THE UREASE ACTIVITY OF *HELICOBACTER PYLORI* AND DUODENAL ULCER DISEASE

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PREFACE

I have had the privilege of working with Dr. Kenneth McColl in the field of duodenal ulcer disease. This collaboration has opened new aspects in the study of duodenal ulcer disease. I am indebted to Dr. McColl for his help and enthusiasm without which this work would not have been completed. This research has illustrated the important principles of scientific investigation.

Some of the work contained in this thesis has been published and reprints of these publications are submitted with the thesis. As the studies span several medical disciplines it has not been possible to complete them without the help of a number of colleagues and this is noted in the formal acknowledgements. The work has otherwise been carried out by myself.

The writing of this thesis is entirely my own work.

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Summary

Helicobacter pylori, a spiral shaped bacterium has recently been identified as one of the most important acquired factors in the development of duodenal ulcer disease.

This organism colonises the gastric antral mucosa, the body of the stomach and areas of gastric metaplasia in the duodenum. Infection of the gastric mucosa by *H pylori* is associated with the development of type B gastritis, but also with the development of gastric ulcers, and more strongly with the development of duodenal ulcers. Almost 100% of individuals who have a duodenal ulcer have infection with *H pylori*.

Eradication of *H pylori* infection of the antral mucosa is associated with a fall in basal plasma gastrin concentration, the meal stimulated gastrin response and gastric acid output suggesting that infection with *H pylori* could lead to increased gastric acidity and cause the development of duodenal ulcer disease.

H pylori possesses unusually high urease enzyme activity. It has been suggested that the organism's urease activity could enable it to survive at low gastric pH by producing a cloud of ammonium which markedly raises the pH of the organism's environment and of the antral mucosal surface. The production of an alkaline microenvironment above the antral gastrin producing G cells could block the normal inhibition of gastrin release by gastric acid. An increased antral pH could

also promote the uptake of amines which promote gastrin release.

To examine the effects of infection on gastric ammonium concentration, plasma gastrin concentration and the creation of an alkaline microenvironment a series of studies were undertaken.

The effect of infection by H pylori on the concentrations of ammonium and urea in gastric juice was investigated. The effect of stimulating and inhibiting urease activity on gastrin release was investigated. The effect of the alkalinisation of the gastric antrum on gastrin release was also investigated. The effect of urease activity on the survival of H pylori was also studied in a series of in vitro environments.

Characteristic changes in the concentrations of ammonium and urea in gastric juice samples were demonstrated. In 27 subjects with duodenal ulcer disease the median (range) ammonium concentration was 3.4 mmol/L (1.0-13.0 mmol/L) when H pylori was present compared with 0.64 mmol/L (0.02-1.4 mmol/L) following eradication. In 16 subjects with chronic renal failure the median gastric juice ammonium concentration when infection was present was 20.0 mmol/L (13.9-43.1 mmol/L) compared with 4.8 mmol/L (0.5-12.3 mmol/L) when infection was absent. Gastric juice urea concentrations were lower when infection with the organism was present, median 0.8 mmol/L (0.5-2.9 mmol/L) compared with 2.1 mmol/L (1.0-3.7 mmol/L) after it had been eradicated. In subjects with chronic renal failure with infection the median gastric juice urea

concentration was 2.2 mmol/L (0.5-8.7 mmol/L) compared with 13.8 mmol/L (5.4-20.8 mmol/L) when the organism was not present. The concentrations of urea and ammonium in the gastric juice samples did not clearly distinguish between the presence or absence of infection. When the urea/ammonium ratio was calculated all subjects with H pylori had a ratio of less than 0.8 while those free of infection had a ratio greater than 0.9. The urea/ammonium ratio could therefore be used to detect the presence of infection.

The effect of stimulating and inhibiting urease activity, on the plasma gastrin concentration was studied. Urease activity was stimulated by the intragastric infusion of dextrose solution containing urea. Subjects with duodenal ulcer disease who had proven infection with H pylori were studied. The same subjects acted as their own controls following eradication of H pylori. The intragastric infusion of 50 mmol/L urea in a dextrose solution increased the median gastric juice ammonium concentration from 2.3 mmol/L (1.3-5.9 mmol/L) to 6.1 mmol/L (4.2-11.9 mmol/L). There was no change in plasma gastrin concentration observed during infusion of urea either before or after eradication of the organism. Inhibition of H pylori urease activity was also attempted. The specific urease inhibitor, acetohydroxamic acid was administered as a single 750 mg oral dose to 6 subjects who had duodenal ulcer disease. Inhibition of enzyme activity was demonstrated by the suppression of a ^{14}C-urea breath test administered shortly after the dose

of acetohydroxamic acid and reversion of the urea/ammonium ratio to that of non-infected subjects. No change in the basal plasma gastrin concentration or meal stimulated gastrin response occurred following inhibition of urease activity.

Some investigators have failed to observe a rise in plasma gastrin concentration in response to alkalinisation of the gastric antrum. This could explain why stimulating and inhibiting urease activity failed to alter plasma gastrin concentrations. The effect of alkalinisation of the gastric antrum on basal and meal stimulated plasma gastrin concentrations in 7 subjects with duodenal ulcer disease was therefore investigated.

Alkalinisation of the gastric antrum to a pH greater than 6.0 by infusion of citrate buffer pH 7.0 produced a similar increase in the integrated meal stimulated gastrin response both before, +73% (-63% to +500%), and after eradication of H pylori, +48% (-26% to +386%) ($p=0.9$, NS). Gastric alkalinisation by itself produced an increase in gastrin concentration both before (62 ng/L, 50-165 ng/L) and after (50 ng/L, 25-75 ng/L) ($p<0.02$) eradication of H pylori. These experiments demonstrated that if alkalinisation of the gastric antrum resulted from H pylori urease activity then stimulation and inhibition of urease activity should have altered plasma gastrin concentration especially as subjects with the infection were more responsive to alkalinisation.

Although the in vivo studies demonstrated that the urease activity of H pylori did not alter plasma gastrin

concentration through alkalinisation of the gastric antrum its urease activity has been shown to protect the organism from acid environments in vitro. In vitro studies of the effect of pH, urea concentration and inhibition of urease activity on survival of the organism were therefore conducted.

In a pilot study H pylori was inoculated into a series of 0.2 mol/L citrate buffer solutions with pH ranging from 1.5 to 7.0, containing 50 mmol/L urea. At the end of a 60 min incubation 89.9% of the initial inoculum survived at pH 2.0, 50.3% at pH 4.0 and 91.3% at pH 7.0. At pH 5.0 and pH 6.0 there was no survival of the organism.

The initial urea concentration in pH 6.0 citrate buffer was a factor in the survival of the organism. The 5 min survival of H pylori decreased from 121% (69-148) in the absence of urea to only 9% (0-22%) with 50 mmol/L urea ($p<0.01$). The addition of the urease inhibitor hydroxyurea to pH 6.0 buffer solution containing 10 mmol/L urea reduced the 5 min ammonium concentration from 2.5 mmol/L (1.9 -7.6 mmol/L) to 1.29 mmol/L (1.03-1.53 mmol/L) ($p<0.001$), but increased survival of the organism from 22% (10-30%) to 35% (range 30-50%), demonstrating that the destruction of the organism was urease dependent.

Enhanced survival of the organism at low pH was also urease dependent as the addition of hydroxyurea to citrate buffer pH 3.0 containing 10 mmol/L urea resulted in the destruction of the organism.

Ammonium production was also a factor in the destruction of H pylori at pH 6.0. The median recovery of

ammonium was 27% (7-94%) with an initial urea concentration of 1 mmol/L, and only 3% (2-22%) at 50 mmol/L. The reduced recovery of ammonium was associated with the fall in the 5 min survival of the organism. Citrate buffer was also required for the destruction of the organism as the 5 min survival improved from 29% (10-64%) in this buffer with 30 mmol/L urea to 80% (26-160%) in acetate, and 100% (72-128%) in phosphate buffers with 30 mmol/L urea ($p<0.01$). The addition of the precursors for ammonium metabolism, isocitric acid and alpha ketoglutarate, to buffer solutions containing 50 mmol/L urea also enhanced the survival of the organism.

These studies indicated that the in vitro destruction of the organism was due to intra-cellular accumulation of ammonium and depletion of alpha ketoglutarate.

In conclusion in vivo studies demonstrated that alkalinisation of the gastric antrum by H pylori urease activity was not responsible for the increased plasma gastrin concentrations found in infected individuals. The in vitro studies demonstrated that uncontrolled urease activity could result in the destruction of H pylori. If this could be harnessed then it may be of great therapeutic potential.

Abbreviations

Acetyl CoA	Acetyl Coenzyme A
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CV	Coefficient of Variation
GDH	Glutamate Dehydrogenase
H pylori	*Helicobacter pylori*
Km	Michaelis constant
NAD^+	Nicotinamide adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
NS	Not statistically significant
NSAIDS	Non-steroidal anti-inflammatory drugs
Paf	Platelet aggregating factor
Pi	Inorganic phosphate
SD	Standard deviation

CHAPTER 1

DUODENAL ULCER DISEASE

DUODENAL ULCER DISEASE

Peptic ulcer disease and in particular duodenal ulcer disease are major causes of illness in the Western world. Autopsy surveys show that more than 20% of all men and 10% of all women have evidence of past or present peptic ulcer disease (1). Estimation of the prevalence of peptic ulcers at autopsies may also significantly underestimate the disease as detection of ulceration depends on the demonstration of an active ulcer or of scarring as a result of the healing of an ulcer (2). Some ulcers may heal without scarring and so escape detection.

It seems likely that the prevalence of duodenal ulcer which was becoming commonly described between 1890 and 1910 rose between 1900 and 1960 (1-4). Initially the prevalence of duodenal ulcer disease may have risen rapidly but for the last 20 years of the period may have risen more slowly. Recently epidemiological data would suggest that duodenal ulcer prevalence has fallen in younger age groups but has remained the same in the elderly, and especially in women.

While the exact aetiology of duodenal ulceration remains unknown some aetiological factors have been identified.

Acid

Some patients with duodenal ulcer disease may have increased gastric acid secretion although there is overlap between gastric acid output of subjects with duodenal ulcer disease and those without ulcers (5,6). The response of peptic ulcers to treatment with agents which block the secretion of acid such as the gastric proton pump inhibitors (Omeprazole, Astra, Bucks, UK) and H_2 Receptor blockers (Ranitidine, Glaxo, UK) also indicates the importance of gastric acidity in the development and persistence of duodenal ulcers.

Pepsin

It was suggested by Schwartz in 1910 that both acid and peptic activity were required for the development of duodenal ulcers (7). The role of gastric peptic activity in the development of duodenal ulcers is not yet established but raised serum pepsinogen I concentrations are a marker for peptic ulcer disease (7,8). Increased pepsinogen I concentrations inherited in an autosomal dominant pattern are also associated with duodenal ulcer disease.

Motility

Abnormalities of gastric emptying are seen in some patients with duodenal ulcer disease (9). The importance of this in the development of ulcers is not known.

Mucus

Patients with peptic ulcer disease have been shown

to have altered gastric and duodenal mucus (10). They have increased portions of low molecular weight dextrans which are associated with weaker mucus and decreased hydrophobicity (11).

Bicarbonate

Decreased secretion of duodenal and pancreatic bicarbonate and impairment of the duodenal mucosal pH gradient have been demonstrated in patients with duodenal ulcers (12). These abnormalities may increase the susceptibility of the duodenal mucosa to damage from gastric acid.

Cigarette Smoking

Cigarette smoking has been linked to the development of duodenal ulcer disease. A dose dependent effect on the rate of ulcer relapse has been demonstrated (13). Other effects of cigarette smoking include acceleration of gastric emptying of liquids, increased duodenal bile reflux, inhibition of pancreatic bicarbonate secretion and decreased duodenal pH (14-17).

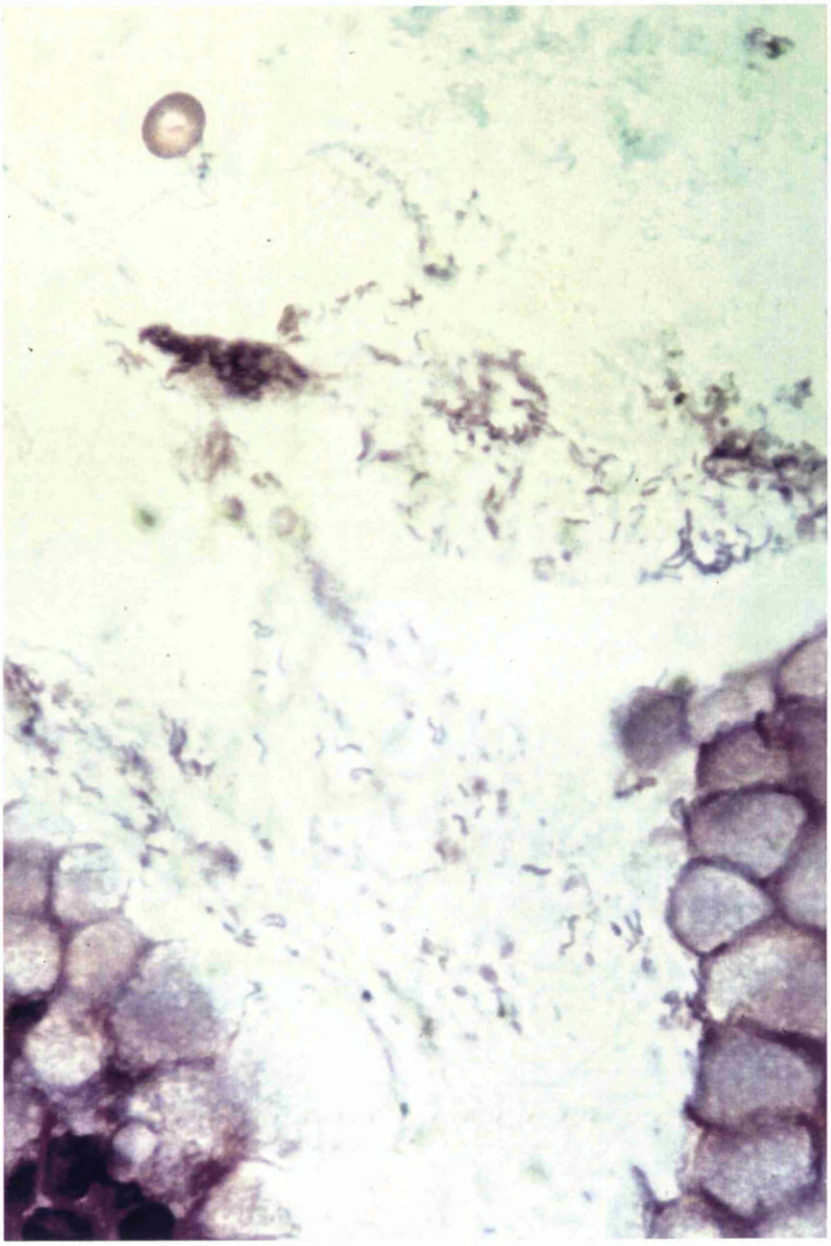
Non-steroidal anti-inflammatory agents (NSAIDS)

The use of non-steroidal anti-inflammatory agents may result in acute mucosal lesions such as haemorrhages and erosions or the development of chronic ulceration. NSAIDS inhibit the production of prostaglandins which have been shown to be necessary for the production of mucus and secretion of bicarbonate and may regulate gastric blood flow (18). The inhibition of these protective mechanisms is likely to be the mechanism through which NSAIDS are involved in the development of peptic ulcer disease.

Helicobacter pylori

Recently, infection of the gastric antral mucosa (Fig 1.1) and areas of gastric metaplasia in the duodenum have been associated with the development of duodenal ulcer disease. Infection with H pylori has also been associated with increased plasma gastrin concentrations, meal stimulated gastrin response and gastric acid output (19-26), serum pepsinogen I concentrations (23), and alterations to gastric mucus (27-31) as well as gastric and duodenal inflammation. Infection by this organism has the potential to explain many of the pathophysiological changes associated with the development of duodenal ulcer disease and the observed change in prevalence of duodenal ulcer disease this century.

Fig. 1.1



Helicobacter pylori colonising the gastric antral mucosal surface.
Haematoxylin and eosin stain, magnification x1000

CHAPTER 2

HELICOBACTER PYLORI AND ITS UREASE ACTIVITY IN THE DEVELOPMENT OF DUODENAL ULCER DISEASE

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2.1 Initial Isolation of Helicobacter pylori

Helicobacter pylori was originally isolated by Warren and Marshall in 1982 (32,33). Since 1980 Warren had observed curved and spiral campylobacter-like organisms in gastric biopsy specimens. These organisms stained well by the Warthin-Starry method but poorly with the haematoxylin and eosin stain (32). A prospective study was initiated which resulted in the isolation of the organism by culture of biopsy material from patients with chronic gastritis (32-36). Following discovery, the organism was initially classified as Campylobacter pyloridis but was later reclassified as Campylobacter pylori (37). Further studies of the organism's ribosomal RNA, its ultrastructure and fatty acid composition showed that it did not belong to the genus campylobacter (38-42). In 1989 it was reclassified as Helicobacter pylori (43).

There had been evidence of spiral bacteria colonising the gastric mucosa since 1938, when Doenges published a study showing that histological examination of the postmortem stomach demonstrated the presence of 'spirochaetes' on the gastric mucosa in 43% of 242 postmortem specimens (44). This original observation was

repeated by Freedberg and Barron in 1940 when they found that 37.5% of 35 gastric specimens they examined contained 'spirochaetes'(45). In 1975 Steer observed bacteria close to the surface of the gastric mucosa and associated with gastritis (46,47). The organisms were also spiral but this was not commented on in the report.

Steer later produced a further study with scanning electron microscopic pictures demonstrating large numbers of curved and spiral bacteria on the surface of gastric type epithelial cells in the prepyloric area of the stomach and in areas of gastric metaplasia in the duodenal bulb but not associated with the intestinal type of epithelial cells (48). Other studies of gastritis had also demonstrated curved bacteria associated with the surface of the mucosal cells but never penetrating the cells (49,50).

Indirect evidence of bacterial colonisation of the gastric mucosa came from studies demonstrating the presence of urease activity in the stomach (51-53). That this urease activity might be bacterial in origin was suggested originally in 1959 by Lieber and Lefevre (52). Studies using germ free animals (54) in 1968 demonstrated that gastric urease activity was bacterial in origin because it was not found in germ free animals. Bacterial urease activity in the stomach was not linked to the reports of spiral bacteria in the stomach until 1984. The original description of Helicobacter pylori (36) reported that it was urease negative but this was later corrected as the organism possesses abundant urease activity which

is now considered to be one of its unique features (55-57).

2.2 Detection of Helicobacter pylori infection

Helicobacter pylori was originally detected by microscopic examination of histologically stained duodenal and gastric biopsies. The organism is often visible on routine haematoxylin and eosin stained preparations. Other stains have been used to help make visualisation easier including the Warthin-Starry silver stain, which results in silver deposition over the organism (33,34). The Giemsa (58), Brown-Hopps (59), Carbolfuchsin (60) and Acridine orange (61) stains have also been used. None of these stains are specific for the micro-organism. Examination of biopsy specimens is not a specific method of detecting the organism. Observation of the organism's characteristic appearance at microscopic examination does not exclude the possibility of other organisms or artefacts (62-64).

The standard by which other methods for the detection of infection with H pylori are assessed remains the bacterial culture of the organism. The bacterium originally proved difficult to isolate and was not identified until culture plates were left for a prolonged period (33,34). The cultures required to be incubated micro-aerophilically at 37°C for 3-4 days before the organisms became visible. The organism was originally isolated using chocolate agar (33,34).

The sensitivity of both bacteriological culture of and histological examination of biopsies depends on the colonisation of the antral mucosa by the bacterium. False negative results could occur when the distribution of H pylori was patchy. In one study of multiple gastric biopsies from 50 patients false negative antral biopsies occurred infrequently (65). Histological examination of biopsies from which the organism had been isolated found a 3% rate of false negative biopsies. The collection of two to four antral biopsies at endoscopy is usually therefore considered sufficient to eliminate sampling error although this view is not universally accepted (65-67).

Other methods of detecting the micro-organism depend on its urease activity. The rapid urease test (CLO Test) has been used to detect urease activity in antral biopsy specimens (68). In this test the biopsy is inserted into a buffered gel. The gel also contains urea and phenol red, which is a pH indicator. Initially the gel is buffered to an acidic pH at which phenol red gives a yellow colour. When a biopsy containing urease activity is inserted the ammonium produced by the enzyme produces an increase in the pH of the gel and results in a change in colour from yellow to pink when the pH rises above 6.0. This test takes a minimum of 3 h when incubated at 30°C before the colour change becomes visible. Some biopsies may take up to 24 h before the colour change becomes visible. A small number of false negative reactions may occur with this test (69).

A number of variations on this principle have been described (70,71). These methods use indicator dyes in buffered solutions to identify specimens which contain urease activity. The reagents used in these tests are easily obtained. Advocates of these tests suggest that they are suitable for the rapid side room detection of the micro-organism although there have been no reports of the sensitivity or specificity of these methods. The advantage of these rapid urease tests is that the H pylori status of patients may be obtained in a few hours compared with 24 h for histology or 3-4 days for microbiology.

The ^{14}C-urea breath test detects H pylori by the production of radiolabelled $^{14}CO_2$ as a result of its urease activity (72,73). A dose of 0.4 Mbq, of ^{14}C labelled urea is administered following a liquid meal which is used to delay gastric emptying. A portion of the labelled CO_2 is exhaled in the breath. Breath samples are therefore collected every 10 min during the 2 h after the radiolabel is ingested. The exhaled CO_2 is trapped by hyamine contained in a vial through which the exhaled breath is bubbled. Scintillant is added to the trapped breath CO_2 samples and the radioactivity counted. The activity is expressed as the percentage of the administered dose per millimole of expired CO_2 multiplied by body weight to allow for endogenous CO_2 production. The ^{14}C-urea breath test is a very sensitive and reproducible method of determining the

H pylori status of a patient. The disadvantage of this test is that it takes at least 24 h before a result is available. In addition a dose of radioactivity has to be administered to the patient. Administration of the radiolabel may make the test unsuitable for repetitive investigation and the investigation of children or pregnant women.

A ^{13}C-urea breath test has also been developed. The principle is the same as the ^{14}C-urea breath test except that a mass spectrometer is required to detect the exhaled ^{13}C labelled CO_2 (74). This method of detecting the micro-organism avoids the use of a radiolabel and is as sensitive and specific as the ^{14}C-urea breath test. The disadvantage is that it requires a mass spectrometer which may not be available outside specialist centres.

Serological tests have also been developed for the detection of H pylori as infection is associated with serological conversion (75-79). Detection of infection by serological methods has the advantage that special precautions prior to collection of the sample are not required. One study suggested that serological testing could reduce endoscopy workload (79). However, during the acute phase of the infection, the organism may not be detected by this method. In addition, serological evidence of infection may persist after the organism has been eradicated (80). While serological detection of H pylori has some advantages over more invasive

investigations the sensitivity and specificity of serology when compared to the ^{14}C-urea breath test was only 83% and 68% in one study of elderly patients (81). It was suggested that this may have been due to reduced immune competence in the elderly.

Other investigators have suggested that differences in specificity of antibodies in enzyme linked immunosorbent assays for H pylori may result in the reduced sensitivity and specificity of the serological tests compared with other methods (80,82-85). This discrepancy between some serological tests may be due to differing sensitivities to different strains of H pylori (85).

The DNA polymerase chain reaction has also been used to detect the presence of H pylori DNA in gastric biopsy samples (86). This method of detecting the organism gives high specificity and sensitivity since the polymerase chain reaction may be used to amplify small amounts of DNA present in biopsy samples. The disadvantage of this method of detecting the organism is that it requires a high degree of technical skill with DNA methodologies which may not be available in district general hospital laboratories.

The development of so many methods for the detection of the micro-organism suggests that none are ideal. The gold standard of detection remains the culture of the organism from a biopsy. The ^{14}C-urea breath test may be as specific as culture of the organism and is potentially more sensitive, more rapidly completed and less invasive

as the collection of a biopsy is not required. The rapid chemical tests based on urease activity are the quickest and most convenient method, although quantitation of infection with these tests is not possible and a biopsy still needs to be collected. Serological identification of infection would appear to be a convenient method but has been shown to be associated with a sufficient incidence of false positives and false negatives to potentially make it one of the least reliable methods of detecting current infection with the micro-organism.

Two methods of detecting the organism are currently advocated in most studies. With the development of suitable treatments which result in the eradication of the organism detection of infection in asymptomatic populations will become essential and will either result in increased usage of these methods for detection of infection with Helicobacter pylori, or the development of other sensitive and specific tests of the presence of the organism.

2.3 Epidemiology of Helicobacter pylori infection

The most extensively studied populations with respect to the prevalence of Helicobacter pylori are those from Western Europe, North America, New Zealand, and Australia. The organism has been identified in association with type B chronic gastritis from most nationalities and populations.

Studies of the prevalence of infection have used a variety of methods of identifying the organism in a wide range of populations.

Autopsy examination of the histological appearance of the gastric mucosa, and tests for urease activity in small numbers of individuals has demonstrated infection in 60% of Polynesians and 20% of Caucasians (87). Endoscopic collection of biopsy specimens from asymptomatic young adult medical students in the Netherlands showed an infection rate of 25% (56). A prevalence of 20% was found in a similar study in the United States of America (88). The prevalence of infection detected using the ^{13}C-urea breath test in Americans rose from 24% in the age range 20-39 years to 36% in the age range 40-50 years and to 82% in the age range 50-84 years (89). Serological testing has also been used and this has again demonstrated an increase in prevalence with age (78). Using serological testing the infection rate for male children was 11% and for female children was 5%. For adults less than 30 years of age the rate was 23% for males and 5% for females. While for adults older than 30 years of age 29% of males and 25% of females had the infection demonstrated by serology. The increase in the rate of infection with age has also been reported in other surveys in which samples from blood donors were used for serological testing or endoscopic biopsies collected for histological examination (90-92).

The prevalence of H pylori specific IgG has also been investigated in a group of North Western Australian

Aborigines (93). It has been discovered that this human population rarely develops duodenal ulcers (94). The prevalence of infection in this population was 1% or less, suggesting that infection is not only important for the persistence of the ulcer but also in the development of a duodenal ulcer.

The available evidence from studies of seropositivity for H pylori specific IgG, ^{13}C-urea breath test, and examination of biopsy material collected at endoscopy or postmortem indicates that the infection is commonly present especially in older groups when over 80% of the population may be infected. The prevalence of infection also rises with age. These epidemiological studies have not demonstrated a relationship between those who only have the infection and those who go on to develop duodenal ulcers. Other factors such as cigarette smoking, peptic activity, and genetic inheritance may also help determine those H pylori positive individuals who go on to develop duodenal ulcers.

2.4 The effects of acute infection with Helicobacter pylori

The effects of acute infection with the organism have been documented in two healthy volunteers, in one research fellow who developed the infection while engaged in research in a gastroenterology unit, and in volunteers in two other studies who probably contracted the infection

accidentally as a result of a failure to sterilise gastric pH electrodes between subjects (95-100). The symptoms reported were of mild epigastric pain or discomfort along with epigastric fullness, malaise, headache, halitosis and vomiting. The symptoms occured 2-7 days after infection and lasted for 1-7 days. One volunteer experienced severe vomiting and symptoms consistent with bowel obstruction of which there was no sign on erect and supine abdominal X-ray (96). Gastric biopsy specimens from these subjects showed an active chronic gastritis with polymorphonuclear inflammation in the antrum and the presence of H pylori (98).

Transient hypochlorhydria was observed in one volunteer, the research fellow, and 9 of the 17 subjects in another study who had been infected with the organism (96,97,100). The gastric juice pH of the other volunteer who ingested the organism was not measured but it was reported that his vomitus did not taste acid, and was not acid on testing with litmus paper (95). The hypochlorhydria appears to be transient but may persist for up to 235 days (median 112, range 53-235 days) before resolving (97). Maximum depression of peak acid output was also noted to parallel the severity of the histological gastritis in one study (97). Chronic gastritis and infection with H pylori persisted in one volunteer despite treatment with bismuth subsalicylate aimed at eradicating the infection.

These studies demonstrate that when H pylori

colonises the gastric mucosa it causes histological gastritis and symptoms associated with this infection. Once established the organism can persist and may cause a chronic gastritis despite attempts to eradicate it. A fall in gastric juice ascorbic acid concentration was observed in samples from the research fellow who became infected (100). The reduction in gastric juice ascorbic acid was attributed to an inhibition of the secretion of ascorbic acid into the gastric juice.

2.5 Helicobacter pylori and disease in children

Infection of the gastric mucosa by Helicobacter pylori in children is associated with an antral gastritis (23,101,102). Recurrent abdominal pain is the common presenting symptom in children (101,102). The pain may be epigastric or periumbilical. Vomiting and haematemesis may also occur.

Infection of children with H pylori is often found in association with gastric or duodenal ulcers (101,103,104). Up to 29% of children infected with the organism may have duodenal ulcers. Recurrence of peptic ulcers in infected children following healing with H_2 receptor antagonists occurs as it does in adults. Besides the development of duodenal ulcers children with this infection have also presented with iron deficiency anaemia (105) and protein losing enteropathy (106). Infection of children with H pylori has also been implicated in the development of malabsorption syndromes in Third World

countries, and East Africa (107).

