defence system to prevent the oxidant induced activation of

NF-κB. This may be due to several factors including an over utilisation of GSH by the continued oxidant load produced during a long standing *H. pylori* infection.

It is suggested that an assessment of the mucosal response to *H. pylori* infection of the mouse is only valid for a comparison with childhood infections. Adult infections would all be long standing and not representative of an early mucosal response to this bacterium. Therefore, this thesis suggests that the mucosal response during childhood infections would possibly show the early mucosal events that were seen in the *H. pylori* infected mice. It is suggested that future studies utilising the *H. felis* model of *Helicobacter* infection may help to explain the differences between the host mucosal response of the SS1 mouse model and that seen during human infection. Additionally, novel agents that regulate the activity of the OPP and/or GSH levels are suggested as future therapies against *H. pylori* associated disease.

chapter 7

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Publications and Presentations

Abstracts:

G.M. Matthews, R.N. Butler, P. Cmielewski, R.B. Johnson and F. Campbell. 1999. Intraindividual Variation Of The ^{13}C-Urea Breath Test In Asymptomatic *Helicobacter pylori* Positive Individuals. *Journal of Gastroenterology and Hepatology*; 14: A164

G.M. Matthews, R.N. Butler, A.G. Cummins, A. Lawrence, R.B. Johnson, M.A.F. Campbell. 2000. The ^{13}C-urea breath test reflects the severity of *Helicobacter pylori* associated antral inflammation. *Gastroenterology* 118 (4): A1272

G.M. Matthews, R.N. Butler, A.G. Cummins, A. Lawrence, R.B. Johnson, M.A.F. Campbell. 2000. The ^{13}C-urea breath test reflects the severity of Helicobacter pylori associated antral inflammation. *Journal of Pediatric Gastroenterology and Nutrition* 31 (suppl 2): S149

Presentations:

G.M. Matthews, R.N. Butler, P. Cmielewski, R.B. Johnson and F. Campbell. "Intraindividual Variation Of The ^{13}C-Urea Breath Test In Asymptomatic *Helicobacter pylori* Positive Individuals". Presented as a poster at the Australian Gastroenterology Week (AGW) conference in Brisbane, October 1999.

G.M. Matthews, R.N. Butler, A.G. Cummins, A. Lawrence, R.B. Johnson, M.A.F. Campbell. "The ^{13}C-urea breath test reflects the severity of Helicobacter pylori associated antral inflammation" presented as a poster at the World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition, 5th - 9th October 2000.

ERRATUM

PENTOSE PHOSPHATE PATHWAY METABOLISM AND GLUTATIONE IN THE HOST MUCOSAL RESPONSE TO *HELICOBACTER PYLORI* INFECTION.

Page/ paragraph	Comments
General	There should be no coma after et al. when the year of publication is given within brackets.
v	And, With, The, Of should be in lower case.
vii	NAC is defined as N-Acetylcysteine.
	GSH is defined as Glutathione.
	G6PDH is defined as Glucose-6-Phosphatedehydrogenase.
	Insert 'to' in the last sentence after 'have'.
25	The mice used within all studies were C57BL/6 females 6 to eight weeks of age.
56	The statistical test used was a 'Student's *t*-test'
63	Should have used 1998b instead of superscript.
	mg should have been spelt as milligrams.
85, line 9	Should have been a space between et al.
106	Kletzien et al (1994) instead of Kletzien and Harris (1994)
109,	'our studies' instead of 'studies within this thesis'
110, last line	Should read figures 25 and 26.
112, last line	Should be a space between 'to' and 'ROS'.

List of references omitted from reference section:

Butler, RN., Butler, W., Antoniou, D. and Pascoe, V. (1993) "Effect of dehydroepiandrosterone on pentose phosphate pathway activity in the rat colon". *Int J Biochem* **25** (11):1601-1607.

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