Infection with H pylori may therefore occur at any age. It is typically associated with antral gastritis and duodenal ulcers in both children and adults.

2.6 Chronic Gastritis and Helicobacter pylori

Persistence of infection of the stomach by Helicobacter pylori results in persistent gastritis (97-100). The multiple appearances of gastritis has made classification of chronic gastritis difficult. Several systems of classification have been proposed (108-110). When chronic gastritis is graded on the basis of the presence or absence of glandular atrophy then in the absence of the loss of glands it is termed chronic superficial gastritis and with the loss of glands chronic atrophic gastritis (108). Chronic atrophy of gastric glands is usually associated with pernicious anaemia but not invariably so (111). Autoimmune gastritis associated with pernicious anaemia has also been termed type A gastritis (110).

Type A Gastritis

Type A gastritis (body-mucosa) affects the body of the stomach and is associated with parietal cell antibodies and achlorhydria (109). Only the acid producing parietal cells of the body and fundus are affected while the mucous secreting epithelial cells lining the entire stomach are not damaged. The gastric

antrum is also spared. The end result of this severe form of gastric atrophy may be intestinal metaplasia. Type A gastritis may be asymptomatic.

A reduced prevalence of H pylori in body and antrum of the stomach of individuals with type A gastritis was found when they were compared with age sex matched controls with peptic ulcer (112). A similar study demonstrated that pylori was rarely present in patients with type A gastritis even when the gastric antrum was involved, suggesting that the organism is not associated with the development of this type of gastritis (113).

Reflux Gastritis

Persistent injury to the surface epithelium of the stomach due to enterogastric reflux following gastric surgery is characterised by compensatory foveolar hyperplasia and a vascular and exudative response evidenced by capillary dilatation, congestion and lamina propria oedema (114). Increased numbers of smooth muscle fibres and collagen bundles may appear in the lamina propria (114).

Agents which injure the gastric epithelial surface and cause this type of gastritis include bile acids and lysolecithin (115). These agents damage the mucous barrier. Exposure of the mucosal surface to bile, a biological surfactant also leads to the degranulation of mast cells and a vascular response mediated by histamine

which causes further damage to the surface epithelium (115). Other chemical insults to the gastric mucosa such as salicylates and other non-steroidal anti-inflammatory agents may also cause the histological appearances of reflux gastritis (116).

Reflux gastritis has not been associated with H pylori (117). The surgical treatment of duodenal ulcers by gastroenterostomy or other procedure that causes gastroduodenal reflux results in a change of the histological appearance of gastritis from that associated with H pylori to that of reflux gastritis (117). The toxicity of bile acids to H pylori may cause the elimination of the organism (118). This could explain the change in histological appearance of gastritis.

Lymphocytic Gastritis

Lymphocytic gastritis is similar to active chronic superficial gastritis but is identified by the infiltration of the surface and foveolar epithelium with lymphocytes (119). At endoscopy nodular prominence of the gastric mucosa with the nodules being surmounted by shallow apthoid erosions may be seen. These changes are usually confined to the body of the stomach. Only small numbers of patients with this type of gastritis have been found to be infected with H pylori (120,121).

Type B Gastritis

Type B gastritis typically affects the mucous secreting epithelium of the gastric antrum but may also involve the acid secreting part of the stomach. It is one of the commonest forms of gastritis and occurs in 78%-100% of patients with duodenal ulcers (35,66,76,122-131). Dyspeptic patients with gastric ulcers also tend to have type B gastritis that is histologically the same as that found in patients with duodenal ulcers (122). Type B gastritis has been shown not to be related to duodenal gastric bile reflux which was at one time thought to be the cause of this type of gastritis. It is highly associated with H pylori infection (66,76,122-131). In type B gastritis the organism is not confined to the antral area but may also be found in the body of the stomach. The natural history of type B gastritis is not known but it is thought that it may commence as a diffuse inflammation in both the body and antrum of the stomach. It may then remain as a static chronic superficial gastritis showing no tendency to progress. In one study of 67 positive patients, 13 became negative on follow-up from one month to 10 years, while 3 of the 41 initially negative became positive (129). Loss of infection was associated with the development of intestinal metaplasia or of lymphocytic gastritis.

The prevalence of type B (antral) gastritis increases with age and this again suggests that once H pylori has

colonised the gastric mucosa it may persist for life (80,89,92). Finally, eradication of antral infection with H pylori using triple therapy which combines a course of tripotassium dicitrato bismuthate (DeNol), metronidazole and amoxycillin, results in resolution of the gastritis indicating that the organism is the cause of the gastritis (23).

Infection with H pylori has been shown to be closely associated with type B chronic antral gastritis. The development of type B antral gastritis has also been shown to be closely associated with the development of duodenal ulcer disease occurring in up to 100% of individuals with duodenal ulcers. Persistent infection of the gastric antral mucosa by H pylori may therefore cause or sustain the development of duodenal ulcers.

2.7 Helicobacter pylori, Gastric Ulcers and Gastric Carcinoma

Infection of the gastric mucosa with Helicobacter pylori results in the development of chronic type B gastritis. Prior to the discovery of H pylori an association had been found between the development of gastritis of a typical type B chronic gastritis pattern affecting the antrum and with the development of gastric ulcers irrespective of the site of the ulcer (132-134). While almost all patients with duodenal ulcers have infection of the gastric antrum with H pylori only 70% of patients with gastric ulcers can be shown to have infection with the organism (35,122). A number of

other factors in addition to Helicobacter pylori have also been found to cause the development of gastritis including cigarette smoking, ingestion of nonsteroidal anti-inflammatory agents and duodenogastric reflux (13,18,135-137).

Examination of the histological type of gastritis associated with the development of gastric ulcers in pylori negative patients has demonstrated that most have features of reflux gastritis (122,138). Biliary reflux is harmful to pylori and the development of this is associated with loss of infection (117).

A multifocal chronic atrophic gastritis is considered to be a precursor lesion in the path of the development of gastric carcinoma (109). Carcinomas which develop from this type of lesion tend to be of the 'intestinal' or 'expansive' type. H pylori does not colonise intestinal epithelium but its presence in surrounding areas of gastritis has recently been associated with the development of intestinal metaplasia of the antrum (139). An association between the development of gastric carcinoma and infection with H pylori demonstrated by the presence of IgG antibodies to the organism has also been recognised (140). This study suggested that between 35% and 55% of all cases may be associated with this infection. Despite this, another recent study of gastric carcinoma in two high risk populations demonstrated that while infection with the organism may cause irritation of

the gastric mucosa it did not appear to have a causative role (141). Type A atrophic gastritis and reflux gastritis have both been associated with the development of gastric carcinoma and are not associated with infection with H pylori (109,117).

Two groups of agents are recognised in the development of cancer. These are initiators and promoters. Initiators cause the pre-requisite cellular genetic or metabolic changes for the development of cancer over a short period of exposure. The initiator may be a complete carcinogen and repeated exposure result in the development of cancer. As prolonged infection with H pylori frequently occurs without the development of malignancy it is unlikely that H pylori is an initiator. Promotors increase the chance of the development of malignancy through repeated exposure to cells already exposed to an initiator. The chronic irritant and inflammatory effects of infection with H pylori might promote the development of malignancy.

In addition, gastric juice vitamin C concentrations are also reduced when H pylori infection is present. It has been suggested that gastric juice vitamin C protects the gastric mucosa from the carcinogenic effects of oxidising agents such as nitrites and free radicals (100,142,143). The fall in gastric juice vitamin C following infection could also explain the association with the development of gastric carcinoma. These effects

could explain the close association of H pylori with chronic gastritis and its association with the development of gastric carcinoma.

2.8 Helicobacter pylori and duodenal ulcer disease

There is a clear association between the development of duodenal ulcer disease and infection of the antral mucosa by Helicobacter pylori. This might merely indicate that the organism is a marker for the development of duodenal ulcer disease. The presence of other factors associated with the development of a duodenal ulcer might themselves make colonisation of the gastric mucosa easier for H pylori. Recently, eradication of infection with H pylori has been shown to reduce the ulcer relapse rate following healing from 80% to 20% or less (144-146). In addition reports of H pylori negative individuals with duodenal ulcers usually identify other aetiological factors, such as the use of non-steroidal anti-inflammatory agents or associated pancreatitis (35,147). Recurrence of the ulcer following eradication of the organism is usually associated with re-infection. Studies of H pylori cultured from individuals with re-infection suggest recrudescence of infection with the original organism rather than the acquisition of a second infection with H pylori (148). This clear association between the development of duodenal ulcers and infection with H pylori and the healing of ulcers following the

eradication of infection by H pylori demonstrates that the organism is likely to be directly involved in the development of, and the relapse of duodenal ulcers. Although the organism is closely associated with the development of duodenal ulcers the means by which it causes the development of an ulcer remains unclear especially as the organism predominantly colonises the gastric mucosa and does not colonise the intestinal mucosa. In some individuals H pylori does colonise the duodenum (147).

2.9 Helicobacter pylori and duodenitis

Individuals from whom the organism has been isolated from the duodenum have histological evidence of duodenitis with areas of gastric metaplasia. Gastric metaplasia in the duodenum is common in normal individuals (149). Gastric epithelium may develop in the duodenum in two circumstances. It develops either as an area of heterotropic gastric body type mucosa or as a metaplastic change of an area of duodenal mucosa (149,150). Gastric heterotropia is characterised by islands of fully developed fundic mucosa including parietal and chief cells. It is found in 1-2% of duodenal biopsies (149,150). It is considered to be congenital in origin and is not associated with duodenitis.

Gastric metaplasia develops as small foci of gastric epithelial cells on the tips of villi. It is extensive in patients with duodenal inflammation and ulceration

(150-156). It occurs in up to 100% of individuals with duodenal ulcer disease. H pylori has been identified histologically in areas of gastric metaplasia in only 55% of duodenal biopsies (150). This may be due to the patchy nature of the lesion.

Gastric metaplasia is thought to represent a response to injury of the duodenal mucosa. Animal experiments have shown that gastric metaplasia may develop in response to duodenal hyperacidity in rats, pigs and monkeys (157-160). It may also occur during the healing of surgically induced defects of the duodenal mucosa in cats (161). In man gastric metaplasia is found extensively in patients with the Zollinger Ellison syndrome (162) and in individuals with a low fasting gastric juice pH (149). Its development has also been correlated with increasing acid output (149,163,164). In addition it has not been found in individuals with atrophic gastritis. Acid secretion in patients with duodenal ulcers may range from normal to increased levels (5,6,19). In patients with duodenal ulcers gastric metaplasia of the duodenum could therefore represent a response to an increased acid load.

Once gastric metaplasia of the duodenum has developed H pylori may colonise the areas of gastric epithelium in the duodenum but it does not colonise the intestinal mucosa. Areas of intestinal metaplasia in the stomach are also spared by the organism (110,156). Although H pylori does not invade metaplastic gastric mucosa in the duodenum

it causes an active duodenitis (149,154). Immune responses to the organism have been demonstrated and putative bacterial exotoxins have also been reported (165-176). Infection of metaplastic gastric mucosa by the bacterium despite the absence of invasion can result in the development of an immune response and could therefore account for the associated inflammation (165,169,170).

Gastric metaplasia may develop in response to increased gastric acidity in patients with duodenal ulcer disease. The development of gastric metaplasia would allow colonisation of the duodenum by H pylori. This could result in the development of duodenitis. If colonisation of the gastric antral mucosa by H pylori leads to the development of gastric metaplasia of the duodenum then colonisation of the antrum would be the primary event in the development of duodenal ulcer disease.

2.10 How Helicobacter pylori Infection might cause Duodenal Ulcer Disease

Infection with Helicobacter pylori results in colonisation of the gastric antral mucosa, the development of chronic type B gastritis and colonisation of areas of gastric metaplasia in the duodenum. These observations do not establish how the organism may cause duodenal ulcer disease.

A number of hypotheses have been proposed to explain how colonisation of the gastric antral mucosa or

colonisation of the duodenal mucosa by this organism could lead to the development of peptic ulcers. It has been suggested that colonisation of areas of gastric metaplasia in the duodenum and the associated inflammation may be a key event leading to ulcerogenesis. Abnormalities of the duodenal mucosa also persist after healing of a duodenal ulcer (177-179). If this is the case then the colonisation of the duodenal mucosa may not be essential for the development of duodenal ulcers. If colonisation of the gastric antral mucosa by H pylori leads to the development of gastric metaplasia of the duodenum then colonisation of the antrum will be the primary event and colonisation of gastric metaplasia in the duodenum a secondary event in the development of duodenal ulcer disease.

It has been suggested that a vigorous immune response to infection of the gastric antrum might result in damage to the duodenal mucosa and the development of ulcers. H pylori can initiate an inflammatory reaction as demonstrated by the expression of Class II transplantation antigens on epithelial cells and increase numbers of T-lymphocytes (169). Studies using a mouse model have shown that infection with H pylori can induce antibodies which cross react with human gastric mucosa (176). Antibodies which cross react with duodenal mucosa have not been reported.

Serological studies show that infection with the organism appears to be widespread and increases with age

(90,91). One study has also shown that the serological response to infection may diminish with age (81), although the prevalence of duodenal ulcers in the elderly may have risen slowly over the past 20 years. Other studies have shown that while 49% of individuals studied had serological evidence of a current immune response to H pylori only 24% had evidence of a current infection. These observations may indicate that a failure to eliminate H pylori may be more important in ulcerogenesis than the development of an immune response to infection (80).

H pylori produces a number of toxins and other substances which have cytotoxic activities (165-175). These include Paf-Acether (166), ammonium (167) and other substances including proteins and enzymes (172-175). The in vitro exposure of tissue culture cells to a concentrated bacterial supernatant in the presence of urea (30 mmol/L) demonstrated vacuolisation of the cells (167). The addition of the urease inhibitor acetohydroxamic acid prevented the development of vacuolisation. Conversely the addition of ammonium to the suspension caused vacuolisation indicating that ammonium produced by the urease activity of the bacterium had a cytotoxic effect in vitro. Studies using a rat model have shown that ammonium can cause a decrease in gastric mucosal potential difference and gastric epithelial damage (180). It has been suggested that the production of

ammonium by the bacterium's urease activity could result in back diffusion of hydrogen ions across the mucosal barrier and cause cellular damage in vivo (181).

A 120 kDa protein has been identified in broth culture supernatants which also has vacuolising cytotoxic activity (172). One other study has suggested that the antibody response of mucosal IgA to a similar 120 kDa protein produced by H pylori may be responsible for the differing susceptibility of individuals to the development of gastric pathology (174).

The production of these toxins and any resulting inflammatory response could be involved in the development of type B gastritis. The effects of these cytotoxic agents may not be essential for the development of duodenal ulcers unless the colonisation of areas of gastric metaplasia in the duodenum was the primary event in the development of duodenal ulcers.

Mucus acts as a barrier to the back diffusion of hydrogen ions and any weakening of this barrier could also lead to inflammation and ulceration. The structure, alignment and turnover of mucous filaments are altered once H pylori is present (10,11,27-31). The mucous layer becomes less viscous and may therefore become more permeable to the organism, and also to the diffusion of hydrogen ions. The proteases and mucinases which the organism secretes may cause the alterations in the structure of the mucus. The production of ammonium by

the organism also alters mucus structure by changing the pH in the environment of the mucus and therefore causes a change in conformation of individual molecules of mucus thereby altering mucous strand alignment (27-31). The alteration of the mucous layer might contribute to the development of antral gastritis or duodenitis. It might not be essential for the development of duodenal ulcers unless the colonisation of areas of gastric metaplasia was the primary event in the development of duodenal ulcers.

It has been proposed that ammonium produced by the organism's abundant urease activity could create an alkaline microenvironment in the vicinity of the organism by the neutralisation of gastric acid (19,181-183). This hypothesis has developed from the observation that the organism does not survive in an acidic environment except when urea is present (118,183-185).

The production of ammonium by the bacterium creating an alkaline microenvironment over the surface of the antral mucosa might block the suppression of gastrin release which occurs with increasing acid secretion. It might prevent the acid sensitive G cells of the antral epithelium sensing a fall in antral pH due to acid production.

The production of ammonium by the bacterium's urease activity therefore has the potential to explain the development of duodenal ulcer disease if the production of an alkaline microenvironment at the gastric antral

epithelial surface results in an increase in plasma gastrin concentration and gastric acidity.

An inappropriate hypergastrinaemia occurs in association with Helicobacter pylori infection (19-26). It has also been demonstrated that eradication of infection of the antral mucosa with H pylori is associated with a fall in basal plasma gastrin concentration and meal stimulated gastrin response (19-26). The inappropriate hypergastrinaemia has also been shown to result in increased intragastric acidity following meals (21,24,26). This failure to switch off gastrin secretion which may result in increased acid production could explain the increased duodenal acidity observed in some patients (19). Increased acid production could also lead to the development of gastric metaplasia of the duodenum and duodenitis with or without colonisation by the organism and ultimately to duodenal ulcer disease.

There is evidence from dogs and rats that the uptake of biological amines by the gastric antral mucosa results in gastrin release by the antral G cells (186-189). Biological amines which cause gastrin release include the ammonium ion (186). Amines would be less likely to be protonated at near neutral or alkaline pH than at acidic pH and would therefore be more able to cross the mucosal barrier into cells. The creation of an alkaline microenvironment in the region of the gastric antral mucosa could therefore result in increased uptake of

biological amines from digested foods or uptake of ammonium produced by urease activity. This could also explain the observed increase in gastrin concentrations in patients with duodenal ulcer disease.

An attempt has been made to relate an increase in gastric juice ammonium ion concentration to plasma gastrin concentration, in order to determine whether ammonium uptake by the antral mucosa is of importance in humans (190). A relationship between the concentrations was not found. This might indicate that ammonium uptake was not a significant cause of gastrin release in humans, but gastric juice ammonium concentrations may not accurately reflect the concentrations to which the gastric antrum is exposed during infection with H pylori. In addition to the possible effects of ammonium on gastrin release gastrin might also be released in response to inflammation of the antral mucosa which might also result from the cytotoxic and mucolytic effects of ammonium produced by the urease activity of H pylori. The urease enzyme activity of H pylori therefore has the potential to explain many of the abnormalities associated with the development of duodenal ulcer disease and may therefore be a key factor in the development of duodenal ulcers.

2.11 The urease enzyme of Helicobacter pylori

Although Helicobacter pylori has been shown to possess many enzymes including gamma glutamyl transferase

activity, alkaline phosphatase activity, oxidase activity, catalase activity and glutamate dehydrogenase activity (55,191) the one characteristic biochemical feature of the organism is its abundant urease activity (55-57). The urease enzyme of this organism has the highest activity of all bacterial ureases tested (192). The amount of ammonium produced in one study was 36 ± 28 umol/min/mg of protein compared with 14.6 ± 11.8 umol/min/mg protein for Proteus mirabilis, 5.5 ± 4.9 umol/min/mg protein for Proteus vulgaris, 5.2 ± 2.6 umol/min/mg protein for Morganella morganii and 4.6 ± 1.4 umol/min/mg protein for Providenci rettgeri (193).

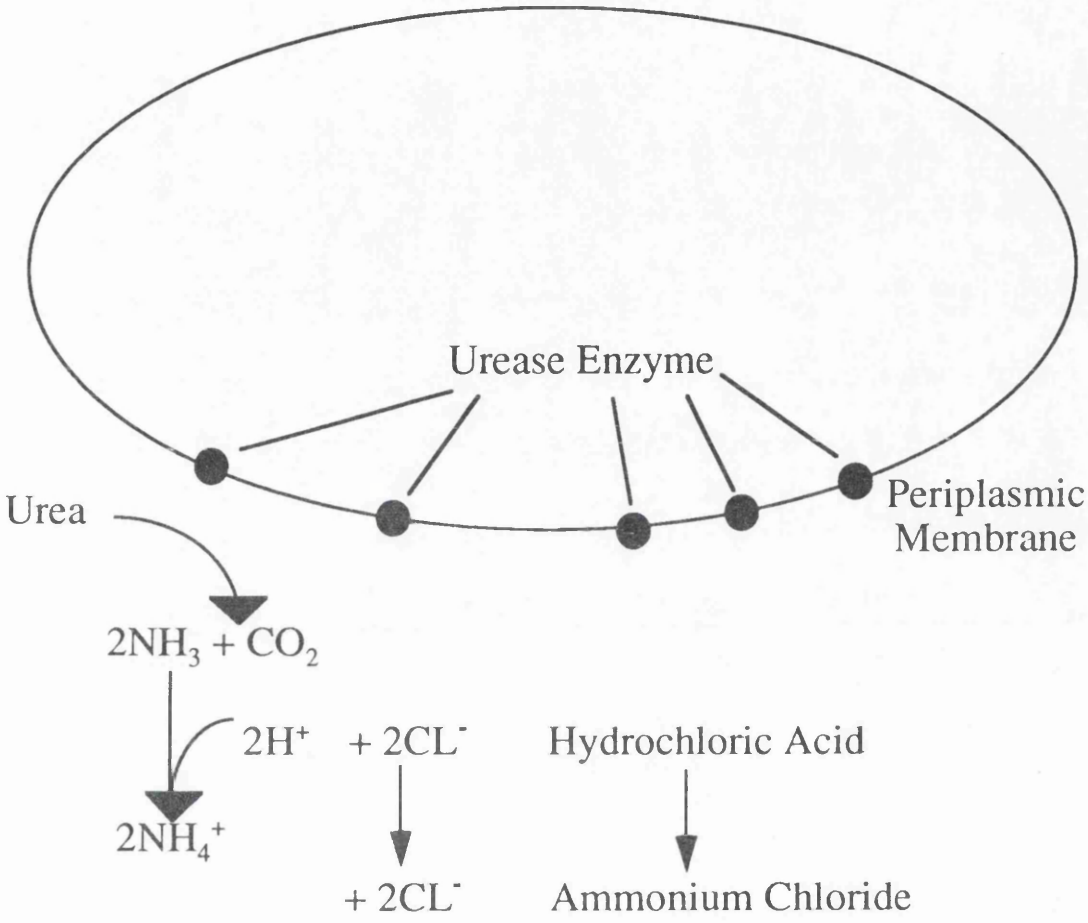
When the bacterium colonises the gastric mucosa its abundant urease activity has been reported to increase the gastric juice ammonium concentration and reduce the gastric juice urea concentration (182). Other investigators have not found any difference in urea concentrations between subjects with the infection and those without the infection (194,195).

It has been suggested that during initial infection with the organism its urease activity could result in the production of sufficient ammonium to effectively neutralise the gastric juice pH and cause achlorhydria (182). This may be of importance during acquisition of this infection as a number of studies have also demonstrated that the presence of urea is essential for the survival of the organism at low pH (118,183-185). In the absence of urea the organism does not survive at a pH

of less than 4.0. These observations have resulted in the suggestion that the organism's urease activity creates an alkaline microenvironment to protect it from gastric acidity which is a potent bacteriocidal agent (185) (Fig 2.1). It has also been suggested that H pylori may secrete urease activity in order to better mop up urea in its immediate vicinity and thereby better create an alkaline microenvironment (181). There is some experimental evidence to indicate that the organism may indeed excrete the enzyme in vitro as culture supernatants may contain urease activity (167). Partially purified urease enzyme for H pylori has a pH optimum of 8.7 which would indicate that the enzyme would not function if it was secreted into gastric juice as the low pH would inactivate it (193). When the organism colonises the gastric mucous layer it is found in close proximity to the epithelial cells below this. The gastric mucous layer acts as a barrier to the back diffusion of H^+ ions and as a result the pH below the mucus close to the epithelial cells may be closer to 6.0-7.0 pH units (196). This pH would be more favourable for the growth of the organism than that of gastric juice, but it still may not be optimal for its urease activity.

The Km for H pylori's urease enzyme has been found to be in the range 0.2 - 0.7mmol/L (193,197). This is considerably lower than other microbial and plant ureases (192). This probably is due to adaptation of the enzyme for the concentrations of urea normally found in gastric juice, which tend to be two thirds of serum urea

Fig. 2.1



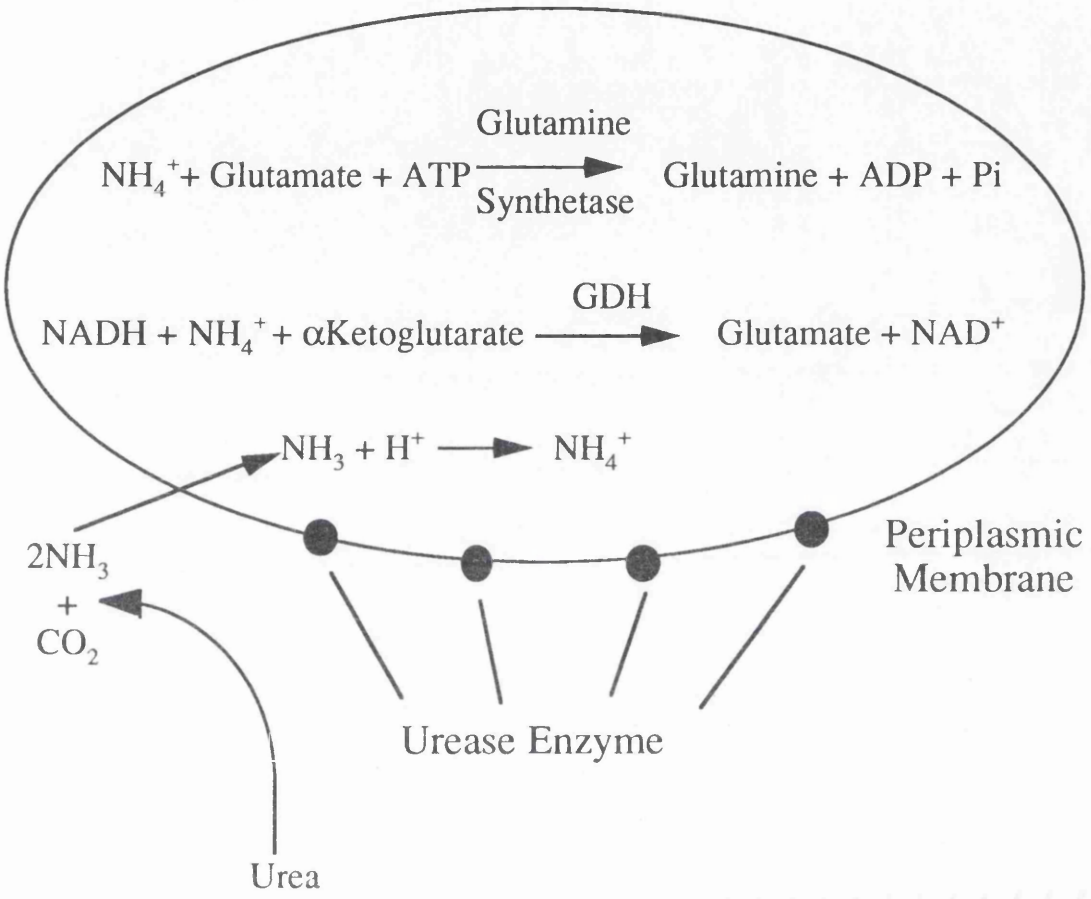
The creation of an alkaline microenvironment using Helicobacter pylori urease enzyme activity.

concentrations. In contrast urease enzymes of proteus species are adapted for the hydrolysis of urea at concentrations of 400-500 mmol/L found in urine (192). Even with a low Km it is unlikely that H pylori urease activity would ever operate at maximum velocity at normal gastric juice urea concentrations.

In other bacteria urease enzyme activity is used to scavenge ammonium from urea (198). Once ammonium has been generated by the hydrolysis of urea it will be taken up by the cell and enter the metabolic pathways of the organism by one of two main routes (Fig 2.2). Glutamate dehydrogenase activity may be used to catalyse the conversion of alpha ketoglutarate and ammonium into glutamate (199). Glutamine synthetase may also be used. This enzyme converts glutamate and ammonium into glutamine which may be used as a labile intracellular pool of metabolically available amino groups (200). H pylori possesses glutamate dehydrogenase activity which would allow it to use urease activity as a method of scavenging nitrogen (55). Even if this is the function of the urease activity the reason it contains such abundant enzyme activity remains unclear.

Most bacterial ureases are considered to be intracellular enzymes although some have been localised in association with the cell's periplasmic membrane (192). Localisation of H pylori urease activity by means of urease linked precipitation of silver followed by visualisation of the precipitate by electron microscopy

Fig. 2.2



Possible methods of assimilation of urea nitrogen by Helicobacter pylori.

has suggested that the urease activity of this organism was associated with its periplasmic membrane (201). Investigation of the location of the urease by using monoclonal antibodies has also demonstrated localisation of this enzyme on the extracellular surface of the organism (202). Similar work with proteus species has also suggested localisation of urease activity in the periplasm and outer membrane of this species (203).

The urease activity of H pylori is expressed constitutively but can also be further induced if the organism is grown in a medium which contains urea (191). The mechanism by which the organism's urease activity is controlled is currently unknown. Product inhibition by ammonium does not inhibit urease activity (193). Whether glutamine or glutamate inhibit the enzyme, or are involved in its control, is unknown (191,193). Similarly, the role of amino acids in the control of this enzyme has not been defined, although the metabolism of these may also produce ammonium.

There are two possible roles for the organism's urease activity. It may be used to create an alkaline microenvironment which could explain the increased gastrin concentrations in patients with the infection. An alkaline microenvironment could also explain the survival of the organism in the gastric acid. The enzyme could also be used to scavenge nitrogen from urea. An alkaline microenvironment would not need to be created and the raised concentrations of gastric juice ammonium would

therefore only represent urea nitrogen surplus to the organism's needs produced as a result of uncontrolled urease activity. Investigation of the function of the organism's urease activity may help explain its survival in the potentially hostile gastric environment and the development of raised gastrin concentrations which occur in association with infection of the antral mucosa with H pylori. The possibility of a new therapeutic approach to the treatment of duodenal ulcers might also exist as the organism is sensitive to low pH in the absence of the protective effect of urease activity.

2.12 Treatment of Infection with Helicobacter pylori

Of the treatments currently available for duodenal ulcers only the use of tripotassium dicitrato bismuthate (DeNol) (Gist Brocades, Surrey, UK) as part of triple therapy with metronidazole and amoxycillin may result in prolonged healing of a duodenal ulcer and also in eradication of Helicobacter pylori (144-146). Treatment with H_2 receptor antagonists such as ranitidine (Glaxo, Glaxo Laboratories, Middlesex, UK) and cimetidine (Smith, Kline and French Laboratories, Welwyn Garden City, UK) and newer agents such as the proton pump inhibitor omeprazole (Astra Pharmaceuticals, Herts, UK) result in a decrease in gastric acid output and also lead to healing of the ulcer. Treatment with H_2 receptor antagonists does not eradicate H pylori. Failure to eradicate the organism is associated with recurrence of the ulcer once treatment has

stopped. Up to 80% of individuals treated with an H_2 receptor antagonist in one study relapsed within a year of stopping treatment (144). As a result maintenance treatment with H_2 receptor antagonists is necessary and adds to the cost of the treatment.

The proton pump inhibitor omeprazole (Astra Pharmaceuticals, Herts, UK) is also effective in healing duodenal ulcers. There is some evidence that treatment with this agent might reduce H pylori infection (204-207). Unfortunately many of these studies only tested for eradication of the organism on completion of treatment and not one month after cessation of treatment. There is no evidence of in vitro activity against H pylori (208). One study which did look for evidence of eradication of H pylori one month after treatment with omeprazole showed no activity against H pylori (209). It was suggested that the previous reports of eradication could have been due to bacterial overgrowth in the stomach as a result of raised gastric juice pH.

Omeprazole (Astra Pharmaceuticals, Herts, UK) is activated in an acidic environment and once activated combines covalently with sulphydryl groups to inactivate the H^+/K^+ ATPase of the gastric epithelial cells (210). The virtually complete stoppage of gastric acid production by omeprazole results in raised plasma gastrin concentrations. There is concern that these high plasma concentrations of gastrin could promote the development of gastrointestinal neoplasia and prolonged treatment of

duodenal ulcers with omeprazole (Astra Pharmaceuticals, Herts, UK) is not recommended (210). Tripotassium dicitrato bismuthate (DeNol) (Gist, Brocades, Surrey, UK) both heals duodenal ulcers and eliminates H pylori in some individuals. The mechanism by which ulcer healing occurs when this is used is not known. It is suggested that DeNol creates a barrier to the back diffusion of hydrogen ions thereby promoting the healing of ulcers (211-214). It also eradicates H pylori (144-146). This may be due to the known toxic effects of heavy metals (215,216). Although the eradication of H pylori results in prolonged remission from duodenal ulcer disease it is not essential for the healing of duodenal ulcers with bismuth preparations as the organism is not eradicated in some individuals treated with bismuth preparations (217). Thereby demonstrating that healing can occur despite continued colonisation with the organism.

Eradication of H pylori in up to 90-95% of subjects can be achieved by the combination of DeNol with amoxycillin and metronidazole, with treatment extended over a minimum of a 2 week period (144,146,218). The extensive use of this triple therapy in all patients with duodenal ulcers could result in the spread of antibiotic resistance among H pylori. There is already some evidence that this is occurring (219). This restricts the use of triple therapy for patients with persistent or complicated ulcer disease.

A new treatment which would help eradicate the organism but avoid the risk of antibiotic resistance would

allow the full potential benefits of treatment of H pylori infection to be achieved. Potential benefits from eradication of the organism would include cessation of lifelong treatment for ulcer disease, and a reduction in the incidence of gastric carcinoma and treatment of H pylori related disease in children.

2.13 Overall summary

While Helicobacter pylori may not be the sole cause of the development of duodenal ulcers there is clear evidence that infection of the gastric antral mucosa by the organism, is one of the most important acquired factors in the development of duodenal ulcers (218). In addition, persistence of infection of the antral mucosa following the healing of a duodenal ulcer almost inevitably results in the relapse of the duodenal ulcer (144-146, 217).

The eradication of the organism has been associated with a reduction in fasting gastrin concentration, meal stimulated gastrin release and acid output (19-26). This increase in gastrin concentration may be the link between the infection and the development of duodenal ulcer disease.

When antral infection is present the urease activity of this organism may cause increased gastric juice ammonium concentrations. Reduced gastric juice urea concentrations have been reported (182), but other investigators have found no difference in urea

concentrations between subjects with the infection and those without the infection (194,195).

The reason the organism possesses abundant urease activity is not known. It has been suggested that because H pylori's urease activity has a protective effect at low pH that possession of this enzyme may enable the organism to survive in the acid gastric environment through the neutralisation of gastric acidity (183-185).

Ammonium produced by urease activity might be used to maintain a favourable extracellular pH by creating an alkaline microenvironment close to the antral mucosal surface where the gastrin producing (G cells) are located. The production of ammonium with an increase in the local pH at this site could result in inappropriate gastrin release leading to the observed increased meal stimulated gastrin response and gastric acidity. If the urease activity was used to alter the pH of the extracellular environment of the organism then large amounts of extracellular ammonium would be produced. The pH of the environment close to the antral mucosal surface may be closer to neutrality than that of the gastric juice. If this is the case then H pylori may not need to possess urease activity to maintain a favourable extracellular pH. The organism might therefore possess urease activity to scavenge nitrogen from urea which diffuses from the bloodstream into the gastric juice. Cellular uptake of ammonium produced by urease activity would therefore be expected.

To distinguish between these possibilities and to clarify the biochemical effects of infection of the gastric antral mucosa with H pylori a series of studies were undertaken.

The effect of the organism's urease activity on gastric juice ammonium and urea concentrations in individuals with normal and increased plasma urea concentrations was studied. A relationship between gastric juice ammonium and urea concentrations and the presence of infection was sought.

The effect of stimulating and inhibiting the organism's urease activity on plasma gastrin concentration and meal stimulated gastrin response was investigated. The effect of increasing intragastric pH by the infusion of a buffer solution on basal plasma gastrin concentration and meal stimulated gastrin response in infected and non-infected subjects was also investigated.

In vitro experiments were also undertaken to investigate alteration in urease activity with pH, the effect of urease activity on survival of the organism in different pH environments, and uptake of ammonium produced from hydrolysis of urea.

These studies were conducted to establish whether the raised plasma gastrin concentrations found in individuals with duodenal ulcers resulted from urease activity creating an in vivo alkaline microenvironment and if the effect of the organism's urease activity on in vitro survival in hostile conditions was also due to the production of an alkaline environment.

CHAPTER 3

MATERIALS AND ANALYTICAL METHODS

MATERIALS AND ANALYTICAL METHODS

3.1 Detection of Helicobacter pylori infection

3.2 Culture of Helicobacter pylori

The method of Miles and Misra was used to establish survival of the organism in the samples (220). Viable colony counts were obtained on blood agar plates after 3 days of incubation at 37°C in a microaerophilic atmosphere (BBL CampyPak Gas Generating System). In the in vitro studies the survival of the organism was expressed as a percentage of the starting inoculum calculated from the dilution of the viable colony count in the initial broth suspension.

3.3 Histological Examination of Antral Biopsies

Antral biopsies were stained using the haematoxylin and eosin stain. They were examined double blind by a single pathologist. An aggregate gastritis score of between 1 and 10 was determined on biopsy samples by the method described by Rauws (125).

3.4 ^{14}C-Urea Breath Test

^{14}C-Urea breath tests were performed in the Department of Nuclear Medicine at the Western Infirmary in Glasgow. Following a 14-hour overnight fast the patient drank 240 ml Ensure Plus (Abbott, Maidenhead, UK) to delay gastric emptying. This was followed by 0.4 MBq ^{14}C-urea

in 20 ml water. Samples of breath carbon dioxide were then collected every 10 minutes for 30 minutes prior to administration of the isotope and for a further 120 minutes following it. Breath carbon dioxide was trapped by asking the patient to exhale through a tube of anhydrous calcium chloride into a vial containing 2 mmol of hyamine in 2 ml of ethanol. Phenolphthalein was added as an indicator and became colourless when 1 mmol of exhaled CO_2 had been trapped. The appearance of ^{14}C labelled CO_2 in the breath samples was followed by liquid scintillation counting of the trapped labelled CO_2 following the addition of 10 ml of scintillant to the vial. The area under the curve of the breath test was calculated using the trapezoid rule for the time periods 0-30 min, 0-40 min, 0-60 min and 0-120 minutes of the test.

3.5 Measurement of urea concentration in gastric juice and buffer solutions

Several methods for the measurement of urea in gastric juice samples and in buffer samples collected during in vitro experiments were used:

A) Chemical methods of urea analysis.

1) o-Pthalaldehyde method:

Urea was analysed in gastric juice and buffer samples collected during the in vitro experiments by the ortho-pthalaldehyde method using an American Monitor Perspective Analyser or an American Monitor Excel Analyser (American Monitor, West Sussex, UK) (221). When

appropriate samples were diluted 1 in 3 with 0.2 mol/L phosphate buffer pH 7.40 prior to analysis.

2) Diacetylmonoxime method: Urea was also measured in gastric juice samples using the diacetylmonoxime method (222). Reagents were purchased from the Sigma Chemical Company (Dorset, UK). No dilution of the gastric juice sample was required. 10 ul of sample was added directly to 5 ml of working diacetylmonoxime reagent, incubated for 10 min in a boiling water bath, cooled for 5 minutes in tap water and the absorbances were read at 575 nm using a SP8-200 UV/VIS scanning spectrophotometer (Pye Unicam, UK).

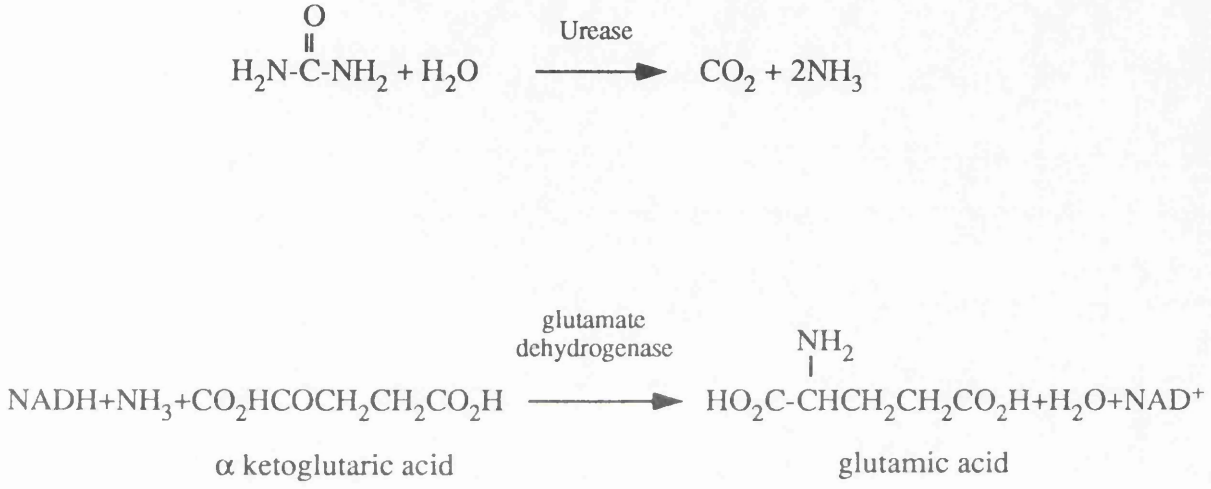
B) Enzymatic methods of urea analysis

1) Automated Urease methods

i) Cobas Bio Analyser

Urea was measured by following the decrease in absorbance at 340 nm due to the consumption of NADH by linked urease and glutamate dehydrogenase enzymatic reactions (Fig 3.1). Ammonia produced by the hydrolysis of urea was used to convert alpha ketoglutarate to glutamate by glutamate dehydrogenase with the consumption of NADH (223). Reagents were purchased from the Sigma Chemical Company (Dorset, UK) and the method automated using the Cobas Bio centrifugal

Fig. 3.1



The enzymatic measurement of urea. The concentration of urea is proportional to the rate of utilisation of NADH. This is followed by the decrease in absorbance at 340nm as NADH is consumed.

analyser (Roche, Welwyn Garden City, UK) following a protocol supplied by the Sigma Chemical Company (Dorset, UK) (Table 3.1).

ii) Continuous Flow Analysis

Samples of gastric juice from the in vivo infusion experiment were analysed for urea concentration using a modification of the urease method on a Technicon SMAC (Technicon, Basingstoke, UK). A blank channel in addition to the test channel was also used. Samples were diluted where appropriate using a 9 g/L saline solution.

2) Urease method linked to the Berthelot reaction

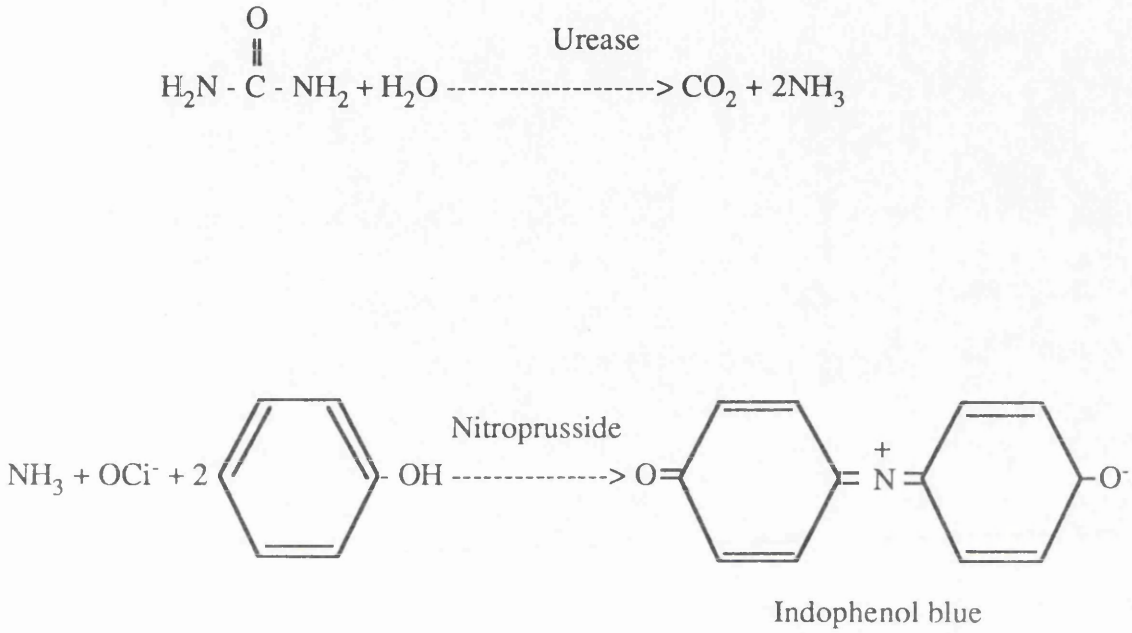
A urease method linked to the Berthelot reaction which detected the ammonia produced as a result of the hydrolysis of urea in the sample was also used (Fig 3.2) (224). Reagents were purchased from the Sigma Chemical Company (Sigma, Dorset, UK). Samples were diluted 1/10 in 0.2 mol/L phosphate buffer pH 7.4 prior to analysis. The urease reagent supplied was reconstituted using deionised water or 0.2 mol/L phosphate buffer pH 7.4. Samples (0.1 ml) were incubated for 30 minutes at room temperature with 0.5 ml of urease reagent in order to hydrolyse all urea present. Following addition of 3 ml of the acid reagent and 2 ml of the phenol reagent the samples were incubated at room temperature for 30 minutes to

Table 3.1

Units	3
Calculation Factor	0
Standard 1	1.75
Standard 2	3.45
Standard 3	15.7
Limit	25.0
Temperature (°C)	30
Type of Analysis	4
Wavelength (nm)	340
Sample Volume (ul)	3
Diluent Volume (ul)	30
Reagent Volume (ul)	250
Incubation time	0
Second reagent	0
Time of 1st Reading	20
Time interval	20
Number of readings	2
Blanking mode	1
Printout mode	2

Instrument settings used with the enzymatic urease method for measuring urea installed on a Cobas Bio centrifugal analyser (Roche, Welwyn Garden City, UK).

Fig. 3.2



The Berthelot reaction for the measurement of urea.
The concentration of urea is proportional to the amount of indophenol blue produced. Indophenol blue may be measured by its absorbance at 575nm.

allow the colour to develop. The concentration of urea was proportional to the absorbance at 575 nm. A blank for each sample was also analysed.

3.6 Measurement of Ammonium Concentration in gastric juice and buffer solutions

The ammonium concentration in these samples was measured by an enzymatic method (Sigma Chemical Company Ltd., Dorset, UK) (225). The detection of ammonium ions was followed by the consumption of NADH during conversion of alpha ketoglutarate and ammonium ions to glutamate by glutamate dehydrogenase. The samples from both in vivo and in vitro experiments were diluted with 0.2 mol/L phosphate buffer pH 7.40 to bring the concentration of ammonium into the linear range of the method (30-1000 umol/L). The analysis of ammonium ions in these samples was automated using a Cobas Bio Centrifugal Analyser (Roche, Welwyn Garden City, UK). The program used to operate the instrument was supplied by Sigma Chemical Co (Dorset, UK) (Table 3.2).

In the in vitro experiments the percentage recovery of ammonium was calculated assuming that the urea lost from the solution had been completely hydrolysed. The final urea concentration in the buffer was subtracted from the initial urea concentration and the result multiplied by 2 to give the expected final ammonia concentration. The measured concentration of ammonium could then be

Table 3.2

Units	12
Calculation Factor	0
Standard 1	5
Standard 2	5
Standard 3	0
Limit	20
Temperature	30
Type of Analysis	6
Wavelength	340
Sample Volume (ul)	40
Diluent Volume (ul)	20
Reagent Volume (ul)	260
Incubation time	300
Start Reagent Volume (ul)	20
Time of first reading	10.0
Time interval	300
Number of readings	2
Blanking mode	1
Printout mode	1

Instrument settings used with the ammonia method installed on a Cobas Bio Centrifugal analyser (Roche, Welwyn Garden City, UK).

expressed as the percentage of the expected final ammonium concentration calculated from the fall in urea concentration to give percentage recovery of ammonia.

3.7 Measurement of Urease activity

A kinetic spectrophotometric assay was developed for the measurement of urease activity in samples from in vitro studies. The urease activity in samples was measured by following the consumption of ammonia produced as a result of urease activity by a linked glutamate dehydrogenase reaction (Fig 3.3). Glutamate dehydrogenase uses NADH in the production of glutamate from ammonia and alpha ketoglutarate. The rate of ammonia production was therefore followed by the consumption of NADH which was measured by the change in absorption of the incubate at 340 nm. The concentrations of the reagents were arranged so that the utilisation of NADH would be directly proportional to urease activity in the sample. Samples containing urease activity were incubated with excess glutamate dehydrogenase, alpha ketoglutarate and NADH. A 2.5 mol/L urea solution was used as the start reagent. A pre-incubation period of 3 min prior to the addition of the urea start reagent was used to eliminate any endogenous ammonia. Jackbean Urease (EC 3.5.1.5) supplied by Sigma was used as a standard (Sigma Chemical Company, Dorset, UK). The urease assay was linear up to a concentration of 500 IU/L. The instrument settings used are listed in Table 3.3.

Fig. 3.3

$$\text{Urea} + \text{Urease activity} \dashrightarrow CO_2 + NH_3$$

$$NADH + NH_3 + \alpha\text{ ketoglutarate} \xrightarrow[\text{Dehydrogenase}]{\text{Glutamate}} NAD^+ + \text{glutamate} + H_2O$$

The urease activity in the sample is measured by the rate of utilisation of NADH in the linked glutamate dehydrogenase reaction.

Table 3.3

Units	10
Calculation Factor	1
Standard 1	0
Standard 2	0
Standard 3	0
Limit	0
Temperature	37
Type of Analysis	3
Wavelength	340
Sample Volume (ul)	2
Diluent Volume (ul)	20
Reagent Volume (ul)	280
Incubation time	120
Start Reagent Volume (ul)	50
Time of first reading	10.5
Time interval	10
Number of readings	18
Blanking mode	1
Printout mode	3

Instrument settings used with the urease assay installed on a Cobas Bio Centrifugal analyser (Roche, Welwyn Garden City, UK).

3.8 Measurement of Gastrin concentration

Blood samples for gastrin determination were collected in lithium heparin tubes containing 100 kIU of trasolol. Following centrifugation at 3,000 g for 10 min in a refrigerated centrifuge at 4°C the plasma was stored at -20°C until analysis. Plasma gastrin was initially measured at Queens University Belfast, by a radioimmunoassay using antibody R98 which has a lower limit of detection of 5-10 ng/L (226). Plasma gastrin concentrations in subsequent studies were measured using a commercially available radioimmunoassay (CIS (UK) Ltd., Buckinghamshire, UK). This gastrin assay reacted 100% with gastrin 17 and 72% with gastrin 34. The minimum detection limit of the assay was 20 ng/L. The inter-assay coefficient of variation was less than 10% from 40-700 ng/L. All samples for each patient were measured with the same assay. All samples from each experiment were measured in the same batch.

3.9 Manufacture of Buffer solutions and other solutions for in vivo experiments

1) Dextrose solution for intragastric infusion:
   The pH of 500 ml of 5% Dextrose solution for intravenous infusion (Baxter, Surrey, UK) was adjusted to 1.80 by the addition of 1 ml of concentrated hydrochloric acid. To add urea, when required, to this solution, 1.5 g of sterile urea powder was dissolved in 30 ml of the

dextrose solution aspirated aseptically from the sealed 500 ml intravenous solution bag and when dissolved was reinjected into the bag.

2) 0.2 mol/L Citrate Buffer pH 7.0.

100 ml of fresh 2 mol/L citric acid solution was added to 315 ml of fresh 2 mol/L sodium hydroxide solution in a 1L volumetric flask. Water was added up to the 1L mark and mixed. The buffer was then sealed in 500 ml glass bottles with a top containing a self-sealing rubber septum. The solution was sterilised by heating. It was stored in the dark at room temperature until use.

3.10 Manufacture of Buffer and other solutions for in vitro experiments

1) A series of 0.2 mol/L citrate buffers with a pH ranging from 2.0 to 7.0 were made using stock solutions of 2 mol/L citric acid and 2 mol/L sodium hydroxide.

To 100 ml of fresh stock 2 mol/L citric acid the amounts of 2 mol/L sodium hydroxide listed in Table 3.4 were added and the final volume of the solution was made to 1L with distilled water.

To make a 0.2 mol/L citrate buffer pH 1.5 10 ml of concentrated hydrochloric acid was added to 100 ml stock 2 mol/L citric acid and the volume of the solution was made to 1L with distilled water.

Table 3.4

0.2 mol/L citrate buffer solutions

Required pH	Volume of 2 molar NaOH (ml)
2.0	0
3.0	60
4.0	130
5.0	210
6.0	280
7.0	315

The volume of 2 mol/L NaOH to be added to 100 ml 2 mol/L citric acid solution to produce 1L buffer with the required pH. The final volume is made up to 1L with distilled water.

In experiments comparing 0.2 mol/L citrate buffer pH 3.0 with 0.2 mol/L citrate buffer pH 6.0, the osmolality of the pH 3 buffer was adjusted to be the same as that of the pH 6.0 buffer by adding 0.641 g sodium chloride per 100 ml pH 3.0 buffer. Isomolar saline solution used in the experiments was 0.25 mol/L sodium chloride solution. The pH of this solution prior to use was adjusted if necessary to 6.0.

2) 0.2 mol/L Isocitrate Buffer

The trisodium salt of isocitric acid was obtained from the Sigma Chemical Company (Poole, Dorset). To make a 0.2 mol/L solution pH 6.0, 12.68 g of powder was dissolved in 250 ml of deionised water. The pH was adjusted to 6.0 by the addition of 1 mol/L hydrochloric acid.

3) 0.2 mol/L Sodium Acetate Buffer

To make 1L 27.2 g sodium acetate (trihydrate) was dissolved in 1L of water and the pH adjusted to 6.0 with glacial acetic acid. The osmolality was adjusted to that of 0.2 mol/L citrate buffer with NaCl solution.

4) 0.2 mol/L Phosphate Buffer

To make 1L 13.68 g of sodium dihydrogen phosphate and 1.75 g of disodium hydrogen phosphate were dissolved in deionised water. The pH was adjusted to 6.0 if necessary and the volume made

to 1L. The osmolality was adjusted to that of 0.2 mol/L citrate buffer with NaCl solution.

3.11 Collection of Fasting Gastric Juice Samples

During endoscopy and just after emptying the stomach 2 ml of gastric juice was aspirated through the suction channel of the endoscope and collected in a trap inserted in the suction line. Samples were sealed in 2 ml tubes and frozen at -20°C until analysis. On thawing samples were centrifuged at 3000 g for 10 min to remove the mucous.

3.12 Chemicals

All reagents and chemicals were of analar grade and were supplied by BDH (Thornliebank, Glasgow) except where otherwise stated.

3.13 Statistical Methods

Non-parametric statistical methods were used for the analysis of the data. The Sign Rank Wilcoxon test was used for paired data and for non-paired data the Mann Whitney U test was used.

3.14 Ethical Permission

All studies involving patients received the approval of the Western Infirmary Ethical Committee, and each patient gave written informed consent.

CHAPTER 4

THE MEASUREMENT OF UREA AND AMMONIUM CONCENTRATIONS IN GASTRIC JUICE

THE MEASUREMENT OF UREA AND AMMONIUM CONCENTRATIONS IN GASTRIC JUICE

4.1 Introduction

There is disagreement concerning the concentrations of urea and ammonium in gastric juice samples from subjects with *Helicobacter pylori* infection. Marshall and colleagues reported mean urea concentrations of 0.45 mmol/L in individuals with the organism compared with 2.9 mmol/L in individuals who were not infected (182). Mean urea concentrations of 3.27 mmol/L and 4.8 mmol/L for *H pylori* positive individuals and 3.4 and 4.3 mmol/L for *H pylori* negative individuals have also been reported by other groups (194,195).

There is also disagreement concerning the concentrations of ammonium ions in gastric juice. Marshall and colleagues found mean ammonium concentrations of 34 mmol/L in infected subjects and 11.5 mmol/L in non-infected subjects (182). The difference in ammonium concentrations between infected and non-infected subjects was considered to be a poor indicator of the presence of infection (182). Kim and colleagues reported mean ammonium concentrations of 5.48 mmol/L in samples from infected subjects and mean concentrations of 1.26 mmol/L in non-infected subjects (196). In this study raised gastric juice ammonium concentrations were considered to

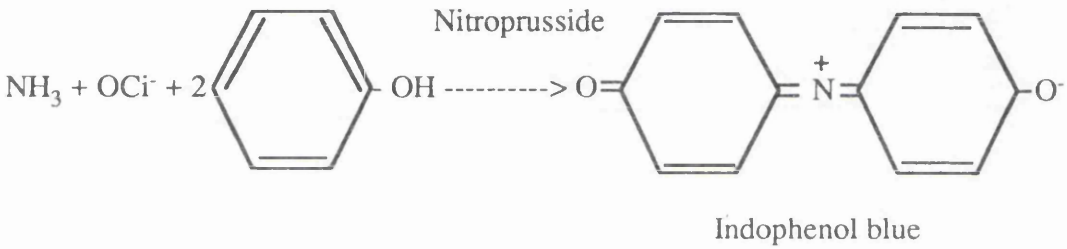
be a useful indicator of the presence of infection with a predictive value of 78.3% if a threshold of 3 mmol/L was exceeded.

In none of these studies were the methods of measuring urea and ammonium concentrations clearly described (182,195,196).

Several different methods may be used for the measurement of urea and ammonium concentrations in biological samples (221-225). There are also well recognised potential interferences in the measurement of urea and ammonium in biological samples. Interference in the measurement of urea may occur as a result of the measurement of ammonia present in samples when urease linked methods such as the berthelot reaction (Fig 4.1) or other urease methods are used (Fig 4.2). The low pH of gastric juice samples could also interfere in the enzymatic methods of measurement of urea by inhibiting the urease enzyme. Differences between the matrix of serum and gastric juice samples such as a lower protein concentration, or lower gastric juice pH, the presence of mucous or high bile acid concentrations might interfere in both urease and chemical methods of urea measurement.

Ammonia concentrations may be difficult to measure accurately as a result of contamination of samples with ammonia from the laboratory environment as methods for the measurement of ammonium are able to detect micromolar concentrations of ammonium. The activity of urease,

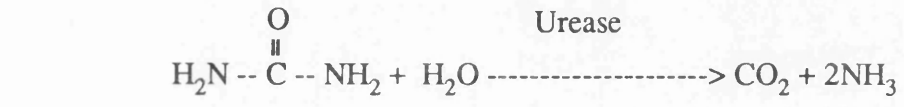
Fig. 4.1

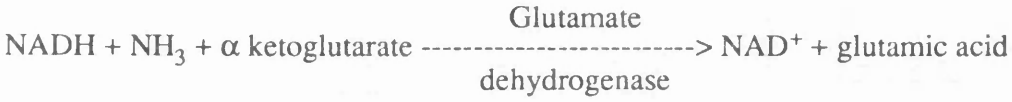


Indophenol blue

The Berthelot reaction for the measurement of urea.
The concentration of urea is proportional to the amount of indophenol blue produced. Indophenol blue may be measured by its absorbance at 575nm.

Fig. 4.2





The enzymatic measurement of urea. The concentration of urea is proportional to the rate of utilisation of NADH. This is followed by the decrease in absorbance at 340nm as NADH is consumed.

protease or transaminase enzymes in samples could also lead to falsely high measured concentrations of ammonia. The low pH of gastric juice samples might also interfere with the activity of glutamate dehydrogenase used in the enzymatic measurement of ammonia (Fig 4.3).

The use of different methods of measuring urea and ammonium concentrations in gastric juice might have resulted in the different concentrations of urea and ammonium reported in gastric juice samples. An assessment of methods of measuring these analytes in gastric juice samples was therefore conducted.

4.2 Methods

Following the passage of a nasogastric tube and pentagastrin stimulation gastric juice was collected from one healthy volunteer who had H pylori infection. The gastric juice samples were stored at -20°C until analysis.

The effects of pH and gastric juice ammonium concentration on the imprecision, inaccuracy and detection limit of 4 methods of urea analysis were investigated. The methods investigated were a rate reaction enzymatic method automated on the Cobas Bio (Roche, Welwyn Garden City, UK), the o-phthalaldehyde method automated on the Excel analyser (American Monitor, West Sussex, UK), a manual diacetylmonoxime method (Sigma Chemical Company, Dorset, UK) and a manual urease method linked to

Fig. 4.3

$$NADH + NH_3 + \alpha \text{ ketoglutarate} \xrightarrow[\text{dehydrogenase}]{\text{Glutamate}} NAD^+ + \text{glutamic acid}$$

The measurement of ammonia. The concentration of ammonia is proportional to the rate of utilisation of NADH. This is followed by the reduction in absorbance of the solution at 340nm

the berthelot reaction (Sigma Chemical Company, Dorset, UK). The effect of pH on the inaccuracy and imprecision of the enzymatic ammonia method automated on the Cobas Bio (Roche, Welwyn Garden City, UK) (Sigma Chemical Company, Dorset, UK) was also investigated.

1) The effect of pH on the measured gastric juice urea concentration

This was examined by taking portions of the gastric juice and adjusting the pH of the juice by the addition of small amounts of 2 mol/L sodium hydroxide solution or 1 mol/L hydrochloric acid. The pH of the gastric juice was measured using a Corning 220 pH meter (Corning, Herts, UK). Once the basal pH of the gastric juice had been measured 75 ul of 2 mol/L sodium hydroxide was added to increase the pH to 4.0, a portion of pH 4.0 gastric juice was set aside and the pH of the remainder was again increased to pH 6.5 by the addition of more sodium hydroxide solution. Again a portion of the gastric juice was set aside. The pH of the remaining gastric juice was then titrated back to the original value by the addition of approximately 50 ul of 1 mol/L hydrochloric acid. Portions (0.9 ml) of the four pH adjusted gastric juice samples then had 0.1 ml of stock 5 mol/L urea solution or deionised ammonia free water added to produce gastric juice samples with a low urea concentration, or 5 mmol/L urea.

The inaccuracy of the measurement of urea in gastric juice samples with a low pH was assessed by calculating

the recovery of added urea in portions of gastric juice to which urea had been added to give concentrations of 2.5, 5, 10 and 20 mmol/L. The samples were assayed by each method in triplicate.

2) The effect of ammonium chloride on measured urea concentration

Gastric juice samples had 0.1 ml of 100 mmol/L solution of ammonium chloride added to increase the total ammonium concentration by 10 mmol/L. These samples containing ammonia were split into 2 portions and stock urea solution added to give a final urea concentration of 5 mmol/L in one portion. The concentrations of urea were then measured in each of the portions of gastric juice by all four methods.

3) The detection limit of urea analysis

Samples of gastric juice were spiked with stock urea solution to give a final urea concentration of 5 mmol/L. The gastric juice was then diluted, 1/2, 1/5, 1/10, 1/20 to give a range of urea concentrations from undetectable to 5 mmol/L. The urea concentrations in these dilutions were measured by all four methods.

4) The effect of pH on the measured gastric juice ammonium concentration

Samples of gastric juice had their pH adjusted as described for urea. Portions of 100 mmol/L ammonium chloride solution were then added to produce 5 mmol/L and 10 mmol/L ammonia concentrations. Samples were then analysed in quintuplicate for ammonium concentration using

the Sigma method adapted for the Cobas Bio centrifugal analyser.

The inaccuracy of the ammonia method was assessed by measuring the recovery of ammonium chloride which was added to portions of gastric juice to give final concentrations of 2.5, 5, 7.5 and 10 mmol/L. Basal gastric juice samples collected from several other subjects who had H pylori infection were also analysed in replicate to assess the imprecision of measurement using native gastric juice.

5) The stability of gastric juice ammonium concentration on storage

Samples were stored in a freezer at -20°C over a 3 week period and repeatedly re-analysed.

4.3 Results

1) The effect of pH on urea measurement

Alteration of the pH of the samples did not markedly interfere in any of the 4 urea methods investigated (Table 4.1 and Table 4.2). Measured urea concentrations compared well with the expected concentrations with all the methods except the berthelot linked urease method (Table 4.2). The use of 0.2 mol/L phosphate buffer as a diluent for the urease enzyme used in the berthelot method did not increase the urea concentration obtained by this method. The enzymatic urease method needed to be blanked as the measured basal gastric juice urea concentrations were

Table 4.1

	Mean Urea concentration in gastric juice (mmol/L)			
	pH 1.56	pH 4.13	pH 6.49	pH 1.60
o-pthalaldehyde method	0.3	0.2	0.2	0.2
Diacetylmonoxime method	0.3	0.2	0.2	0.3
Urease method unblanked	1.9	1.9	1.7	1.8
Berthelot linked Urease method	0.3	0.2	0.2	0.1

The effect of gastric juice pH on the measurement of basal urea concentrations prior to the addition of urea to samples.

Table 4.2

	Mean Urea concentration in gastric juice mmol/L			
	pH 1.56	pH 4.13	pH 6.49	pH 1.60
o-pthalaldehyde method	4.8	4.6	4.6	4.9
Diacetylmonoxime method	4.5	4.3	4.3	4.9
Urease method				
unblanked	7.0	6.9	6.9	6.6
(blanked)	(5.2)	(5.1)	(5.2)	(4.8)
Berthelot linked Urease method	4.3	3.0	3.0	4.0

The effect of gastric juice pH on the measurement of urea concentrations after the addition of 5 mmol/urea.

higher with this method than in the other methods (Table 4.1).

The percentage recovery of added urea is shown in Table 4.3. The recovery of urea was best with the blanked enzymatic urease method (97-105%) and with the o-pthalaldehyde method (92-98%).

The inter-assay coefficient of variation (CV) of analysis at each of the urea concentrations investigated demonstrated that the blanked urease enzymatic method and o-pthalaldehyde methods were least imprecise (Table 4.4). The bertholet reaction had unacceptable imprecision (20.4%-59.2%).

2) The effect of ammonium on the measurement of urea

The addition of ammonium chloride did not interfere with the measurement of urea by either the diacetyl monoxime method or the o-pthalaldehyde method (Table 4.5). There was interference in the enzymatic urease method when it was not blanked. This method depends on the measurement of ammonia to determine urea concentration. If appropriate blanking was used then this was not a problem (Table 4.2).

3) The detection limit of urea analysis

The automated o-pthalaldehyde method had the lowest detection limit (Table 4.6).

Table 4.3

Percentage recovery of added urea

	pH 1.56	pH 4.13	pH 6.49	pH 1.60
o-pthalaldehyde method	96	92	92	98
Diacetylmonoxime method	89	86	86	98
Urease method (blanked)	104	101	105	97
Berthelot linked Urease method	86	60	60	80

Recovery of 5 mmol/L urea added to portions of gastric juice.

Table 4.4

	Coefficient of Variation at a range of urea concentrations			
	2.5mmol/L	5mmol/L	10mmol/L	20mmol/L
o-pthalaldehyde method	5.4	6.0	4.6	3.9
Diacetylmonoxime method	16.0	12.0	9.8	8.3
Urease method blanked	5.4	2.8	10.2	10.6
Berthelot linked urease method	45.6	20.4	33.5	59.2

Inter-assay imprecision in the measurement of urea in gastric juice samples.

Table 4.5

	Measured Urea concentration (SD) mmol/L
o-pthalaldehyde method	0.2 (0.05)
Diacetylmonoxime method	0.0 -
Urease method not blanked	4.2 (0.14)
Berthelot linked urease method	0.4 (0.3)

The effect of adding 10 mmol/L ammonium chloride on the measurement of urea in fasting gastric juice samples pH 1.38.

Table 4.6

	Detection limits (mmol/L)
o-pthalaldehyde method	0.3
Diacetylmonoxime method	0.5
Urease method blanked	0.7
Berthelot linked urease method	0.6

The detection limits of the 4 urea methods.

4) The effect of pH on the measurement of gastric juice ammonium

Alteration of gastric juice pH over the range 2.29 to 7.20 did not affect the measurement of ammonium (Table 4.7). The samples were diluted 1/4 or 1/20 (prior to analysis) depending on the ammonium concentration with 0.2 mol/L phosphate buffer pH 7.4. This reduced the concentration of ammonia in the sample to that of the linear range of the assay which was between 30 umol/L and 1 mmol/L. The phosphate buffer neutralised the effects of gastric juice acidity. If distilled water was used as the diluent then the measured concentrations of ammonia were reduced. The inter-assay coefficient of variation of the enzymatic ammonia method (including dilution) using gastric juice samples with added ammonium chloride ranged from 17.2% for 2.5 mmol/L ammonium to 6.5% with 7.5 mmol/L ammonium (Table 4.8). The inter-assay coefficient of variation from a range of samples from several individuals ranged from 8.5% at 2.3 mmol/L to 1.5% at 11.1 mmol/L ammonium (Table 4.9).

The intra-assay coefficient of variation of the standard with 10 mmol/L ammonium concentration was 1.0% while the inter-assay coefficient of variation was 2.0%. Using samples from patients the intra-assay CV including dilution varied from 8.5% at an ammonium concentration of 2.3 mmol/L to 1% at an ammonium concentration of 13.0 mmol/L.

Table 4.7

	Gastric juice ammonium concentration (SD) at a range of sample pH			
	pH 2.29	pH 4.04	pH 7.20	pH 2.29
Ammonium chloride not added	0.98 (0.010)	0.97 (0.016)	0.93 (0.016)	0.90 (0.016)
5 mmol/L added ammonium chloride	5.5 (0.14)	5.0 (0.05)	5.4 (0.07)	5.2 (0.26)
10 mmol/L added ammonium chloride	9.8 (0.16)	9.8 (0.07)	9.8 (0.51)	9.4 (0.26)

The absence of any effect of the pH of gastric juice samples on the measurement of ammonium concentration (n=5) using the enzymatic method adopted for the Cobas Bio (Roche, Welwyn Garden City, UK).

Table 4.8

	Added concentration of ammonium			
	2.5mmol/L	5mmol/L	7.5mmol/L	10mmol/L
Measured concentration of ammonium	3.0	5.6	7.9	10.3
Inter-assay CV	17.2%	9%	6.5%	9.9%
% recovery (mean initial ammonium concentration was 0.75 mmol/L)	94%	97%	95%	96%

The inter-assay coefficient of variation and recovery of the measurement of gastric juice samples to which ammonium chloride had been added.

Table 4.9

	Gastric juice samples from patients			
	mmol/L			
Gastric juice Ammonium concentration	2.3	4.7	6.5	11.1
Inter-assay coefficient of variation (%)	8.5%	1.9%	2.1%	1.5%

The inter-assay coefficient of variation in the measurement of gastric juice samples from several individuals.

The measured ammonium concentration in the samples was linear with serial dilution. The detection limit of the assay was 30 umol/L.

5) The Stability of ammonium during storage

Samples kept in a freezer at -20° also demonstrated no significant change in ammonium concentration over a 21 day period.

4.4 Discussion

The o-pthalaldehyde method and the enzymatic urease method, when blanked proved the most suitable methods for the measurement of urea. Both of these were automated methods and suitable for large numbers of samples. The berthelot method of measurement of urea did not prove suitable for use with gastric juice samples. Its imprecision, when compared with the manual diacetyl-monoxime method suggested an interference in the method. This was not due to the pH of the sample because reconstituting the urease enzyme with 0.2mol/L phosphate buffer pH 7.4 instead of the deionised water in order to neutralise the pH of the samples did not eliminate the interference. It may have been due to a matrix effect of the gastric juice, such as the concentration of bile acids.

The diacetylmonoxime method of measurement of urea may also be suitable for use with gastric juice samples if care were to be taken to improve the imprecision of the method.

The enzymatic ammonium method had acceptably low imprecision and high sensitivity. The pH of the sample did not interfere in measurement of ammonium concentration. Samples needed to be diluted with 0.2 mol/L phosphate buffer pH 7.4 prior to analysis to bring the ammonia concentration to within the linear range of the method. Gastric juice ammonium concentrations also proved stable on storage at -20°C. This may have been due to the reduced protein content of gastric juice compared with plasma, the low level of enzymatic activity as a result of the acid pH of the sample and the trapping of ammonium ions in solution due to the acidity of gastric juice.

The improved imprecision of ammonium measurement observed when samples from subjects with H pylori infection were compared with samples with added ammonium may have been the result of error in the addition of ammonium to replicate samples. When these samples were diluted prior to analysis the effect of the error in dilution on the result would have been magnified.

This study has demonstrated that the differing reports of urea concentration in gastric juice samples (182,194,195) should not have been the result of the use of different well controlled methods. It is possible that the report of a mean urea concentration of 4.8 mmol/L in samples from subjects who had infection may have been due to failure to use blanks to control the interference of

endogenous ammonia in the urease method used (194). The method of measuring urea used by the second group which found mean urea concentrations of 3.27 mmol/L in samples from subjects with H pylori infection was not well described (195). The differences in reports of gastric juice urea and ammonium concentrations might also have been due to a failure to properly distinguish between individuals with H pylori infection and those free from infection.

Care also needs to be taken during the analysis of samples by manual methods to ensure that there is not unacceptable imprecision in the method. High imprecision could lead to apparent differences between samples due to analytical error.

In conclusion, differences in reports of gastric juice urea and ammonium concentration should not be the result of the use of different methods when well controlled. Other reasons for the reported differences must be sought.

CHAPTER 5

THE DETECTION OF HELICOBACTER PYLORI INFECTION OF THE GASTRIC MUCOSA BY MEASUREMENT OF GASTRIC ASPIRATE AMMONIUM AND UREA CONCENTRATIONS

THE DETECTION OF HELICOBACTER PYLORI INFECTION OF THE GASTRIC MUCOSA BY MEASUREMENT OF GASTRIC ASPIRATE AMMONIUM AND UREA CONCENTRATIONS

5.1 Introduction

The remarkably high urease activity of *Helicobacter pylori* has been used to detect infection of the gastric antral mucosa by the organism. At present there are two methods of diagnosing *H pylori* infection based on its urease activity. One uses the detection of ammonia formation following incubation of an antral biopsy in a urea containing medium (68-71). There are several variants of this method. The second method requires the oral administration of ^{14}C or ^{13}C labelled urea and subsequent analysis of labelled CO_2 excreted in the breath (72-74). The isotopic breath tests take 1-2 h to complete and the final result may not be available for several days, while the methods based on the detection of urease activity may take from a few minutes to 24 h to complete.

The organism's high urease activity would be expected to cause characteristically raised gastric juice ammonium concentrations and reduced gastric juice urea concentrations when infection was present. These findings have been reported by Marshall and his colleagues (182) but not by others (194,195).

If there are characteristic changes in gastric juice then these might be used for detecting presence of the infection. The ability to detect this infection and to confirm its eradication is likely to become increasingly important in the management of patients with duodenal ulcer disease. Any new method of detection of the organism which used currently available laboratory based analytical methods and did not rely on the purchase of new equipment, or reagents could therefore prove useful.

The concentrations of urea and ammonium in gastric juice samples from infected subjects and after the bacterium's eradication were measured to establish the characteristic changes associated with infection. The usefulness of the measurement of these concentrations in the detection of the presence of the organism was also investigated. Subjects with the infection who had normal renal function, or who had chronic renal failure and also following treatment with H_2 receptor antagonists were investigated.

5.2 Patients

1. Subjects with Duodenal Ulcers

Twenty-seven patients (21 male, age range 18-64 years) with a history of endoscopically proven duodenal ulceration were examined. Two of them were taking ranitidine but none had been prescribed omeprazole. All had normal renal function. Twelve were only examined on one occasion having never received any anti-*H pylori*

treatment. Twelve were examined before and one month after completing a 4 week course of tripotassium dicitrato bismuthate 120 mg tid, metronidazole 400 mg tid and amoxycillin 250 mg tid. Three patients were examined only one month after the same treatment. At each time they were examined an upper gastrointestinal endoscopy and ^{14}C-urea breath test were performed. All the patients fasted for 16 h prior to endoscopy.

During endoscopy and just after entering the stomach 2 ml of gastric juice was aspirated through the suction channel of the endoscope and collected in a trap inserted in the suction line. Routine inspection of the upper gastrointestinal tract was then performed and following this an antral biopsy was obtained for histological examination. The biopsy was fixed in formalin prior to staining with haematoxylin and eosin. The severity of histological gastritis was scored as described by Rauws (125) and the presence of H pylori noted.

2. Subjects with Chronic Renal Failure

A further 16 patients (9 male) with established chronic renal failure and dyspeptic symptoms were investigated to examine the effect of chronic renal failure on gastric juice urea and ammonium concentrations. Two were examined before and after treatment to eradicate the infection while the other 14 patients were examined only on one occasion.

As previously described 2 ml gastric juice samples

and antral biopsies were collected during routine upper gastrointestinal endoscopic examination. A ^{14}C-urea breath test was also performed on each occasion a patient was examined. The biopsy was fixed and examined and inflammation scored as previously described. Only the peak 10 or 20 min ^{14}C-urea breath test value was recorded.

3. Subjects treated with Ranitidine

A further 10 patients (3 *H pylori* positive, 7 *H pylori* negative) were investigated for the effect of H_2 receptor antagonist treatment on the concentrations of urea and ammonium in gastric juice. A fasting sample of gastric juice was obtained from each patient before and following a 7 day course of ranitidine 300 mg nocte. The second gastric juice sample was collected within 14 h of the last dose of ranitidine.

5.3 Methods

The gastric juice samples were stored frozen at -20°C until analysed. Prior to analysis the samples were centrifuged at 3000 g for 10 min to remove the mucous. The concentration of ammonium was measured in the supernatant following dilution in 0.2 mol/L phosphate buffer pH 7.4 using the enzymatic method (Sigma Chemical Co., Dorset, UK) adapted for the Cobas Bio (Roche, Welwyn Garden City, UK).

Urea concentrations were measured using an automated urease method with a blank channel to control endogenous

ammonia concentrations (SMAC I, Technicon, Basingstoke, UK). All samples were analysed blind without the knowledge of the presence of infection by the organism in individuals studied.

The ^{14}C-urea breath tests were undertaken as previously described. Each breath test was performed within 5 days of endoscopic examination.

5.4 Results

5.5 Helicobacter pylori status of subjects studied

1) Subjects with Duodenal Ulcers

In the 24 duodenal ulcer patients who had not received anti-*H pylori* treatment, the organism was present in each on examination of their antral biopsy. In each patient with the organism antral gastritis was present and the median score for severity of the gastritis was 5 (range 2-8). In 14 of the 15 patients examined one month after completing a course of anti-*H pylori* treatment *H pylori*-like organisms were not identified in the antral biopsies. None had gastritis scores greater than 1. In one patient treatment failed to eradicate the infection and the bacterium was still present in the mucosa and was associated with persistent antral gastritis with a severity score of 5.

Analysis of the area under the 2 hours of the breath test showed clear separation of the patients with histological evidence of eradication of the infection from

patients with evidence of the infection and who had never received anti-H pylori treatment (Table 5.1). The values in the former were all less than 20 (range, 3-18) and in the latter all were greater than 60 (range, 63-267). In the one patient with histological evidence of failure of eradication the value was 114 before treatment and 90 after treatment. She was therefore included only as a positive for H pylori in the further analysis. The higher single value of either the 10 min or 20 min breath test result was also found to clearly separate the patients into the two groups indicating that a shortened 20 min breath test could be as useful as the complete 2 h test (Table 5.1).

2) Subjects with Chronic Renal failure

H pylori like organisms were detected by antral biopsy from 4 of the 14 patients with chronic renal failure investigated on only one occasion. Infection was associated with antral gastritis with a median score of 4 (range, 3-6). In the remaining 10 patients examined on only one occasion H pylori was not detected on examination of antral biopsy or by culture for the organism. None had gastritis scores greater than 1.

Two patients were examined before and after treatment to eradicate H pylori. Following treatment the organism had been eradicated from both individuals who had antral gastritis scores of 1 and 2.

In the H pylori positive individuals the median peak

Table 5.1

Area under breath test curve

Median (Range)

(% dose/mmol CO_2 x kg x min)

	0-30 min	0-40 min	0-60 min	0-120 min	Peak of 10/20 min breath test result (% dose/ mmol CO_2 x kg x 100)
Pre-eradication of H pylori	25 (6-42)	41 (12-69)	76 (23-127)	152 (63-267)	137 (39-225)
Post-eradication of H pylori	0.6 (0.1-1.1)	0.7 (0.3-2.5)	1.7 (0.8-7.2)	5.0 (3.0-18.0)	2.6 (0-5.1)

^{14}C-urea breath test results from individuals with normal renal function.

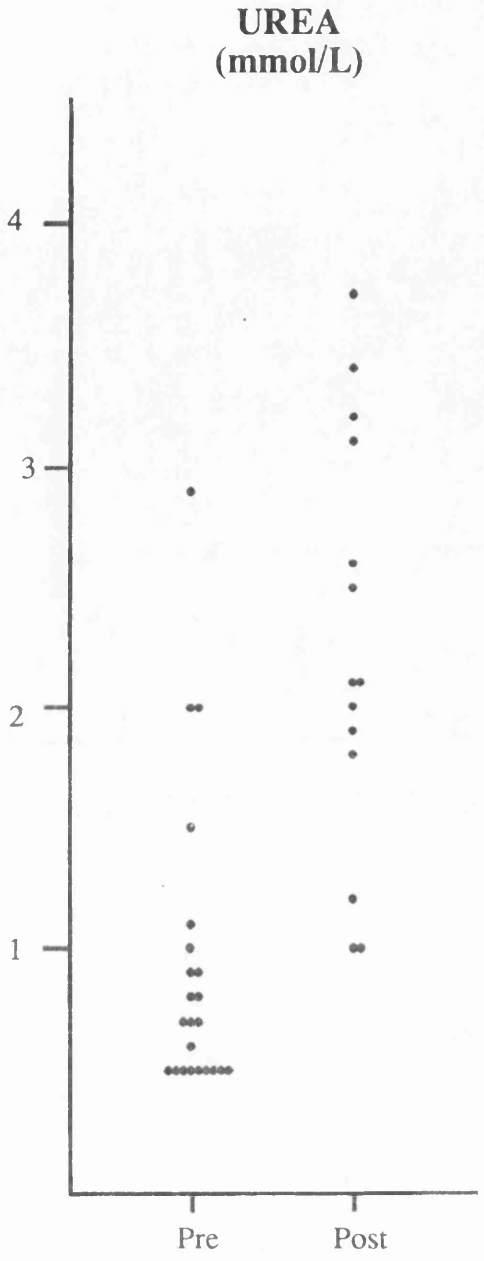
10/20 min breath test value was 146 (range, 61-584). In H pylori negative individuals the median value was 8 (range, 0.09-38). The values clearly separated the two groups and were similar to those found in the patients with duodenal ulcers and normal renal function. The range of values for the peak 10/20 min breath test for H pylori negative individuals with chronic renal failure was greater than that of the patients with normal renal function but did not overlap with the values in either group of H pylori positive subjects. Despite this the highest peak 10/20 min breath test value from subjects with chronic renal failure who were H pylori negative was similar, 38, to the lowest value found in individuals with normal renal function, 39, who had H pylori infection. The 10 or 20 min breath test value although useful in subjects with chronic renal failure may require the use of a second method of detecting H pylori infection for subjects with borderline results.

5.6 Concentrations of urea and ammonium in gastric juice samples

1) Subjects with Duodenal ulcers

The median (range) gastric juice urea concentration was 0.8 mmol/L (0.5-2.9 mmol/L) in those with the infection compared with 2.1 mmol/L (1.0-3.7 mmol/L) in patients in whom it was eradicated ($p<0.001$) (Fig 5.1). The median (range) gastric juice ammonium concentration was 3.4 mmol/L (1.0-13.0 mmol/L) in infected individuals

Fig. 5.1



Urea concentrations (mmol/L) in gastric juice samples from individuals before and after eradication of H pylori.

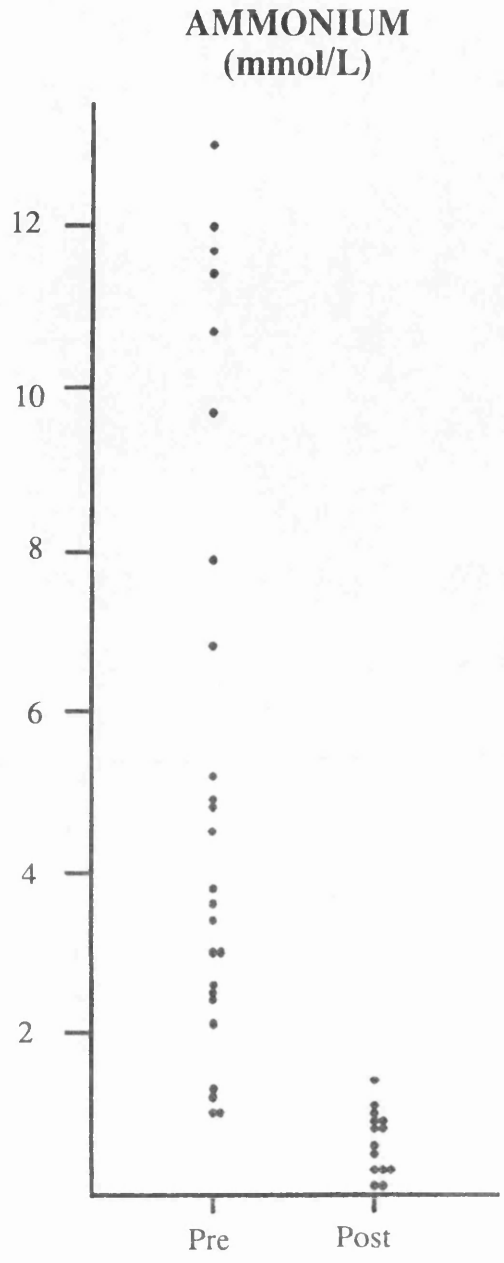
and 0.64 mmol/L (0.02 - 1.4 mmol/L) following eradication ($p<0.001$) (Fig 5.2). Although there was considerable overlap between the two groups with respect to their urea and ammonium concentrations, there was complete separation of the groups with the urea/ammonium ratio (Fig 5.3). The median ratio in gastric juice of infected subjects was 0.26 (0.04 - 0.7) compared with 3.4 (1.1 - 113) in those eradicated of the infection ($p<0.001$). Thus, all subjects with H pylori infection had a urea/ammonium ratio of less than 0.80 and all those eradicated of the infection had a ratio of more than 0.90. There was no correlation of the area under the 2 h breath test values and the gastric urea concentration, ammonium concentration or urea/ammonium ratio (Fig 5.4).

2) Subjects with Chronic renal failure

The median (range) serum urea concentration in those with the infection was 19.7 mmol/L (12.0 - 31.2 mmol/L) compared with 23.8 mmol/L (10.7 - 32.2 mmol/L) in patients without the infection ($p >0.5$, NS) (Table 5.2).

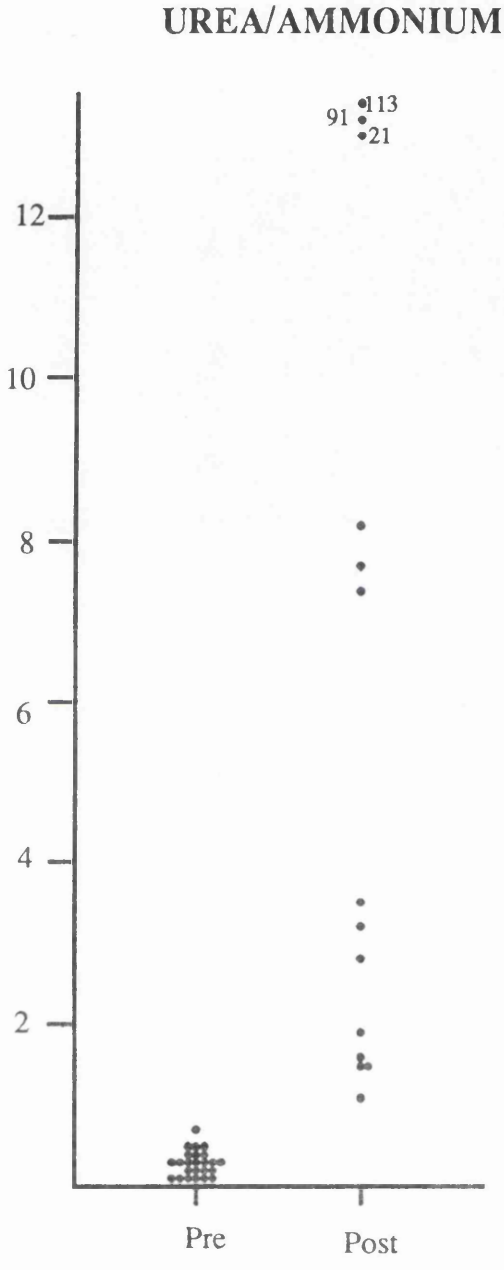
The median (range) gastric juice urea concentration in those with the infection was 2.2 mmol/L (0.5 - 8.7 mmol/L) compared with 13.8 mmol/L (5.4 - 20.8 mmol/L) in non-infected individuals ($p<0.001$) (Table 5.2). The median (range) gastric juice ammonium concentration was 20.0 mmol (13.9-43.1 mmol/L) in those with the infection compared with 4.8 mmol/L (0.5-12.3 mmol/L) in patients without the infection ($p<0.001$). The median concentrations of urea and ammonium were different in the

Fig. 5.2



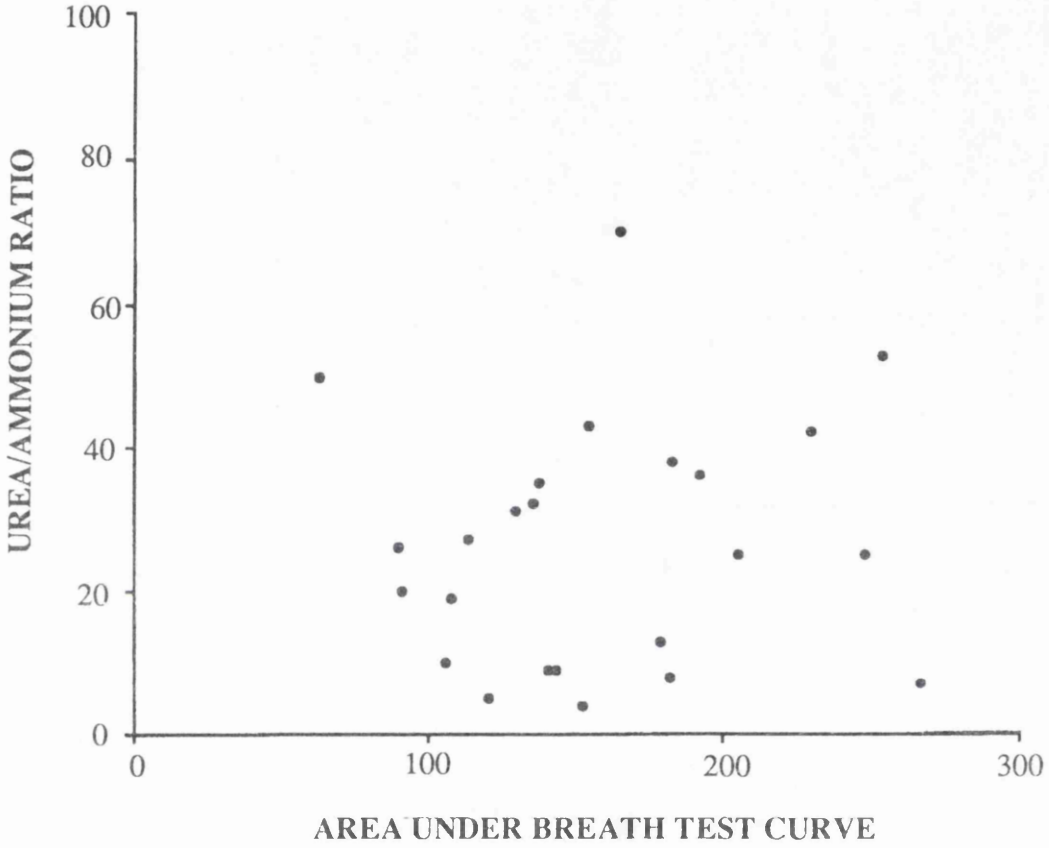
Ammonium concentrations (mmol/L) in gastric juice samples from individuals before and after eradication of H pylori.

Fig. 5.3



Urea/ammonium ratio in gastric juice samples from individuals before and after eradication of H pylori.

Fig. 5.4



Correlation of the area under the 120 min breath test curve (% dose/mmol CO_2 kg body weight x min) with urea/ammonium ratio in gastric juice (r = 0.004).

Table 5.2

H pylori -ve subjects	Gastric Juice Ammonium	Urea	Ratio	Serum Urea	20 min breath test value (% dose/mmol CO_2 Kg x 100)
1	10.9	19.3	1.8	23.1	38
2	11.4	14.1	1.2	16.7	0.3
3	2.0	10.6	5.3	19.4	14
4	0.5	20.8	-	32.2	0.09
5	4.2	7.0	1.7	25.2	10
6	0.8	15.1	18.9	28.3	18
7	12.3	16.9	1.4	27.1	27
8	5.4	5.4	1.0	10.7	14
9	11.5	18.5	1.6	23.6	5
10	2.6	12.7	4.9	22.5	1
11	3.3	9.3	2.5	20.5	34
12	11.4	13.4	1.2	24.0	5
H pylori +ve subjects					
1	13.9	0.5	0.01	28.3	584
2	43.1	3.0	0.07	31.2	150
3	16.1	0.6	0.04	12.0	174
4	30.0	8.7	0.30	18.3	106
11	18.9	1.4	0.07	14.2	61
12	21.0	7.3	0.35	20.5	87

The effect of chronic renal failure on gastric juice urea and ammonia concentrations and the urea/ammonium ratio.

two groups of patients with renal failure but there was again overlap between those with the infection and patients in whom it was not present or had been eradicated. There was also overlap between the concentrations of urea in gastric juice from H pylori negative individuals with normal renal function and H pylori positive individuals with chronic renal failure.

When the urea/ammonium ratio was calculated the median value for H pylori positive subjects was 0.16 (range, 0.01-0.35) compared with 1.63 (range, 1.0 - 18.9) for H pylori negative subjects ($p<0.001$).

Again, all subjects with infection with H pylori had a urea/ammonium ratio of less than 0.80 and all those in whom it had been eradicated or was not present had a ratio of 0.90 or greater.

3. Subjects treated with Ranitidine

In the 10 patients studied before and following 7 days treatment with ranitidine there was no change in the gastric juice concentrations of urea or ammonium or their ratio (Table 5.3). Using a urea/ammonium ratio of 0.80 or less as indicative of infection treatment with ranitidine did not alter the classification of H pylori status in any subjects studied.

5.7 Discussion

The ^{14}C-urea breath test has been used to detect the presence of H pylori by its urease activity (72,73). This test is reproducible and is a sensitive method of

Table 5.3

	Pre-treatment			During Ranitidine Treatment		
H pylori -ve Subjects	Ammonium	Urea	Ratio	Ammonium	Urea	Ratio
1	2.0	2.9	1.4	1.9	2.6	1.3
2	2.2	3.1	1.4	2.9	2.6	0.9
3	2.0	4.3	2.1	2.5	3.0	1.2
4	0.8	2.2	2.6	2.0	2.7	1.3
5	1.6	2.5	1.6	2.8	3.0	1.1
6	1.4	2.0	1.4	1.2	1.9	1.6
7	2.2	2.1	0.9	2.5	2.6	1.0
H pylori +ve Subjects						
1	6.1	1.2	0.2	5.7	1.4	0.2
2	8.8	2.0	0.2	5.2	1.6	0.3
3	7.2	0.6	0.1	8.4	0.2	0.1

The effect of 7 days' treatment with Ranitidine 300 mg nocte on the concentrations of urea and ammonium and their ratio in gastric juice.

detecting infection of the antral mucosa with H pylori. The test does have several disadvantages. It takes 2 h to complete using the standard protocol although a shortened 40 min test has been proposed (227). The data from the patients with duodenal ulcers indicated that using the highest of the 10 or 20 min values would be as effective as the calculation of the area under the 120 min of the breath test curve. This was confirmed by the data from patients with chronic renal failure in whom the separation of the 2 groups identified by biopsy and culture of the organism was reproduced by the 10 or 20 peak breath test value. The highest 10/20 min peak breath test value from H pylori negative individuals with chronic renal failure was similar to the lowest value from H pylori positive individuals with normal renal function. This suggests that the 10/20 min peak breath test value could not be used on its own for the detection of the organism. Two methods of detecting the bacterium would be needed but this is standard practice even for the complete breath test.

This study has also demonstrated that the urease activity of the organism results in reduced urea and increased gastric juice ammonium concentrations. The range of concentrations found overlapped between individuals with the organism and individuals who were not infected. The concentrations of these two analytes also varied considerably with the availability of urea. Higher concentrations of both urea and ammonium were found in individuals with chronic renal failure. The gastric juice

urea concentrations in some samples from patients with H pylori infection and renal failure were higher than those found in some individuals without the infection but with normal renal function. Treatment with the H_2 receptor antagonist ranitidine did not alter the concentrations of urea and ammonium in gastric juice samples.

Calculation of the ratio of urea to ammonium in gastric juice did clearly discriminate between those with the infection and patients clear of infection. The ratio also distinguished between these groups in the presence of chronic renal failure and also following treatment with ranitidine. The discriminating ability of this ratio can be explained by the inverse effect that the organism's urease activity exerts on urea and ammonium concentrations in gastric juice.

Although the urea/ammonium ratio was as effective as the ^{14}C-urea breath test at distinguishing between patients with the infection and those in whom it had been eradicated there was no correlation between urease activity as assessed by the urea breath test and the ratio of urea to ammonium concentrations in gastric juice. The absence of a correlation may be explained as follows. The ^{14}C-urea breath test is a dynamic test measuring the response of the enzyme to a urea load, while the urea/ammonium ratio is a measure of basal H pylori urease activity. The rate of ammonia production in infected

individuals is mainly dependent on the availability of urea. This was demonstrated by the higher gastric juice ammonia concentrations found in patients with chronic renal failure.

The raised gastric juice ammonium concentrations in samples from subjects with the infection (1.0-13.0 mmol/L) and especially in those with chronic renal failure (13.9-43.1 mmol/L) indicated that the organism's urease activity might possibly produce sufficient ammonia to raise the surface pH of the antral epithelium. This might be responsible for the increase in plasma gastrin concentration with infection which has been demonstrated following eradication of the organism (19-26).

In conclusion infection with the organism results in reduced gastric juice urea concentrations and raised ammonium concentrations. The concentration of ammonium in gastric juice depends on the availability of gastric juice urea. The measurement of the ratio of gastric juice concentrations of urea and ammonium in fasting gastric juice samples provides another simple method of determining H pylori status in patients with duodenal ulcers.

CHAPTER 6

THE EFFECT OF STIMULATING OR INHIBITING HELICOBACTER PYLORI UREASE ACTIVITY ON GASTRIN CONCENTRATION

THE EFFECT OF STIMULATING OR INHIBITING HELICOBACTER PYLORI UREASE ACTIVITY ON GASTRIN CONCENTRATION

6.1 Introduction

The eradication of chronic Helicobacter pylori infection of the gastric antrum which is present in more than 95% of patients with duodenal ulcer disease reduces the duodenal ulcer relapse rate from 80% to 20% (144-146). The eradication of this infection is also accompanied by a lowered circulating gastrin concentration (19-26). The fasting gastrin concentration falls by 27%-33% and the integrated gastrin response to a meal by 30-58%. The increase in circulating gastrin concentration associated with infection is associated with increased intragastric acidity during meals (25) and may therefore be the link between chronic H pylori infection and the development of duodenal ulcer disease.

The mechanism by which H pylori raises serum gastrin concentration is not known. It is possible that H pylori causes increased gastrin concentrations through its abundant urease activity. The organism's urease activity produces high gastric juice ammonium concentrations (median 3.4, range 1.0-13.9 mmol/L). Ammonium concentrations in the region of the gastric antral epithelial surface may be higher than in gastric juice samples as the organism colonises the surface of the

antral mucosa. The production of large amounts of ammonia by the bacterium at the antral epithelial surface could increase gastrin release by any of three theoretical mechanisms. Ammonia is a moderately strong base and could therefore prevent the physiological inhibition of gastrin release by neutralisation of gastric acid (228). In addition, elevation of antral surface pH by ammonia could facilitate the entry of dietary amines into the antral G cells and thereby their stimulation of gastrin release (186-190). Thirdly, ammonia could directly stimulate gastrin release as has been shown to occur in the rat (186). In order to determine whether the raised plasma gastrin concentration was due to ammonia production by H pylori the effect of stimulating ammonia production by intragastric infusion of urea, and the effect of inhibiting ammonia production by administering acetohydroxamic acid, on plasma gastrin concentration was studied.

6.2 Intragastric Urea infusion experiments

6.3 Patients

Eight patients (5 male) with a history of endoscopically confirmed duodenal ulceration within the previous year were studied. Their ages ranged from 26-62 years. Each of the patients had H pylori infection of the gastric antrum confirmed by histology of antral biopsies, rapid urease test (CLO test) and ^{14}C-urea breath test. None had taken any acid inhibiting agents or bismuth preparations in the month prior to commencement of the study.

6.4 Methods

The plasma gastrin response to increasing ammonia production was investigated by infusing a urea solution into the gastric antrum. Each patient acted as their own control by having the urea infusion repeated one month after a three week course of tripotassium dicitrato bismuthate (DeNol tab) 120 mg qid, and metronidazole 400 mg tid designed to eradicate H pylori. Confirmation of eradication was obtained by repeating the endoscopic antral biopsies and ^{14}C-urea breath test one month after completion of therapy.

Following a 16 h overnight fast a dual-lumen size 16F gastric tube (Anderson Inc., New York) was passed nasogastrically and positioned radiographically so that its tip lay in the distal part of the stomach. An intravenous cannula was inserted into the antecubital vein. At the end of a 30 min basal period, the stomach was emptied and over the following hour dextrose solution (328 mmol/L) which did not contain urea was infused into the stomach at a rate of 2 ml per minute. Following this control period, dextrose solution containing urea, 50 mmol/L was infused at a rate of 2 ml per minute for 4 hours. The concentrations of dextrose in the urea solution was reduced by 50 mmol/L so that the osmolalities of the two solutions were the same. In addition the pH of the solutions were reduced to 1.8 by adding 1 ml concentrated HCL per 500 ml dextrose to prevent them raising intragastric pH.

Venous blood samples were obtained at 30 minute intervals through the experiment for measurement of plasma gastrin concentration. The blood was centrifuged at 3000 g for 10 minutes at 4°C and the plasma stored at -20°C. During the intragastric infusions 10 ml samples of gastric juice were collected every 15 minutes for the measurement of ammonium and urea concentrations and determination of pH. At the end of each hour all the gastric contents were aspirated to prevent accumulation of infusate.

Plasma gastrin concentrations were measured as previously described. The concentration of urea in gastric juice was measured by a blanked automated urease method using the SMAC I (Technicon, Basingstoke, UK). The ammonium concentration was measured by an enzymatic method adapted for the Cobas Bio (Sigma, Dorset, UK). Statistical analysis was by means of the Wilcoxon signed rank-sum test. The study had the approval of the Western Infirmary Ethical Committee and each patient gave written informed consent.

6.5 Results

6.6 The effect of stimulating urease activity by intragastric infusion of urea

The infection by H pylori was successfully eradicated in 7 of the patients. Clearance of the infection was confirmed by resolution of gastritis on antral biopsies and a fall in the ^{14}C-urea breath test 20 min value to

less than 20 percentage dose/mmol CO_2 x kg body wt x 100 at four weeks after completion of the antibacterial treatment. The urea/ammonium ratios were also all greater than 0.9 in the basal samples collected during the post-eradication infusion studies. The patient in whom the organism was not cleared was excluded from further analysis.

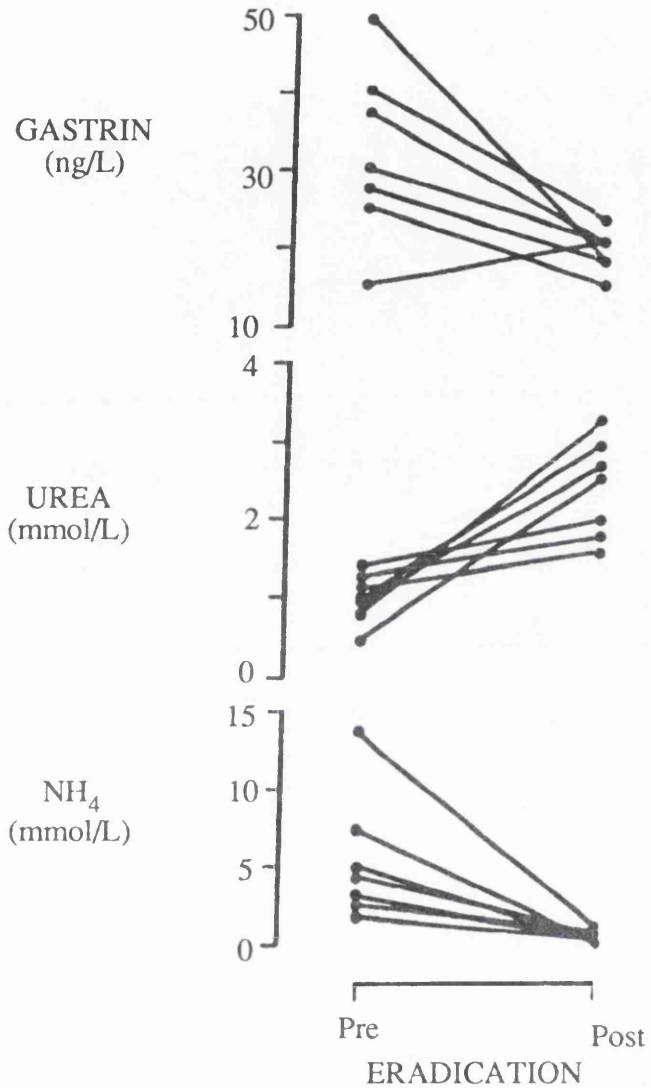
6.7 Basal Values

The median concentration of ammonium ions in the basal gastric aspirate prior to eradication of H pylori was 4.4 mmol/L (range 1.8-14.7) and this fell following eradication to 0.7 mmol/L (range 0.3-1.4) ($p<0.02$) (Fig 6.1). The median concentration of urea in the basal gastric aspirate was 1.1 mmol/L (range 0.3-1.6) and rose to 2.5 mmol/L (range 1.0-3.4) following eradication ($p<0.02$) (Fig 6.1). The median plasma concentration of gastrin was 30 ng/L (range 15-60) and this fell to 20 ng/L (range 15-25) ($p<0.05$) following eradication (Fig 6.1). The median pH of the basal gastric aspirates did not change significantly being pH 1.7 (range 1.2-1.9) before and 1.6 (range 1.2-1.7) following eradication.

6.8 The effect of the intragastric infusions

During the 60 minute control infusion of dextrose solution both before (Fig 6.2) and after eradication (Fig 6.3) there was a progressive fall in urea and ammonium concentrations in the gastric aspirate due to dilution by the infusate. Plasma gastrin concentration did not change over this period.

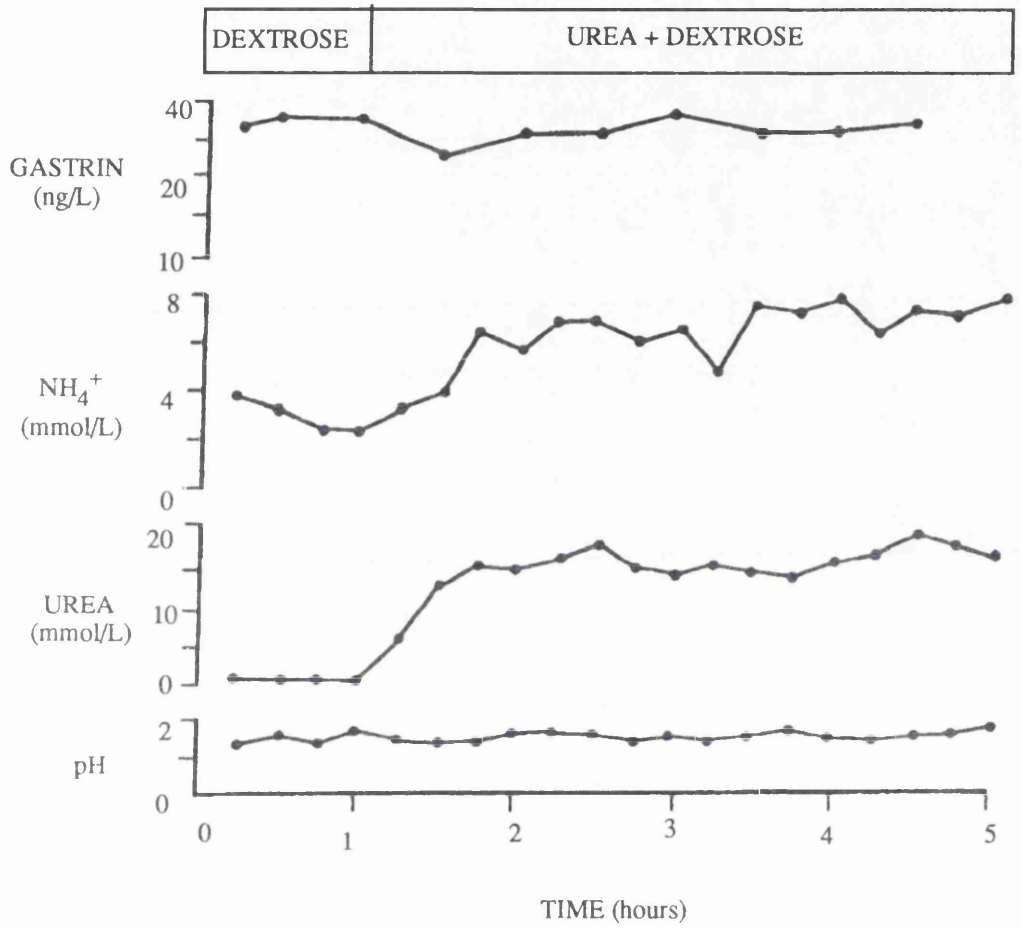
Fig. 6.1



Gastric juice concentrations of urea and ammonium and plasma gastrin concentrations before and after eradication of H pylori infection in 7 duodenal ulcer subjects.

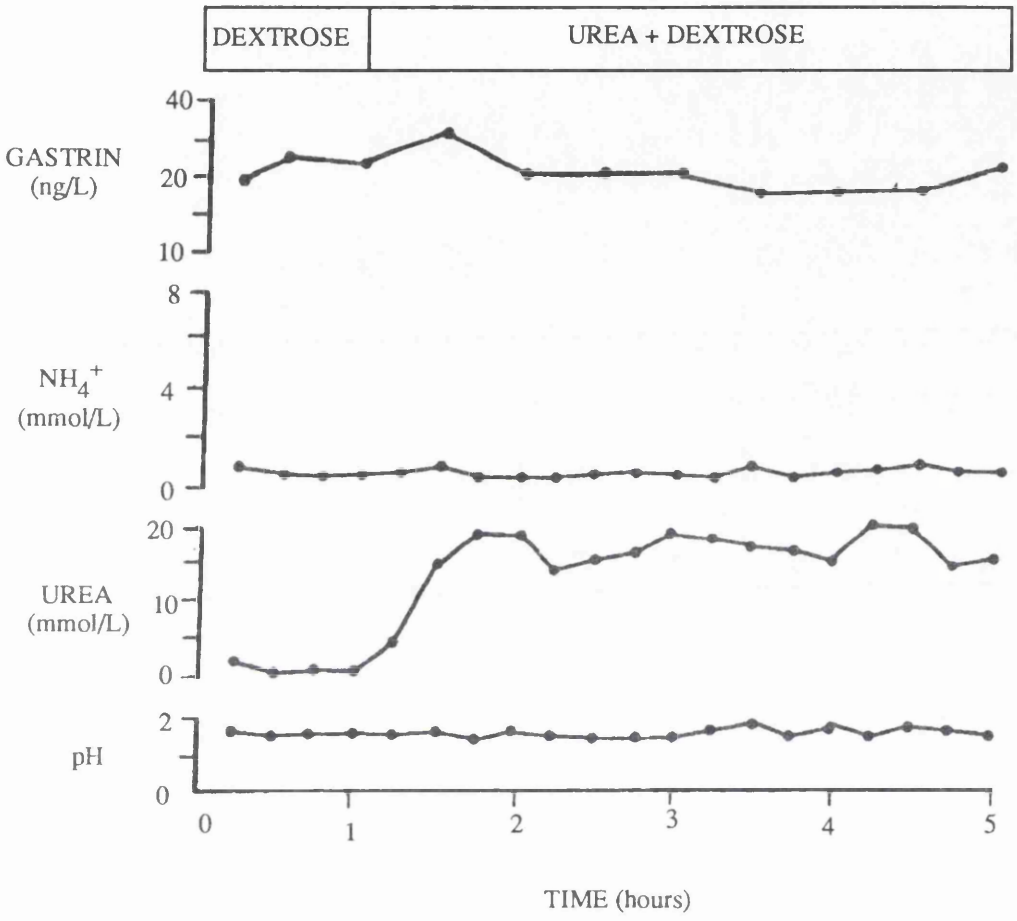
Following eradication plasma gastrin concentration and gastric juice ammonium concentrations were lower ($p < 0.05$, $p < 0.02$ respectively) and gastric juice urea concentration increased ($p < 0.02$).

Fig. 6.2



Effect of intragastric infusion of urea on gastric juice concentrations of urea and ammonium, intragastric pH and plasma gastrin concentration in 8 patients with H pylori infection of the gastric antrum. Values are medians.

Fig. 6.3



Effect of intragastric infusion of urea on gastric juice concentrations of urea and ammonium, intragastric pH and plasma gastrin concentration in 7 patients following eradication of H pylori infection. Values are medians.

Prior to eradication of H pylori the urea infusion resulted in a rise in the gastric aspirate urea concentrations which reached a plateau after 60 min at a median value of 15.5 mmol/L (range 7.9-21.3) (Fig 6.2).

The median gastric juice ammonium concentration immediately prior to commencing the urea infusions was 2.3 mmol/L (range 1.3-5.9) and rose over 90 minutes to reach a median plateau value of 6.1 mmol/L (range 4.2-11.9). This rise in ammonium production was not accompanied by any change in plasma gastrin concentration (Fig 6.2). Following eradication of H pylori the rise in gastric juice urea concentration during urea infusion was similar to that prior to eradication (Fig 6.3). On this occasion there was no rise in the ammonium concentration with the median value immediately prior to commencing the infusion being 0.4 mmol/L (range 0.1-0.9) and 0.4 mmol/L (range 0.3-1.2) at the end of the infusion. The median gastrin concentration which was lower following eradication of H pylori was unaffected by the urea infusion.

The pH of gastric aspirates remained between 1.5 and 2.0 throughout the pre and post eradication studies.

6.9 Discussion of the intragastric infusion study

In agreement with previous work this study demonstrated that there was a reduction in plasma gastrin concentrations following eradication of H pylori infection in duodenal ulcer subjects (19-26). Increased H pylori ammonia production failed to alter plasma gastrin

concentration. This observation does not lend support to the hypothesis that hypergastrinaemia is caused by the ammonia. It seems likely that the increase in ammonia production resulted in an adequate stimulus as the rise in ammonium concentration at the antral epithelial surface where the bacteria are found close to the gastrin secreting G cells would have been greater than the 3 fold rise observed in the gastric aspirate. However, failure to cause a further increase in gastrin concentration does not exclude the possibility that the elevated basal gastrin concentrations were due to the bacterium's ammonia production. The amount of ammonia produced by the organism under normal circumstances might be sufficient to produce the maximum gastrin response through alteration of the antral surface pH or by any direct effect on the gastrin producing cells. The mechanism through which H pylori raises plasma gastrin concentrations, therefore, remained to be determined.

6.10 The effect of inhibition of urease activity by acetohydroxamic acid

6.11 Patients with Helicobacter pylori infection

Six patients (3 males) who had endoscopically confirmed duodenal ulceration within the previous year but were in remission at the time of the study were investigated. Their ages ranged from 26-52 years. In each patient an antral biopsy obtained endoscopically

within the preceding 3 months had shown gastritis associated with H pylori-like organisms. None had taken any acid inhibitory agents or bismuth preparations in the month prior to commencement of the study.

6.12 Method

On the first morning of the study the patients having fasted for 16 h overnight had a venous blood sample removed at 0800 h for gastrin measurement. Immediately following this they drank 50 ml of water and 4 further blood samples were collected at 30 min intervals for the next 2 hours. At 1000 h they took a standard meal consisting of 2 beef cubes (OXO Ltd., Croydon, England) dissolved in 200 ml water at 50°C. Further blood samples were collected at 10 min intervals for 70 min and a final sample was collected at 90 min following the OXO drink. Immediately following this sample a ^{14}C-urea breath test was conducted to measure H pylori urease activity.

On the following day the study was repeated in an identical fashion except that the patients received 750 mg acetohydroxamic acid (Lithostat, Mission Pharmacol, USA) with a 50 ml drink of water at 0800 h.

Ten days later a third ^{14}C-urea breath test was performed to determine whether the temporary inhibition of urease activity had resulted in clearance of the infection.

6.13 Patients without Helicobacter pylori infection

Two male patients (aged 25 and 52 years) with a past history of duodenal ulcer disease but in whom H pylori had been eradicated within the previous year were studied in an identical fashion to that described above. This was conducted in order to exclude the possibility that acetohydroxamic acid might have a direct effect on gastrin release.

6.14 Study in Healthy Volunteer with Helicobacter pylori infection

The effect of acetohydroxamic acid on the concentrations of urea and ammonium in gastric juice was examined in a single healthy volunteer (AN aged 32 years) with H pylori infection. Endoscopic antral biopsy 3 months earlier had demonstrated gastritis and H pylori-like organisms. A ^{14}C-urea breath test performed one month prior to the study gave a 20 min value of 109 percentage dose/mmol CO_2 x kg body wt x 100 which is in the range for infected subjects (60-267) found in the previous studies.

He reported fasted at 0800 and a nasogastric tube was passed per orally. The resting gastric juice was aspirated and discarded and then constant suction was applied. At 30 min suction was discontinued to allow for the manual aspiration of a 5 min gastric juice sample.

A further gastric juice sample was similarly collected at 60 min. Following this 750 mg

acetohydroxamic acid was taken with 50 ml of water and no suction was applied until 1 h later when all the resting volume was aspirated but a 5 ml portion was retained for ammonium and urea analyses. Suction was recommenced but temporarily discontinued every 30 min over the following 3.5 h to allow manual aspiration of 5 ml samples of gastric juice.

6.15 Analytical methods

Plasma gastrin concentrations were measured by a radioimmunoassay (CIS (UK) Ltd, Buckinghamshire, UK). All samples from each patient were analysed in the same batch. The concentration of urea in gastric juice samples was measured by the o-pthalaldehyde method using a Perspective analyser (American Monitor, West Sussex, UK). The concentration of ammonium was measured by an enzymatic method (Sigma, Dorset, UK) adapted for the Cobas Bio centrifugal analyser. The integrated gastrin response to the OXO meal was calculated using the trapezoid rule. Statistical analysis used the Wilcoxon signed rank-sum test. The study was approved by the Western Infirmary Ethical Committee and each subject gave written informed consent.

6.16 Results of the inhibition of urease activity with acetohydroxamic acid

The initial median breath test value was 152 percentage dose/mmol CO_2 x kg body wt x 100 (range

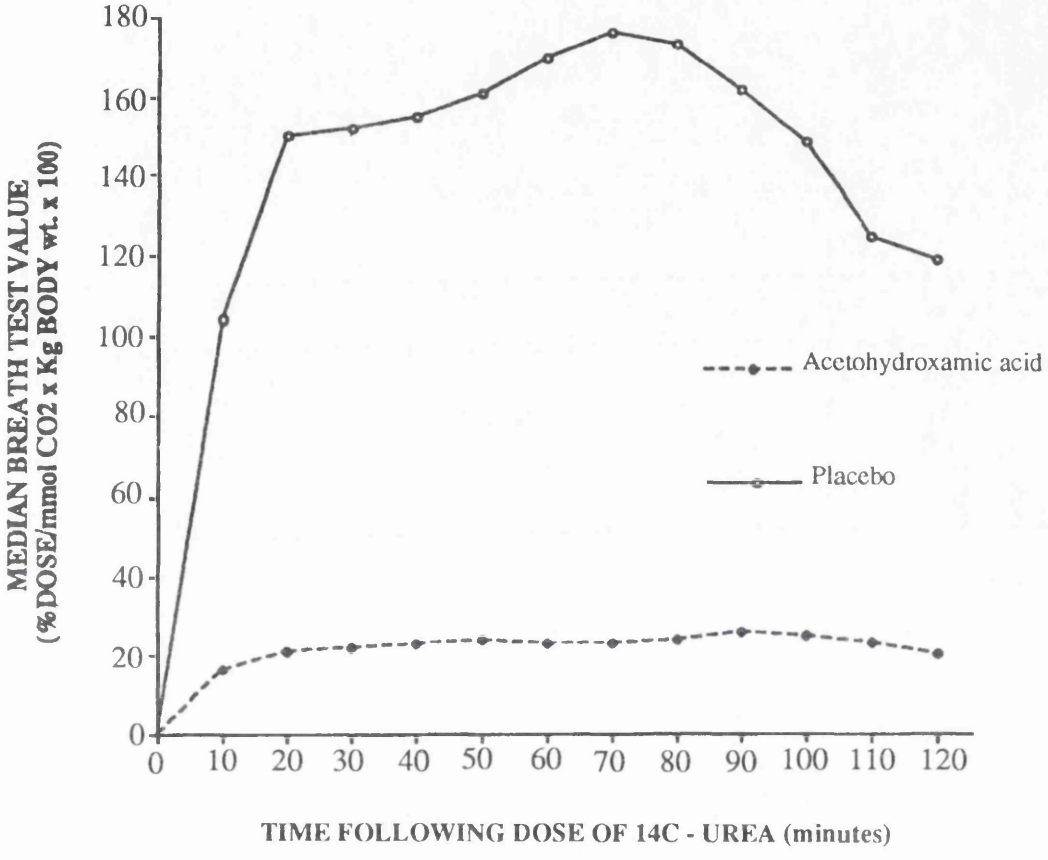
111-335) compared with 22 (range 14-95) on the second day of the study following the administration of acetohydroxamic acid ($p<0.03$) (Fig 6.4). Ten days after the administration of acetohydroxamic acid the median breath test value was 149 (range 126-257) which was similar to the pre-treatment value (Fig 6.5).

Although there was suppression of H pylori urease activity following the administration of acetohydroxamic acid there was no difference between the two study days with respect to the basal or meal stimulated plasma gastrin concentrations (Fig 6.6). The initial median integrated gastrin response to the OXO meal was 78 ng/L.h (range 21-222) compared with 79 ng/L.h (range 33-207) following acetohydroxamic acid (Fig 6.7).

The basal and meal stimulated gastrin responses in the patients without H pylori infection were unaffected by administration of acetohydroxamic acid.

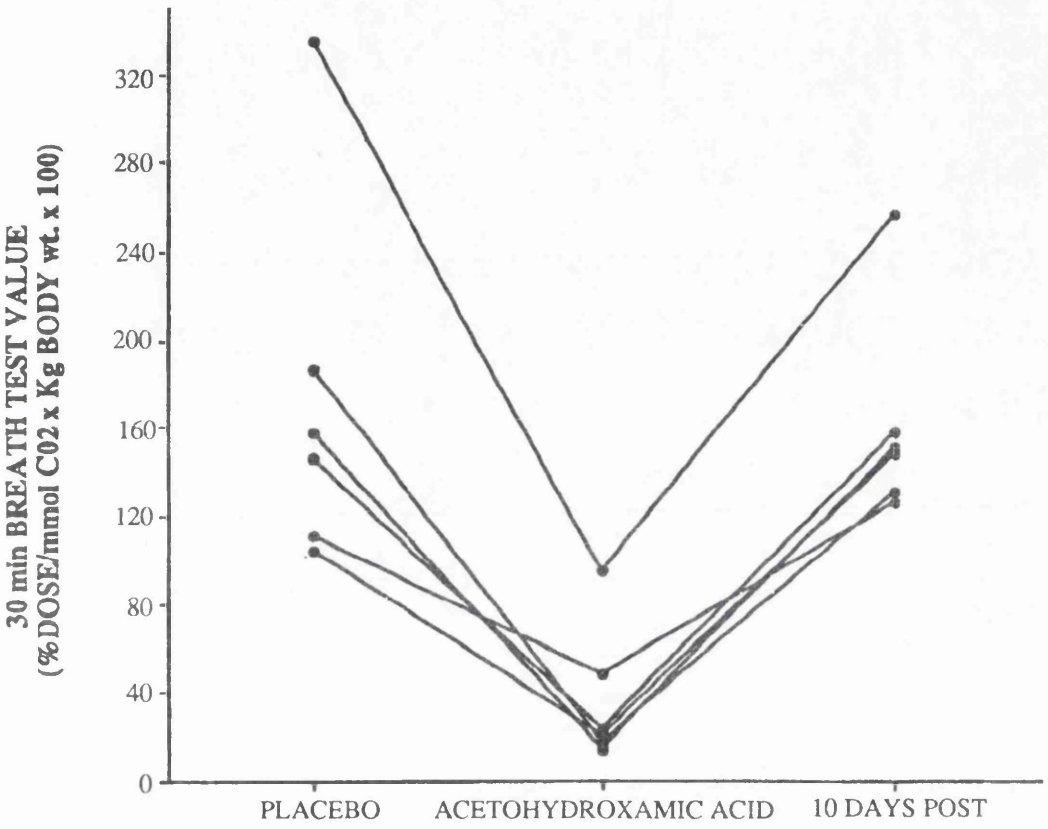
The administration of acetohydroxamic acid to the healthy volunteer who had H pylori infection resulted in a rise in the urea concentration in the gastric juice. The ammonium concentrations prior to administration of the inhibitor were 5.2 mmol/L and 5.5 mmol/L in the two basal gastric juice samples. One hour following administration of the inhibitor the gastric juice ammonium concentration had fallen to 2.0 mmol/L and then ranged from 1.9 - 3.6 mmol/L (Fig 6.8). The ratio of urea/ammonium in gastric juice prior to the urease inhibitor was 0.2 but in 5 of

Fig. 6.4



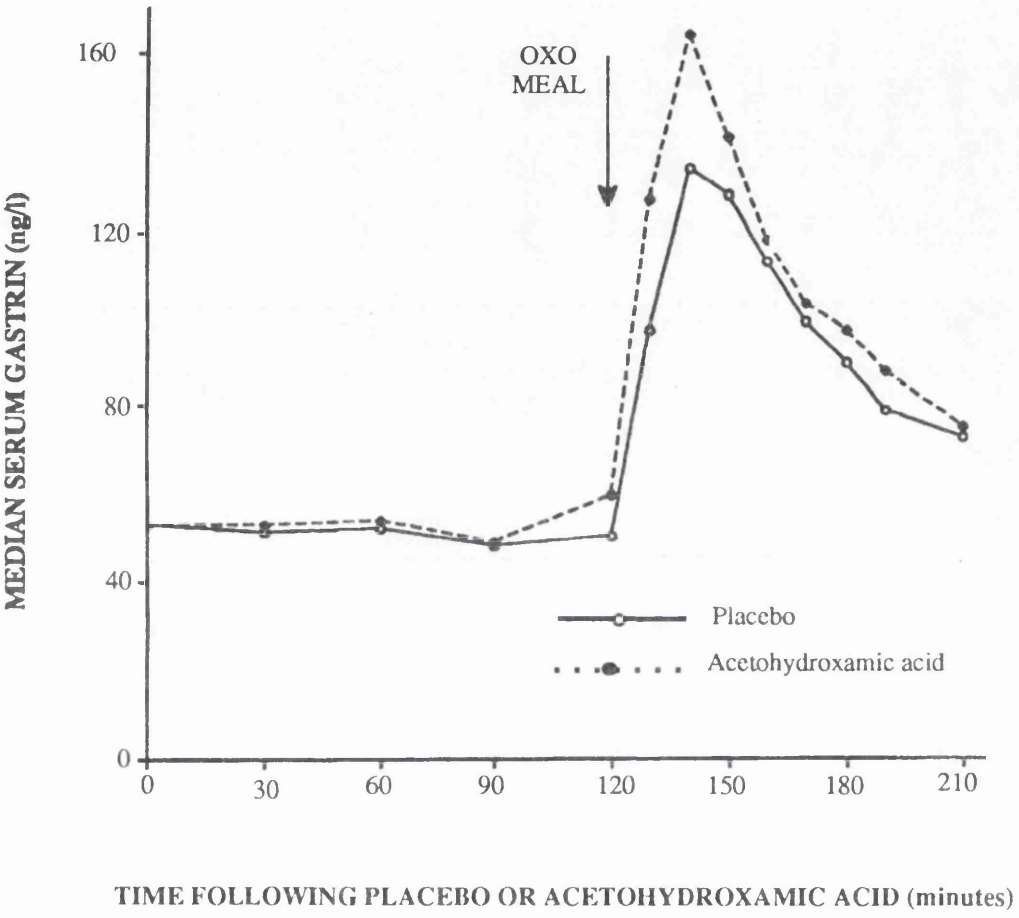
Effect of acetohydroxamic acid on H pylori urease activity assessed by the ^{14}C-urea breath test. The values are medians.

Fig. 6.5



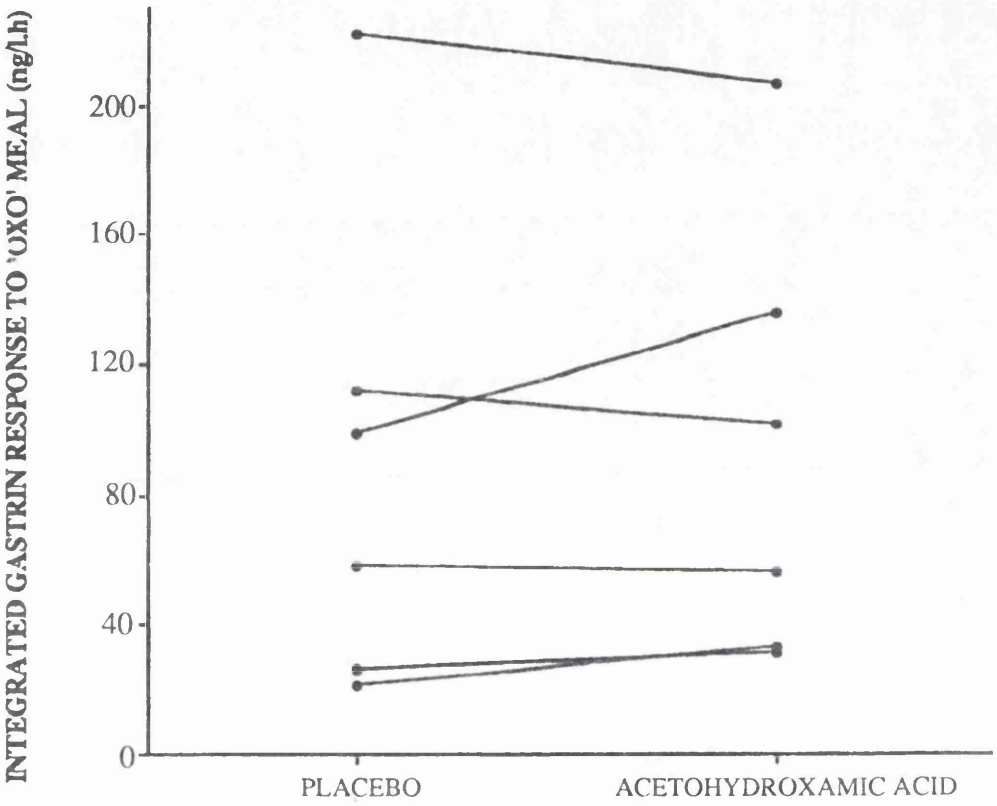
Individuals 30 min ^{14}C-urea breath test values on placebo, 3.5 hours after single 750 mg dose of acetohydroxamic acid and 10 days later.

Fig. 6.6



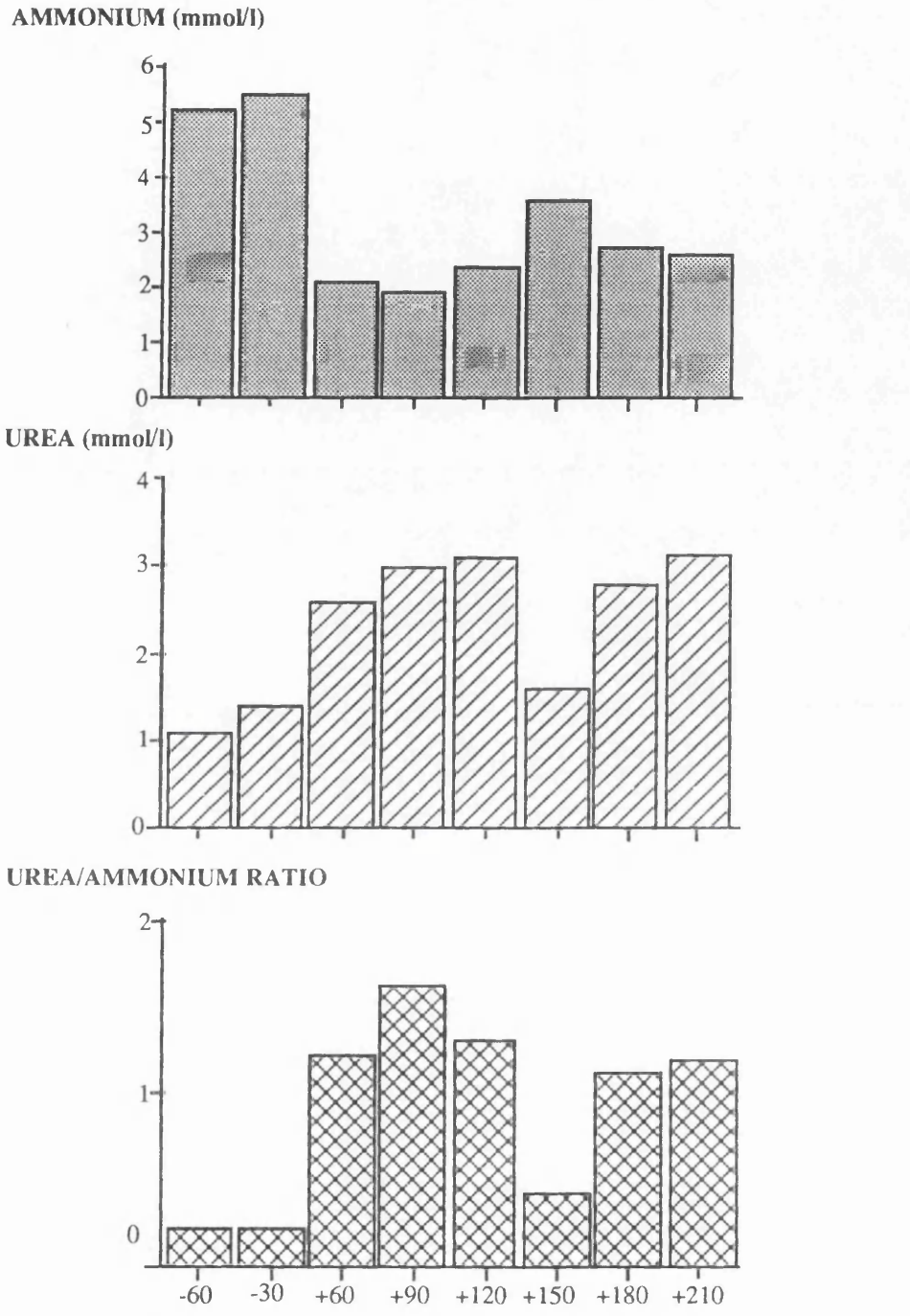
Basal and meal-stimulated serum gastrin concentrations on placebo and following 750 mg acetohydroxamic acid.

Fig. 6.7



Integrated gastrin response of each patient to the OXO meal on placebo and acetohydroxamic acid.

Fig. 6.8



Effect of 750 mg acetohydroxamic acid on gastric juice concentrations of ammonium, urea and their ratio in healthy volunteers with H pylori infection.

the 6 samples collected following the inhibitor was greater than 1.0. None of the patients experienced side effects following the administration of acetohydroxamic acid.

6.17 Discussion of inhibition of urease activity with acetohydroxamic acid

Acetohydroxamic acid is usually used to reduce bacterial ammonium production in patients with renal calculi due to chronic urinary tract infection by inhibiting urease activity (229). Acetohydroxamic acid inhibits urease activity non-competitively with two molecules binding to each urease molecule (230). This binding results in conformational change and loss of activity. Usually the drug is prescribed in a dose of 250 mg four times a day to achieve prolonged inhibition. In this study the drug was administered as a single dose of 750 mg to achieve rapid and effective inhibition of urease activity.

Effective inhibition of urease activity was demonstrated by the 5-fold reduction in the 30 minute values of the ^{14}C-urea breath test. Gastric juice concentrations of ammonium also fell and urea concentrations increased. The urea/ammonium ratio changed from 0.2 prior to administration of the inhibitor, which is in the range of infected subjects (0.04-0.8), to 1.2 which is in the range for non-infected subjects (0.9-113).

The altered urea/ammonium ratio occurred within 1 hour of administration of the inhibitor and persisted for the remaining 3.5 h of the study. Acetohydroxamic acid is rapidly absorbed from the gastrointestinal tract and reaches peak plasma concentrations at 60 minutes with a plasma half life of 3.5-5 hours (231).

Despite the fall in urease activity no change in the basal gastrin concentration or the meal stimulated gastrin response was observed following the administration of acetohydroxamic acid. There was also no change in gastrin concentrations in the two H pylori negative subjects which excluded the possibility that a fall in gastrin had been masked by a direct gastrin stimulatory effect of the acetohydroxamic acid.

This further observation that inhibition of H pylori ammonia production does not lower plasma gastrin concentration does not lend support to the hypothesis that the hypergastrinaemia is a direct result of bacterial urease activity.

6.18 Conclusion

6.19 The result of stimulating or inhibiting Helicobacter pylori urease activity

Neither the intragastric infusion of urea to stimulate urease activity nor the inhibition of urease activity with acetohydroxamic acid had any effect on plasma gastrin concentration.

It was unlikely that any late gastrin response in either of these experiments had been missed. The urea infusion was continued for 4 hours and a rise in gastrin response to direct alkalinisation of the antrum has been observed by this time (232). A fall in serum gastrin concentration would be expected within the 3.5 h period of inhibition of urease activity studied following administration of acetohydroxamic acid.

If alkalinisation of the gastric antrum by H pylori urease activity caused increased plasma gastrin concentrations then these should fall rapidly following inhibition of bacterial urease activity. Patients with hypergastrinaemia secondary to achlorhydria have a fall in serum gastrin concentration within 5 to 15 minutes of intragastric instillation of hydrochloric acid (233). Similarly, in healthy subjects the intragastric administration of acid at the same time as a meal suppresses or abolishes the gastrin response to the meal (228).

These two studies indicate that H pylori associated hypergastrinaemia is unlikely to be due to the creation of an alkaline environment in the region of the antral G cells. This suggests that either the organism does not produce sufficient ammonia to significantly alter the pH of the antral epithelial surface or that alkalinisation of the gastric antrum by any means should not change plasma gastrin concentration.

The low concentrations of urea found in gastric juice prior to eradication of the organism and the rapid rise in gastric ammonium concentration during urea infusion indicated that the generation of ammonia by the organism's urease activity was limited by the concentration of urea in the stomach and its rate of diffusion into the stomach. If the function of the urease activity is to create an alkaline environment to protect the organism from low gastric pH then such a protective mechanism should be able to respond to changes in gastric pH rather than be limited by substrate availability. Similarly in spite of marked suppression of urease activity by acetohydroxamic acid the infection was not cleared in any of the subjects studied as shown by the breath test 10 days after administration of acetohydroxamic acid.

In conclusion ammonia production does not seem to be important in protecting the organism once infection is established in the deep mucous layer. Stimulation and inhibition of ammonia production by the organism also failed to produce any change in basal or meal stimulated gastrin concentrations. The mechanism by which H pylori raises plasma gastrin concentrations, therefore, remains unclear.

CHAPTER 7

THE EFFECT OF DIRECT ALKALINISATION OF THE GASTRIC ANTRUM ON HELICOBACTER PYLORI RELATED HYPERGASTRINAEMIA

THE EFFECT OF DIRECT ALKALINISATION OF THE GASTRIC ANTRUM ON HELICOBACTER PYLORI RELATED HYPERGASTRINAEMIA

7.1 Introduction

It has been proposed that the ammonia produced by Helicobacter pylori urease activity raises antral surface pH and as a result stimulates gastrin release (19). In previous studies the stimulation of ammonia production through intragastric infusion of urea and the inhibition of ammonia production by the administration of the urease inhibitor acetohydroxamic acid, failed to alter basal gastrin concentrations or the meal stimulated gastrin response. These observations fail to support the hypothesis that an increase in antral surface pH resulting from urease activity causes the increased gastrin concentrations found in subjects with duodenal ulcers. However, it is possible that the means used to alter ammonia production did not sufficiently alter antral pH and as a result did not produce an observable change in gastrin concentration.

If the amount of ammonia produced by the bacterium was, under ordinary circumstances, sufficient to produce a maximal gastrin response then increasing ammonia production would not cause a further increase in gastrin concentration. In the inhibition experiments the administration of acetohydroxamic acid while producing a

significant reduction in the response to the ^{14}C-urea breath test did not suppress it to the values found in non-infected individuals. It is possible therefore that sufficient urease activity remained uninhibited and as a result there was no observed change in basal gastrin concentrations or the meal stimulated gastrin response.

The effect of acute alkalinisation of the antrum on gastrin release is also not clear because some studies show an increased release of the hormone (232-234) but others no change (235-239). The reason for this is not known, but the conflicting reports might be explained by differing H pylori status of the groups studied.

If H pylori infection does cause gastrin release through increasing the antral pH then the difference between infected and non-infected subjects should be reduced or eliminated by the alkalinisation of the gastric antrum. The influence of H pylori infection on the gastrin response to antral alkalinisation under both basal and meal stimulated conditions in duodenal ulcer patients was therefore investigated.

7.2 Patients

Eight patients (7 male, age range 32-60 years) with endoscopically confirmed duodenal ulceration within the previous year were entered into the study. Infection with H pylori was confirmed by the rapid urease test (CLO test), histology of antral biopsy and ^{14}C-urea breath

test. None of the patients had taken acid inhibitory agents for 2 weeks before entry into the study.

7.3 Methods

In all patients the gastrin response to a standard test meal at uncontrolled pH and at a pH greater than 6.0 was compared before and after eradication of *H pylori*. In six patients the gastrin response to 5 hours of alkalinisation of the gastric antrum was studied before and after eradication of *H pylori*.

Initially, the gastrin response to a standardised OXO meal was measured. The meal was prepared by dissolving 2 OXO cubes (OXO Ltd. Croydon, England), in 200 ml of water at 50°C. This was drunk over 5 minutes. Three basal venous blood samples were collected at 15 minute intervals prior to the meal. Further venous blood samples were collected 5 minutes after completion of the meal and at 10 minute intervals after that for 90 minutes. On the second day of the study within 7 days of the initial OXO meal the effect of gastric alkalinisation with the same standardised test meal was measured. A 16F dual lumen tube was passed orogastrically (Andersen Inc., New York), the stomach was emptied and a sample of gastric juice retained for measurement of pH. At the same time a blood sample was collected for measurement of plasma gastrin. Further samples of gastric juice and venous blood were collected at 15 minute intervals over the next 30 minutes. At the end of this period the stomach was

emptied and an intragastric infusion of 0.2 mol/L pH 7.0 citrate buffer (530 mmol/L) was commenced at a rate of 4 ml/min. One hour after this was started the standard OXO meal was consumed over 5 minutes. The infusion was continued for a further 10 minutes and 20 ml samples of gastric juice were collected over 20 minutes for pH measurement with a glass electrode (Radiometer ETS 822). Venous blood samples were collected every 15 minutes for gastrin measurement.

In 6 of the 8 patients the gastrin response to gastric alkalinisation alone was assessed. An orogastric tube was passed and basal gastric juice and venous blood samples collected as described above. An intragastric infusion of 0.2 mol/L pH 7.0 citrate buffer was commenced at a rate of 4 ml/min and continued for 5 h. Samples of blood and gastric juice were collected every 30 minutes for measurement of plasma gastrin and gastric juice pH. The stomach was completely emptied at the end of each hour to prevent distension.

Following completion of these studies patients were commenced on a 3 week course of tripotassium dicitrato bismuthate 120 mg four times per day, metronidazole 400 mg three times per day and amoxycillin 500 mg three times per day, designed to eradicate H pylori. One month after completion of anti-H pylori treatment evidence of infection was sought by the rapid CLO test, antral biopsy and histology and the ^{14}C-urea breath test. After this the plasma gastrin response to the standard OXO meal with

and without gastric alkalinisation and to gastric alkalinisation alone was reassessed.

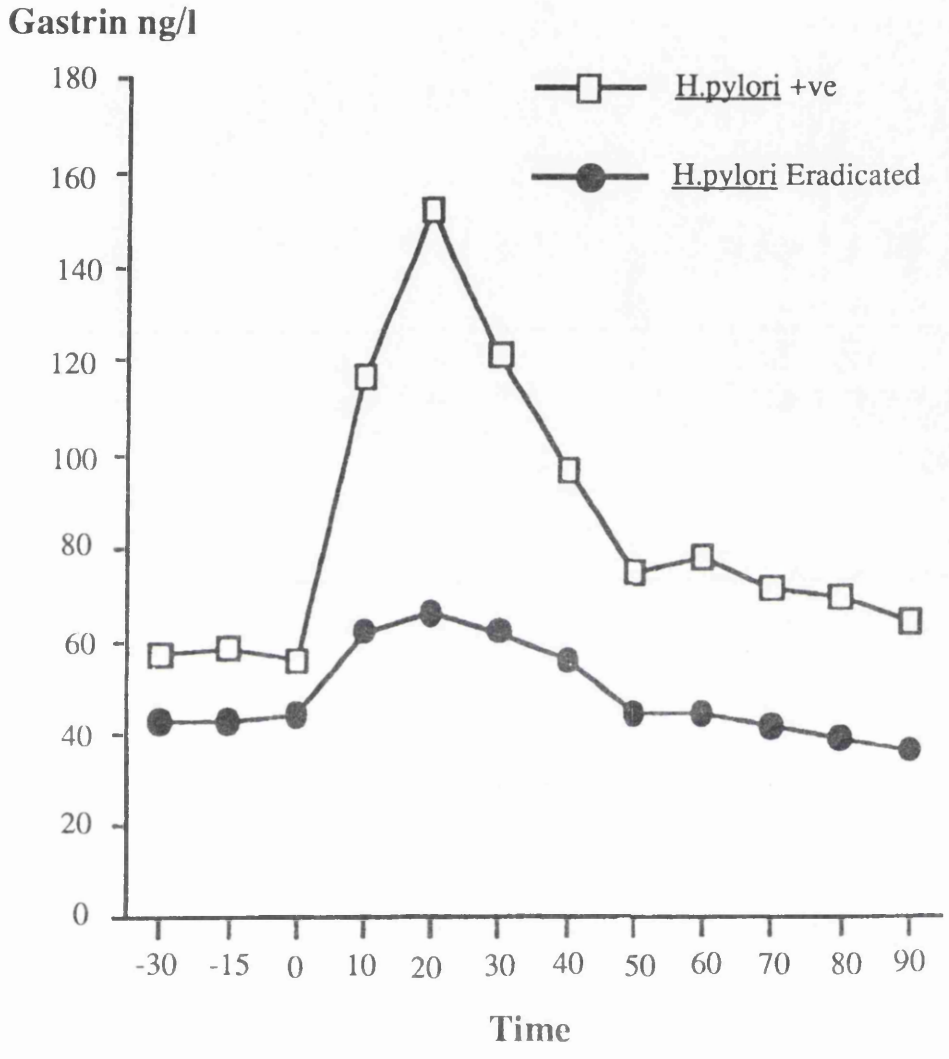
Samples for gastrin analysis were analysed in the same batch as previously described (226). Statistical significance was tested using the Wilcoxon signed rank sum test. The study was approved by the Western Infirmary Ethical Committee and each patient gave written informed consent.

7.4 Results

Eradication of H pylori had been achieved in all the patients when they were re-examined one month after completing triple therapy. The ^{14}C-urea breath test 20 minute value fell from a median pre-treatment value of 126 (range 55-266) to 4 (range 2-8) one month after completion of treatment. The rapid urease test was positive within 5 hours in all eight patients prior to treatment and negative at 24 hours in all of the patients when reassessed one month after completion of treatment.

The basal concentration of plasma gastrin measured on the day the test meal was given without alkalinisation fell from a median of 57 ng/L (range 13-103) pre-treatment to 42 ng/L (range 12-80) ($p<0.05$) post-treatment. The median integrated gastrin response to the test meal without alkalinisation was 2525 ng/L/min (range 55-8725) pre-treatment and fell to 725 ng/L/min (range 250-2925) following eradication ($p<0.01$)(Fig 7.1). On the day the

Fig. 7.1



Plasma gastrin response to oxo meal at uncontrolled pH, before and one month after eradication of H pylori in 8 patients.

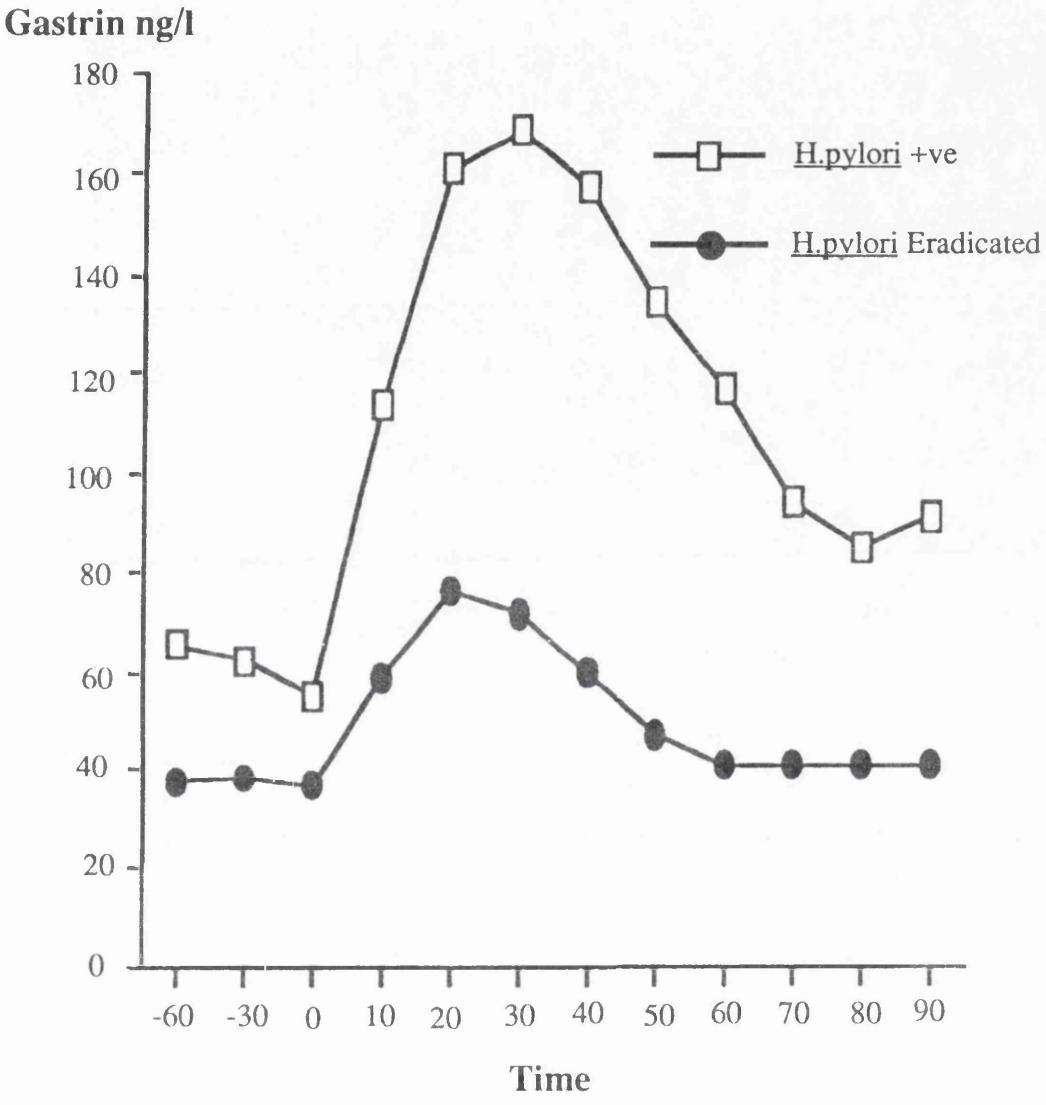
meal was administered with alkalinisation the infusion of citrate buffer raised the intragastric pH to greater than pH 6.0 within 30 minutes of commencement and this was maintained for the duration of the study. The median integrated gastrin response to the test meal with alkalinisation was 3700 ng/L.min (range 1900-14100) pre-treatment and fell to 1400 ng/L.min (range 400-3400) one month after completion of treatment ($p<0.01$)(Fig 7.2).

Alkalinisation of the stomach resulted in a rise in the integrated gastrin response to the test meal in 6 of the 8 patients both before and after eradication of H pylori. The median percent change in the 8 patients was +73% (range -63 to + 500%) pre-treatment compared with + 48% (range -26 to +386%) (NS, $p=0.9$) post treatment.

After eradication of H pylori the median fall in the integrated gastrin response to the test meal alone was 69% (range 36-89%) and was similar to that seen with the test meal plus gastric alkalinisation, 61% (range 0-97%).

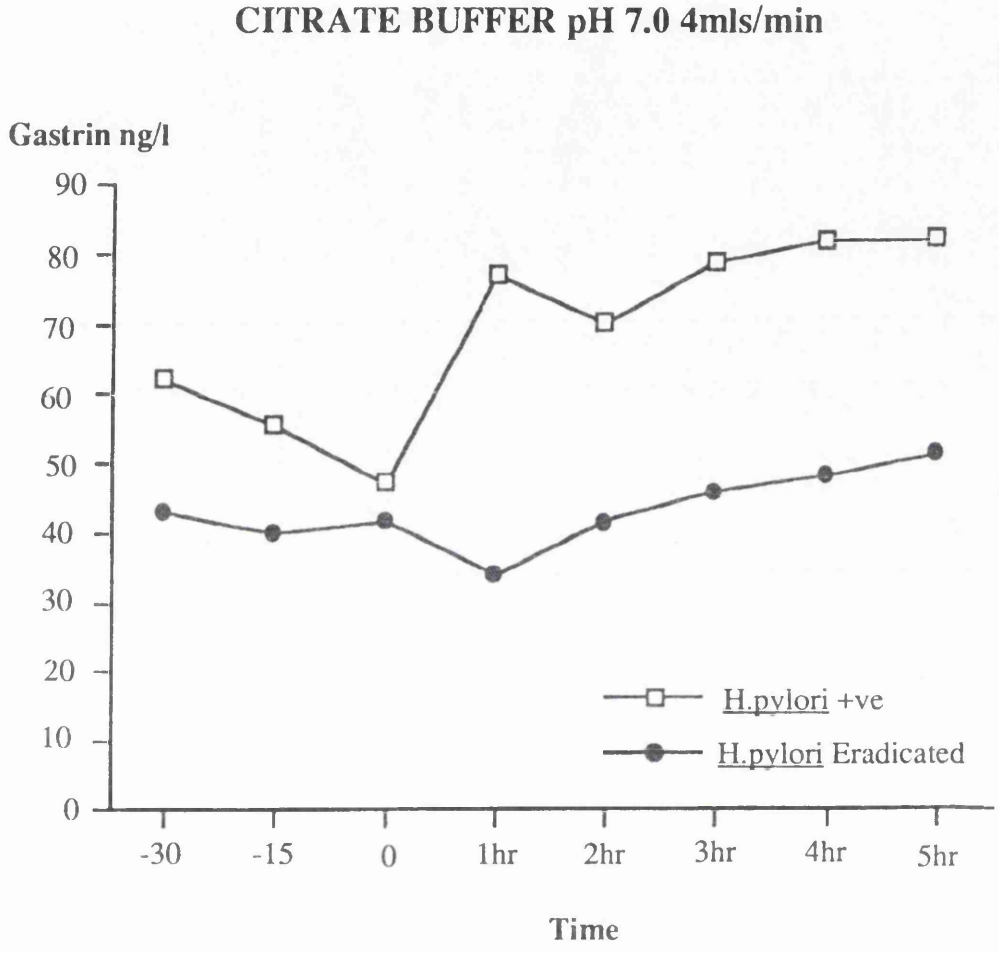
When the effect of gastric alkalinisation alone was studied there was a rise in the plasma gastrin concentration in 4 of the 6 patients before and in the same number after eradication of H pylori. Two patients did not respond to gastric alkalinisation with a change in plasma gastrin concentration. The median plasma gastrin concentration after 5 hours of alkalinisation was greater before, (62 ng/L, range 50-165), than after, (50 ng/L, range 25-75), eradication of H pylori ($p<0.02$)(Fig 7.3). The median percentage change in gastrin with

Fig. 7.2



Plasma gastrin response to oxo meal at near neutral pH, before and one month after eradication of H pylori in 8 patients.

Fig. 7.3



Plasma gastrin response to gastric alkalinisation before and one month after eradication of H pylori in 6 patients.

alkalinisation alone was similar before, 47% (range 0-267) and after 16% (range 0-100) eradication of H pylori (NS, p=0.2).

7.5 Discussion

There are conflicting reports on the effect of alkalinisation of the antrum during fasting on gastrin release in man. Several investigators have reported that acute elevation of antral pH does not alter plasma gastrin concentration (235-238). Others have failed to observe any change in gastrin following more prolonged antral alkalinisation for 3 hours (239) and for up to 10 hours (240) in duodenal ulcer subjects. Peters and colleagues reported that maintaining antral pH above 6.0 for 5 hours produced an increase in serum gastrin of 23-30% (from 53 pg/ml to 71 pg/ml) in duodenal ulcer subjects and 50% (from 25 pg/ml to 38 pg/ml) in healthy controls (232). Similarly Hausky and colleagues reported an increase in serum gastrin in duodenal ulcer subjects and healthy controls which occurred after only 30 minutes of alkalinisation (234). These differing studies occurred before the effect of H pylori infection on gastrin release was appreciated.

In this study the observation that eradication of H pylori infection results in a fall in both basal and meal stimulated gastrin concentrations was again confirmed (19-26). In addition the different response to the test meal from H pylori infected and treated individuals was

not eliminated by maintaining the intragastric pH greater than pH 6.0 by buffer infusion. Administration of a standard OXO meal without infusion of citrate buffer only raised the intragastric pH to above pH 2.0 for less than 15 minutes (241). It has been hypothesised that H pylori raises plasma gastrin concentrations by the blocking of the inhibitory effect of gastric acid on gastrin release through the production of large quantities of ammonia close to the gastric epithelial surface (19). If the hypothesis was correct then the difference between H pylori positive and negative individuals should be largely or completely eliminated when a near neutral intragastric pH is maintained. The observation that this does not occur indicates that infection with H pylori does not result in a raised antral surface pH. It also confirms the earlier observations that stimulating ammonia production by urea infusion, or inhibiting ammonia production by the administration of acetohydroxamic acid did not alter gastrin concentrations.

Five hours of gastric alkalinisation alone caused a similar increase in plasma gastrin concentration before 47% and after 16% eradication of the organism. The demonstration of a gastrin response to prolonged alkalinisation indicates that it is unlikely H pylori creates a significant alkaline microenvironment by production of ammonia. It also demonstrates that if the production of an alkaline microenvironment caused gastrin

release then the stimulus from ammonia production through urea infusion should have been sufficient to cause an increase in gastrin concentration as there was sufficient gastrin reserve to respond to direct alkalinisation using the buffer.

If the raised gastrin concentrations found in subjects with H pylori infection does not occur through alteration of the antral mucosal pH, or promotion of amine uptake then other mechanisms need to be considered. Eradication of infection results in resolution of antral gastritis and it is possible that it is the inflammatory cell infiltrate in the region of the G cells which is responsible for the increased gastrin concentrations. One study has suggested that gastrin concentrations correlate better with antral gastritis than with H pylori infection (242). The gastritis associated with the infection could also be partly due to the damaging effects of ammonia making it difficult in practice to differentiate the effects of the two (167,181).

These observations demonstrate that the effect of infection with H pylori on plasma gastrin concentration was not the result of an antral alkaline microenvironment. The fact that plasma gastrin was also shown to respond to prolonged alkalinisation indicated that it is unlikely that H pylori creates an alkaline microenvironment under the antral mucosal layer in order to survive when it colonises the stomach. As a result

other possible functions of the organism's urease activity need to be examined. The usual role of urease activity in many micro-organisms is to allow the organism to scavenge nitrogen from a urea rich environment.

CHAPTER 8

THE EFFECT OF PH AND UREASE ACTIVITY ON THE IN VITRO SURVIVAL OF HELICOBACTER PYLORI

THE EFFECT OF PH AND UREASE ACTIVITY ON THE IN VITRO SURVIVAL OF HELICOBACTER PYLORI

8.1 Introduction

It has been proposed that Helicobacter pylori possesses abundant urease activity in order to survive low intragastric pH (181-183). It has been suggested that the urease activity might create an alkaline microenvironment around it and thereby neutralise gastric acidity which would otherwise destroy the organism. The previous studies of gastric juice urea concentrations and the effects of urea infusion have shown that when the organism colonises the gastric antral mucosa ammonium production by its urease activity is limited by the availability of urea (243). Direct alkalinisation of the gastric antrum by the infusion of a buffer solution results in an increased plasma gastrin concentration but stimulation or inhibition of ammonium production to alter any urease produced alkaline microenvironment did not change basal or meal stimulated gastrin responses (243-245). These studies demonstrated that H pylori does not produce an alkalinisation of its immediate in vivo environment sufficient to alter plasma gastrin concentrations. Although these in vivo studies suggest that urease activity may not be necessary for the survival

of H pylori in the stomach some in vitro studies have shown that urease activity protects the bacterium from low pH (183-185).

If the function of the organism's urease activity is to protect it from acidity then it might be expected that increased urease activity and ammonium production would occur when the pH of the medium around the organism was reduced. The survival of the organism, its utilisation of urea and production of ammonium were therefore investigated in a series of in vitro studies using buffer solutions ranging from pH 1.5 to 7.0. The effects increasing urea concentration and inhibition of urease activity with hydroxyurea were also examined.

8.2 Methods

The survival of H pylori at a range of buffer pH was investigated. The effect of varying the initial urea concentration in the buffer and of adding the urease inhibitor hydroxyurea was studied. In each experiment 1 ml of a 72 hour broth culture suspension (BHI broth + 0.25% yeast extract + 10% horse serum) of H pylori was added to 9 ml of 0.2 mol/L citrate buffers or to isomolar unbuffered saline and incubated at 37°C. At intervals portions were removed for viable bacterial counts and where appropriate for measurement of ammonium (Enzymatic method Cobas Bio) and urea (o-pthalaldehyde method Perspective analyser) concentrations. The pH was

monitored using a combined glass electrode (Radiometer GK 2802C).

Using the above protocol the following studies were undertaken:

1. The survival of the organism with 50 mmol/L urea at varying pH

The survival of the organism was studied in 0.2 mol/L citrate buffer in the presence of 50 mmol/L urea at pH 1.5,2.0,3.0,4.0,5.0,6.0 and 7.0 during a 60 minute incubation.

2. The survival of the organism at pH 6.0 during a 5 min incubation with 10 mmol/L urea

The survival of the organism during a 5 minute incubation was compared in citrate buffer pH 6.0 and isomolar unbuffered saline both with and without 10 mmol/L urea.

3. The effect of increasing urea concentration on survival at pH 6.0

The effect of varying the starting concentration of urea on survival at pH 6.0 in citrate buffer was assessed.

4. The effect of the addition of hydroxyurea on survival at pH 6.0 in the presence of urea

The effect of adding the competitive urease inhibitor, hydroxyurea on the survival of H pylori at pH 6.0 in the presence of 10 mmol/L urea was examined.

5. The effect of the addition of hydroxyurea on survival at pH 3.0 in the presence of urea.

The effect of adding the competitive urease inhibitor hydroxyurea on the survival of H pylori at pH 3.0 in the presence of 10 mmol/L urea was also examined.

A microtitre modification of the method of Miles and Misra (220) was used to establish survival. The portions removed for biochemical analysis were immediately filtered to remove the organism (Gelman Sciences, Acrodisc 0.2 um) and stored at -20°C. Experiments were performed with both NCTC 11637 culture of H pylori and with fresh clinical isolates. Statistical analysis was performed using the Mann-Whitney U test.

8.3 Results

The starting inoculum was determined separately for each experiment and was consistently 10^{5-6} cfu/ml. The pH of the incubating solution remained constant in each of the experiments employing citrate buffer. In the saline experiments, the pH at the start ranged from 6.26-6.60 and at 5 minutes had risen to pH 7.04-7.50.

1 The survival of the organism with 50 mmol/L urea at varying pH

At pH 1.5 the survival of the organism was impaired although the buffer contained 50 mmol/L urea (Table 8.1). As the pH increased the survival of the organism improved until pH 5.0 and 6.0 were reached when little or no

Table 8.1

Median % survival at a range of pH
in the presence of 50 mmol/L urea

Time	pH 1.5	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
10 min	0	4.9	17.6	65.6	1.2	1.9	71.6
30 min	5.1	51.1	112.0	56.3	0.3	0	85.0
60 min	9.1	89.9	55.0	50.3	0.1	0	91.3

The median percentage of initial inoculum of H pylori surviving in 0.2 mol/L citrate buffer with 50 mmol/L urea at a range of pH from 1.5 to 7.0.

survival of the organism was found following an incubation of only 10 minutes. When the pH of the buffer was increased to pH 7.0 survival of the bacterium returned to close to 100% of the initial inoculum These experiments were conducted using isolate 788 of the organism.

2 The survival of the organism at pH 6.0 during a 5 minute incubation with 10 mmol/L urea

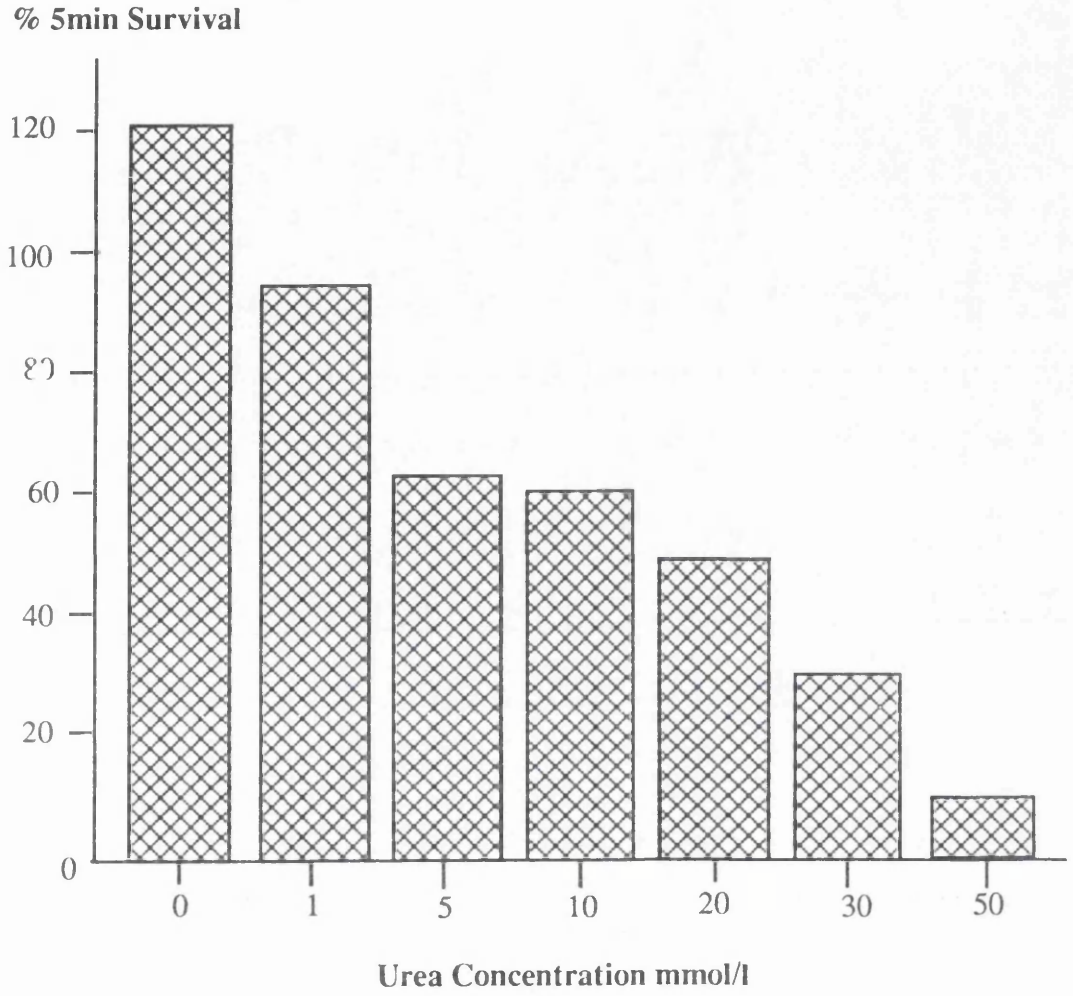
The median 5 minute survival in citrate buffer pH 6.0 was reduced when only 10 mmol/L urea was present in the incubation medium, 26%, (range 0-87%) with urea, compared with 96%, (range 28-179%) without urea, ($p<0.001$). Similar results were obtained when fresh clinical isolates of H pylori were also tested confirming that this was not solely a feature of the NCTC 11637 strain.

The median 5 minute survival in isomolar saline was not affected by the addition of 10 mmol/L urea (81% without urea compared with 77% with urea). The saline experiments were allowed to run for up to 2.5 hours with no difference in survival.

3 The effect of increasing urea concentration on survival at pH 6.0

The mean 5 minute survival at pH 6.0 decreased with increasing concentration of urea from 121% (range 69-148) in the absence of urea to only 9%, (range 0-22) with 50 mmol/L urea (Fig 8.1).

Fig. 8.1



Survival of H pylori in citrate buffer pH 6 with different urea concentrations.

Each value represents the mean of 5 experiments.

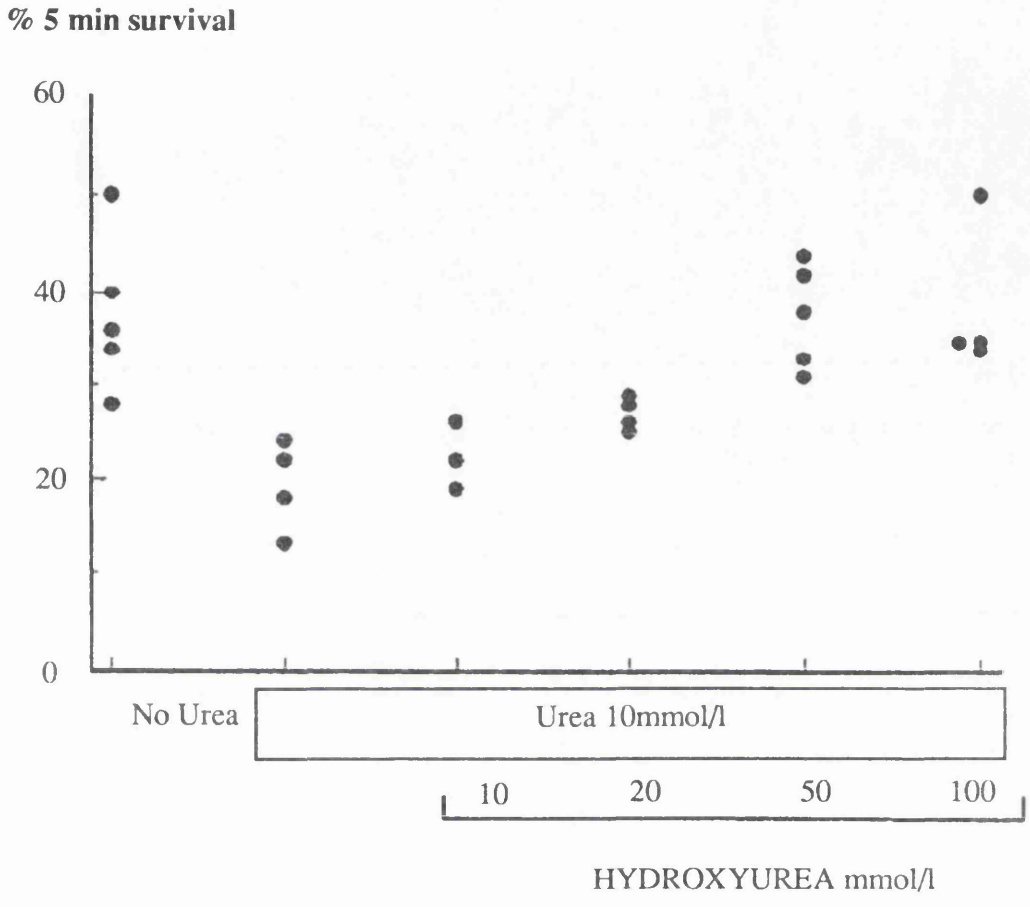
4 The effect of the addition of hydroxyurea on survival at pH 6.0 in the presence of urea

The addition of hydroxyurea inhibited the killing effect at pH 6.0. The protective effect increased with increasing concentration of hydroxyurea (Fig 8.2). The median 5 min ammonium concentration at pH 6.0 in the presence of 10 mmol/L urea was 2.5 mmol/L (range 1.9-7.6) compared with 1.13 mmol/L (range 0.8-1.3) in the blank incubation. When 10 mmol/L hydroxyurea was added the median 5 minute ammonium concentration was reduced to 1.29 mmol/L (range 1.03-1.53)($p<0.001$) indicating effective inhibition of the enzyme.

5 The effect of hydroxyurea on survival at pH 3.0 in the presence of urea

The addition of hydroxyurea in the presence of urea at pH 3.0 reduced the survival of the organism. The median 5 min survival of the organism in 0.2 mol/L citrate buffer pH 3.0 was 0% (range 0-2) compared with 179% (range 86-197) in the presence of 10 mmol/L urea ($p<0.05$). The median 5 min survival in the presence of 10 mmol/L urea and 100 mmol/L hydroxyurea was reduced, 15% (range 0-64%), compared with survival in citrate buffer containing 10 mmol/L urea ($p<0.05$). The median final ammonium concentration in the buffer was greater in the presence of urea alone (1.30 mmol/L, range 1.19-1.36) compared with the presence of both urea and hydroxyurea (1.15 mmol/L, range 1.12-1.16) indicating inhibition of urease activity.

Fig. 8.2



Effect of increasing concentrations of hydroxyurea on survival of H pylori in citrate buffer pH 6 plus 10 mmol/L urea.

8.4 Discussion

The rapid death of *H pylori* can be induced in vitro by altering both the pH and urea concentration of the organism's environment. Paradoxically urea protects the organism from the lethal effects of exposure to a pH of 4.0 or less and causes the organism's destruction at pH 5.0 and 6.0. Similarly the addition of the urease inhibitor hydroxyurea to the incubate results in the destruction of the organism at pH 3.0 and its survival at pH 6.0 despite the presence of urea. The rapid death of *H pylori* without the use of conventional antibacterial agents indicates that its destruction may be the consequence of the metabolic effects of uncontrolled urease activity. The use of isomolar saline, and citrate buffers without any urea added shows that the effect is not merely a result of altered osmolality of the environment. The protection of *H pylori* from destruction at pH 6.0 in the presence of urea by the addition of the specific urease inhibitor hydroxyurea suggests that its destruction is a result of over-production of ammonium resulting in irreversible metabolic damage. The activity of the urease enzyme is not suppressed by ammonium as a result of the absence of a protective feedback control mechanism (191).

Although the addition of urea protected the organism from pH 3.0 it did not alter the buffer pH or produce large quantities of ammonium which could create an

alkaline microenvironment. Inhibition of urease activity by hydroxyurea was fatal at low pH although buffer ammonium concentrations were only slightly reduced. This suggested that intracellular neutralisation of acidity by the production of ammonium might be more important for survival at low pH than the creation of an extracellular alkaline microenvironment.

Whether it will be possible to induce the destruction of H pylori in vivo by altering the pH and urea concentration of the gastric contents remains to be seen. Ammonium production can be increased by the infusion of urea (243). The pH of gastric juice can be altered by treatment with acid inhibitory agents. Patients with increased gastric pH due to pernicious anaemia or due to duodenogastric alkaline reflux following gastric surgery have a low prevalence of H pylori infection (113,246). In addition treatment with the powerful acid inhibitory agent omeprazole which raises intragastric pH to near neutral values has been reported to clear H pylori infection by some investigators (204,205). Omeprazole does not have any direct toxic or inhibitory effect on H pylori in vitro (208). It is also of interest that patients with uraemia have a lower prevalence of H pylori (247,248).

These studies might indicate a potential new approach to the treatment of H pylori infection. The mechanism of its urease mediated destruction deserves further investigation.

CHAPTER 9

UREASE MEDIATED DESTRUCTION OF HELICOBACTER PYLORI IS DUE TO INTRACELLULAR ACCUMULATION OF AMMONIUM

UREASE MEDIATED DESTRUCTION OF HELICOBACTER PYLORI IS DUE TO INTRACELLULAR ACCUMULATION OF AMMONIUM

9.1 Introduction

In the previous in vitro studies the paradoxical effects of urease activity on the survival of H pylori in citrate buffer were discovered. High concentrations of urea while protecting the organism in acid buffer at pH 3.0 caused its destruction at pH 6.0. Administration of the specific urease inhibitor hydroxyurea in the presence of urea resulted in the destruction of the organism at low pH and its survival at pH 6.0, thereby demonstrating that the survival of the organism at low pH and its destruction at pH 6.0 were mediated by its urease activity. The speed of death in citrate buffer pH 6.0 with a urea concentration of 50 mmol/L suggested that this was the result of a catastrophic metabolic failure. The reduction in colony forming units of the bacterium following a 5 minute incubation in citrate buffer with urea was greater than which could be produced with microaerophilic campylobacters during a 2 hour in vitro incubation with metronidazole (249). The speed and extent of the destruction of the organism suggested that if it could be harnessed in vivo it could provide a new therapeutic approach for the treatment of duodenal ulcers. In order

to investigate the biochemical basis of the suicidal process the organism's urea metabolism in environments causing its rapid death has been compared with that in environments in which it survived.

9.2 Methods

The earlier studies had indicated that pH, urea concentration and type of buffer may have contributed to the metabolic destruction of the organism. The effect of each of these on the organism's urea metabolism and survival was examined. The effect of adding metabolic intermediates used in intracellular ammonium metabolism was also examined.

1. The effect of pH

The survival of the organism its urea consumption and ammonium release in 0.2 mol/L citrate buffer pH 6.0 containing 50 mmol/L urea were compared with that in the same solution at pH 7.0. Similar studies were also conducted with unbuffered isomolar saline at pH 6.0 containing 50 mmol/L urea and 0.2 mol/L citrate buffer pH 6.0 without urea.

2. The effect of urea concentration

The effect of increasing the initial urea concentration on urea consumption, ammonium release and survival of the organism in 0.2 mol/L citrate buffer pH 6.0 was investigated. The following urea concentrations were examined:- 1 mmol/L, 5 mmol/L, 10 mmol/L, 20 mmol/L, 30 mmol/L and 50 mmol/L.

3. The effect of buffer ammonium ion concentration

The effect of the addition of 10 mmol/L ammonium chloride to 0.2 mol/L citrate buffer pH 6.0 on the survival of the organism was investigated.

4. The effect of citrate buffer

The survival of the organism in the presence of 30 mmol/L urea in 0.2 mol/L citrate buffer pH 6.0 was compared with survival in 0.2 mol/L acetate buffer pH 6.0 containing 30 mmol/L urea, and with 0.2 mol/L phosphate buffer pH 6.0 also containing 30 mmol/L urea. Urea consumption and percentage ammonium released were also measured.

5. The effect of isocitric acid buffer

The survival of the organism in the presence of 50 mmol/L urea in 0.2 mol/L isocitrate buffer pH 6.0 was compared with its survival in 0.2 mol/L citrate buffer with the same concentrations of urea.

6. The effect of the addition of alpha ketoglutarate

The survival of the organism following a 3 day pre-incubation in broth with 10 mmol/L alpha ketoglutarate, and then during a subsequent 5 min incubation in 0.2 mol/L citrate buffer pH 6.0 with a urea concentration of 50 mmol/L was compared with its survival in the buffer with urea but without pre-incubation with alpha ketoglutarate.

7. The recovery of urea and ammonium in citrate buffer in the absence of the organism.

The recovery of ammonium and loss of urea from the experimental system was investigated by incubating 1, 5

and 10 units of Jackbean urease enzyme (Sigma Chemical Company, Dorset, UK) in 5 ml of 0.2 mol/L citrate buffer pH 6.0 with 50 mmol/L urea at 37°C in a waterbath for 10 min. Following the incubation the enzymatic reaction was stopped by the addition of 2 ml of 1 mol/L HCl which reduced the pH of the buffer to 3.0. Samples were stored frozen at -20°C until analysis. The urea and ammonium concentrations in these samples were measured as previously described.

8. Removal of urease activity by filtration of samples

The urease activity in the filtered samples was measured by the urease method previously described.

The method of Miles and Misra (220) was used to establish survival of the organism. The 1 ml sample collected for urea and ammonium analyses was filtered (Gelman Sciences, Acrodisc 0.2 um) to remove the organism, snap frozen and stored at -20°C until analysis. The urea concentration was measured by the o-pthalaldehyde method and the ammonium concentration using the enzymatic method described for the Cobas Bio.

The stock buffer solutions used were 0.2 mol/L sodium citrate buffer pH 6.0 and pH 7.0, 0.2 mol/L sodium isocitrate buffer pH 6.0, 0.2 mol/L sodium acetate buffer pH 6.0 and 0.2 mol/L sodium phosphate buffer pH 6.0. Isomolar sodium chloride solution was adjusted to pH 6.0 prior to use. To obtain a range of urea concentrations,

stock solutions with urea concentrations of 0.1 mol/L, 0.5 mol/L, 1 mol/L, 2 mol/L, 3 mol/L and 5 mol/L were made. All the stock solutions were stored at -20°C until use. To set the initial urea concentration in the buffer 100 ul of the appropriate stock urea solution was added to 8.9 ml of the required buffer, to give final urea concentrations of 1 mmol/L, 5 mmol/L, 10 mmol/L, 20 mmol/L, 30 mmol/L, or 50 mmol/L in the final suspension. Buffers were equilibrated at 37°C and their pH checked prior to each experiment. To commence each experiment 1 ml of a 72 h broth culture suspension was added to 9 ml of buffer as previously described.

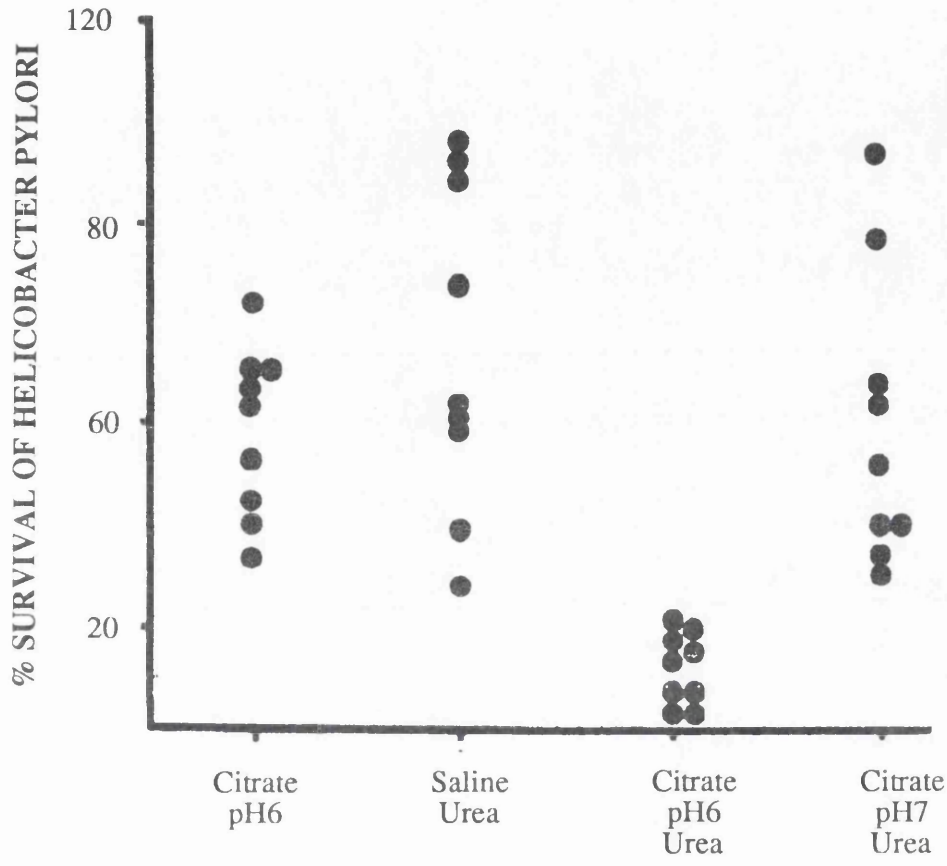
9.4 Results

1 The effect of pH

The median 5 min survival of H pylori when incubated at 37°C in 0.2 mol/L sodium citrate buffer pH 6.0, with an initial urea concentration of 50 mmol/L was only 14% (range 0-22%) compared with 53% (range 31-112) when incubated in the same solution but at pH 7.0 ($p<0.01$) (Fig 9.1). The organism's survival in citrate buffer at pH 6.0 with 50 mmol/L urea was also reduced in comparison with survival in isomolar saline containing 50 mmol/L urea, (median 65%, range 29-113%) ($p<0.01$) and with survival in 0.2 mol/L sodium citate buffer pH 6.0 without any added urea (median 60%, range 33-114%) ($p<0.01$)(Fig 9.1).

Urea utilization was similar in pH 6.0 buffer and in pH 7.0 buffer, being 154 umol/5 min (126-162) and 162

Fig. 9.1



The percentage survival of H pylori NCTC 11637 at the end of a 5 min incubation (n=9), using 0.2mol/L citrate buffer pH 6.0, isomolar saline containing 50 mmol/L urea, 0.2mol/L citrate buffer pH 6.0 containing 50 mmol/L urea, and 0.2mol/L citrate buffer pH 7.0 containing 50 mmol/L urea.

umol/5 min (73-188) respectively (Table 9.1). The median amount of ammonium found at 5 min in the incubate at pH 7.0 was 18 umol, (range 8-21) which was significantly greater than at pH 6.0, 9 umol, (range 7-10) ($p<0.01$) (Table 9.1).

2 The Effect of Urea Concentration

The median 5 min survival of the organism in 0.2 mol/L citrate buffer pH 6.0 progressively fell with increasing initial urea concentrations from 89% (range 19-124%) at 1 mmol/L to 39% (range 13-87%) at 10 mmol/L, 29% (range 10-64%) at 30 mmol/L and to 14% (range 0-22%) at 50 mmol/L. Although the median percentage survival of the organism fell as the initial urea concentration increased, the amount of urea used by the organism during the 5 min incubation increased from 9 umol (range 7 - 9) at an initial urea concentration of 1 mmol/L to 32 umol (range 14-92 umol) at 10 mmol/L ($p<0.05$), 98 umol (range 15-281 umol) at 30 mmol/L ($p<0.01$), and 146 umol (range 11 - 171) at an initial urea concentration of 50 mmol/L ($p<0.01$) (Fig 9.2). The percentage recovery of ammonium from the hydrolysis of urea fell as the initial urea concentration increased (Fig 9.3). With an initial urea concentration of 1 mmol/L, the median 5 min recovery of ammonium was 27% (range 7-94%) compared with 18% (range 15-80%) at 10 mmol/L, 16% (range 4-315%) at 30 mmol/L, and only 3% (2-22%) at 50 mmol/L urea ($p<0.01$ versus 1 mmol/L urea).

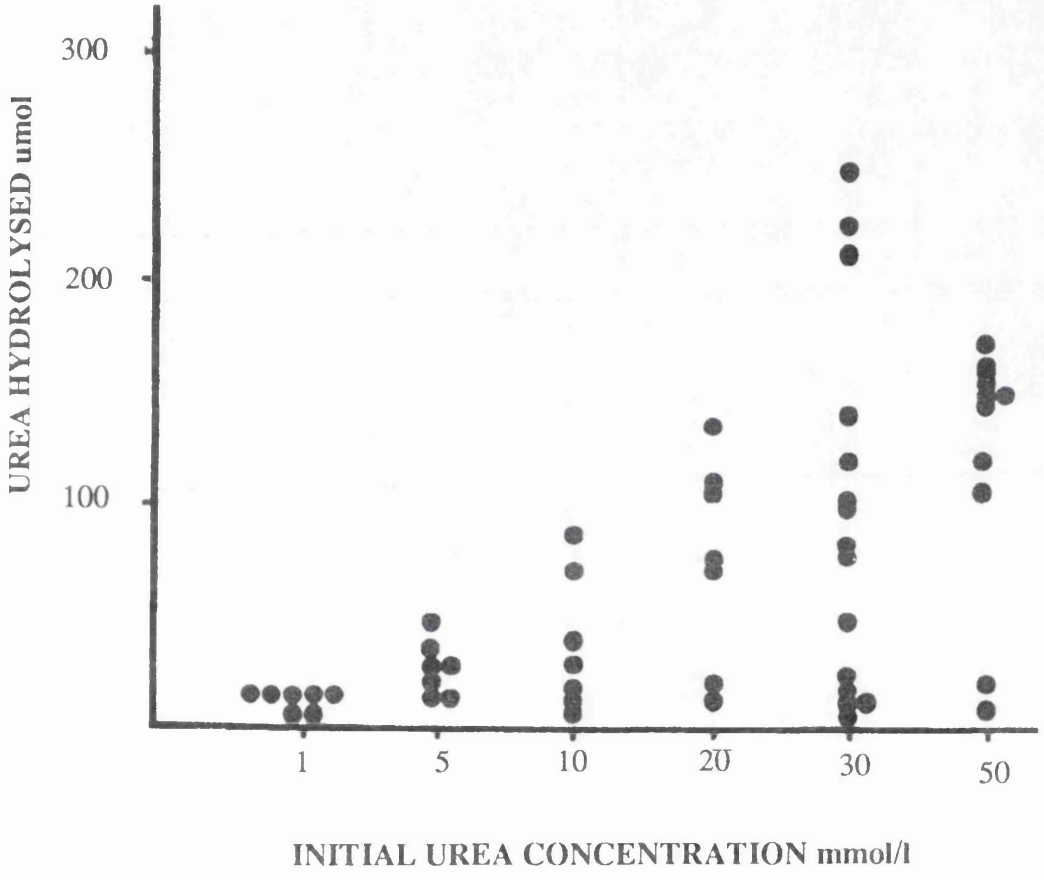
Table 9.1

Buffer	Urea hydrolysed umol/5 min median (range)	NH_4+ produced umol/5 min (range)	% Survival median (range)
Citrate pH 6.0 50 mmol/L urea n=8	154 (126-162)	9 * (7-10)	14 * (0-22)
Citrate pH 7.0 50 mmol/L urea n=8	162 (73-188)	18 (8-21)	53 (31-112)
Isomolar saline pH 6.0 50 mmol/L urea n=8	200 (182-225)	10 (7-12)	65 (29-113)
Citrate no urea pH 6.0 n=8	-	-	60 (33-114)

* $p<0.01$

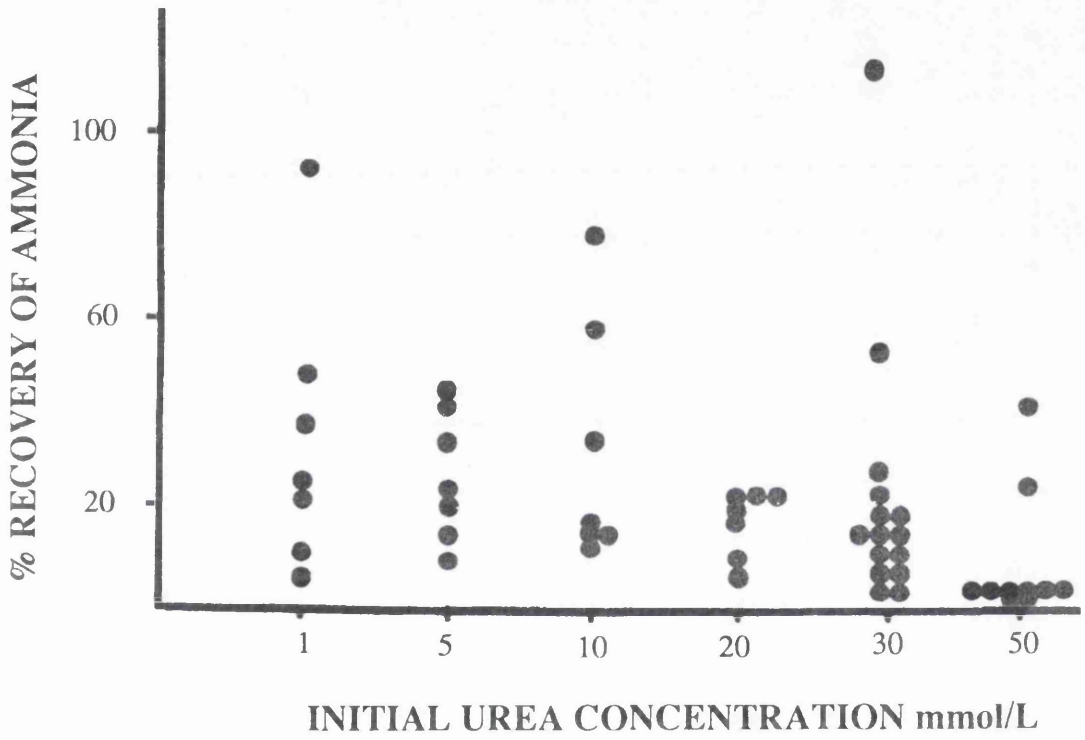
Survival, urea utilisation and ammonium production in 0.2 mol/L citrate buffer pH 6.0 and pH 7.0.

Fig. 9.2



The amount of urea used by H pylori during the 5 min incubation in 0.2mol/L citrate buffer pH 6.0 containing initial urea concentrations from 1 to 50 mmol/L. For urea concentrations of 1 to 20 mmol/L, n=7 and for 30 mmol/L urea n=17 and for 50 mmol/L urea n=9.

Fig. 9.3



Recovery of ammonia, expressed as a percentage of the amount of ammonia expected to have been formed as a result of hydrolysis of urea by H pylori in 0.2mol/L citrate buffer pH 6.0 with initial urea concentrations from 1 to 50 mmol/L. For urea concentrations of 1 to 20 mmol/L, n=7 and for 30 mmol/L urea n=17 and for 50 mmol/L urea n=9.

3 The Effect of Buffer Ammonium Ion Concentration

The survival of the organism in 0.2 mol/L citrate buffer pH 6.0 in the presence of 10 mmol/L ammonium chloride was investigated. The median ammonium concentration of the 12 replicate experiments when measured at the start was 10.7 mmol/L (range 9.7-13.1) and at the end it was 10.9 mmol/L (range 10.6-11.3). The 5 min survival of the organism in buffer with ammonium chloride, (median 64%, range 20-137%) was similar to its survival in 0.2 mol/L sodium citrate buffer pH 6.0, without any additions (median 60%, range 33-148%).

4. The Effect of Citrate Buffer

The median 5 min survival in buffer pH 6.0 in the presence of 30 mmol/L urea when citrate buffer was used was 29% (range 10-64%) compared with 80% (range 26-160%) when acetate buffer was used ($p<0.01$) (Table 9.2). The amount of urea used by the organism in the acetate buffer, (median 215 umol, range 18 - 272) was not statistically different from that in the citrate buffer, (median 97 umol range 5-282). The median 5 min ammonium concentration was similar in the acetate buffer, (33 umol, range 7-132) to that in the citrate buffer (19 umol, range 7-41). The percentage of ammonium recovered from the hydrolysis of urea in the acetate buffer (12.1%) was also similar to that (8.6%) in the citrate buffer. In the absence of urea the 5 min survival in citrate buffer pH 6.0 was still reduced (68%, range 33 - 163%) when compared with survival in the acetate buffer pH 6.0 (95%, range 36 - 211%) ($p<0.05$).

Table 9.2

Buffer	Urea hydrolyzed umol/5 min median (range)	NH_4^+ produced umol/5 min median (range)	% recovery of NH_4^+ median (range)	% survival median (range)
Citrate buffer pH 6.0 with 30 mmol/L urea n=18	97 (5-282)	19 (7-41)	8.6% (2.6-61)	29* (10-64)
Acetate buffer pH 6.0 with 30 mmol/L urea n=18	215 (18-272)	33 (7-132)	12.1% (3.5-90)	80 (26-160)

*$p<0.01$

Survival, urea utilisation and ammonium production in 0.2 mol/L citrate buffer compared with 0.2 mol/L acetate buffer.

The median 5 min survival of H pylori in the presence of 30 mmol/L urea in phosphate buffer, at pH 6.0 (100%, range 72-128) was also greater than that in citrate buffer (42%, range 27-55) ($p<0.05$) (Table 9.3). In the absence of urea survival was similar in citrate (83% range, 75-103%) and phosphate buffers (90%, range 82-126%). A similar amount of urea was consumed in the phosphate buffer, (83 umol, range 0-296) and in the citrate buffer, (120 umol, range 19-264). The median percentage recovery of ammonium from urea hydrolysed was again similar for both buffers, 17% for citrate and 15% for phosphate (Table 9.3).

5 The Effect of Isocitric Acid buffer

The median 5 min survival of the organism in 0.2 mol/L isocitrate buffer containing 50 mmol/L urea was 37% (range 0-274%), compared with 9.9% (range 0-146%) in 0.2 mol/L citrate buffer with 50 mmol/L urea (Table 9.4) ($p<0.01$). The utilisation of urea was similar in the isocitrate buffer, median 3.9 mmol/L (range 0-24) to that in the citrate buffer median 3.8 mmol/L (range 0-28) (Table 9.5). There was no significant difference in ammonium production between the two buffers. The median final ammonium concentration in the isocitrate buffer was similar, 5.34 mmol/L (range 1.83-27.6) compared with 4.24 mmol/L (range 2.11-27.1) in citrate buffer (Table 9.5).

6 The Effect of the addition of Alpha Ketoglutarate

The median 5 min survival of the organism in 0.2 mol/L citrate buffer containing 50 mmol/L urea following a

Table 9.3

Buffer	Urea hydrolyzed umol/5 min median (range)	NH^{4+} produced umol/5 min median (range)	% recovery of NH_4^+ median (range)	% survival median (range)
Citrate buffer pH 6.0 with 30 mmol/L urea n=7	120 (19-264)	39 (32-41)	17% (7.4-21)	42 * (27-55%)
Phosphate buffer pH 6.0 with 30 mmol/L urea n=7	83 (0-296)	30 (5-36)	15% (4.3-19%)	100 (72-128)

*$p<0.05$

Survival, urea utilisation and ammonium production in 0.2 mol/L citrate buffer compared with 0.2 mol/L phosphate buffer.

Table 9.4

Buffer	Survival of H pylori % of initial inoculum median	range
Citrate buffer pH 6.0	88	18-384
Isocitrate buffer pH 6.0	88	15-274
Citrate buffer pH 6.0 + 50 mmol/L urea	9.9*	0-164
Isocitrate buffer pH 6.0 + 50 mmol/L urea	37	0-274

* p <0.01 vs isocitrate buffer with urea

Survival in 0.2 mol/L citrate buffer pH 6.0 compared 0.2 mol/L isocitrate buffer pH 6.0.

Table 9.5

	Decrease in Urea concentration mmol/L (range)	Final Ammonium concentration mmol/L (range)
citrate buffer + 50 mmol/L urea	3.8 (0-28.0)	* 4.24 (2.11-27.1)
isocitrate buffer + 50 mmol/L urea	3.9 (0-24.0)	* 5.34 (1.83-27.6)

* p >0.5 : NS

Urea utilisation and ammonium production in 0.2 mol/L citrate buffer pH 6.0 with 50 mmol/L urea compared with 0.2 mol/L isocitrate buffer pH 6.0.

72h pre-incubation with 10 mmol/L alpha ketoglutarate, was greater, (43%, range 3 - 256%), than its survival in 0.2 mol/L citrate buffer with the urea but without pre-incubation with alpha ketoglutarate, (14%, range 0-22%)($p<0.01$).

Following pre-incubation with alpha ketoglutarate the median 5 min survival in the citrate buffer pH 6.0 containing the urea (43%, range 3-256%), was similar to the organism's 5 min survival in the pH 6.0 citrate buffer but without urea (70%, range 3-323%) ($p>0.5$ NS).

7 The recovery of urea and ammonium in citrate buffer in the absence of the organism

Following a 10 min incubation the recovery of ammonium in citrate buffer containing 50 mmol/L urea was proportional to the amount of urease enzyme added. When 1 unit of enzyme was added the mean ($\pm$ SD) 10 minute ammonium concentration was 5.6 mmol/L ($\pm$ 0.62) with 5 units of enzyme it was 21.0 mmol/L ($\pm$ 2.4) and when 10 units of enzyme were added the 10 minute ammonium concentration was 46.2 mmol/L ($\pm$ 4.40). The amount of urea hydrolysed by the enzyme was accounted for by the production of ammonium. The mean urea consumed was 2.7 mmol/L with 1 unit of urease, 12 mmol/L with 5 units of enzyme and 22.9 mmol/L with 10 units of enzyme, giving recoveries of 103%, 91% and 101% respectively.

8 Urease activity in filtered samples

Urease activity was not detected in the filtered samples when checked prior to urea and ammonium measurement.

9.5 Discussion

The rapid destruction of H pylori in citrate buffer was due to a combination of the pH of the solution, urea concentration and citrate. The speed of the bacterium's destruction indicated that the effect was mediated through its metabolism.

The importance of the pH of the citrate buffer was demonstrated by the organism's improved survival in citrate buffer solution pH 7.0 containing 50 mmol/L urea compared with its survival in the same solution at pH 6.0. Although similar amounts of urea were utilised less ammonium was released into the incubate at pH 6.0 than at pH 7.0. This might explain the organism's impaired survival at pH 6.0 in the presence of urea.

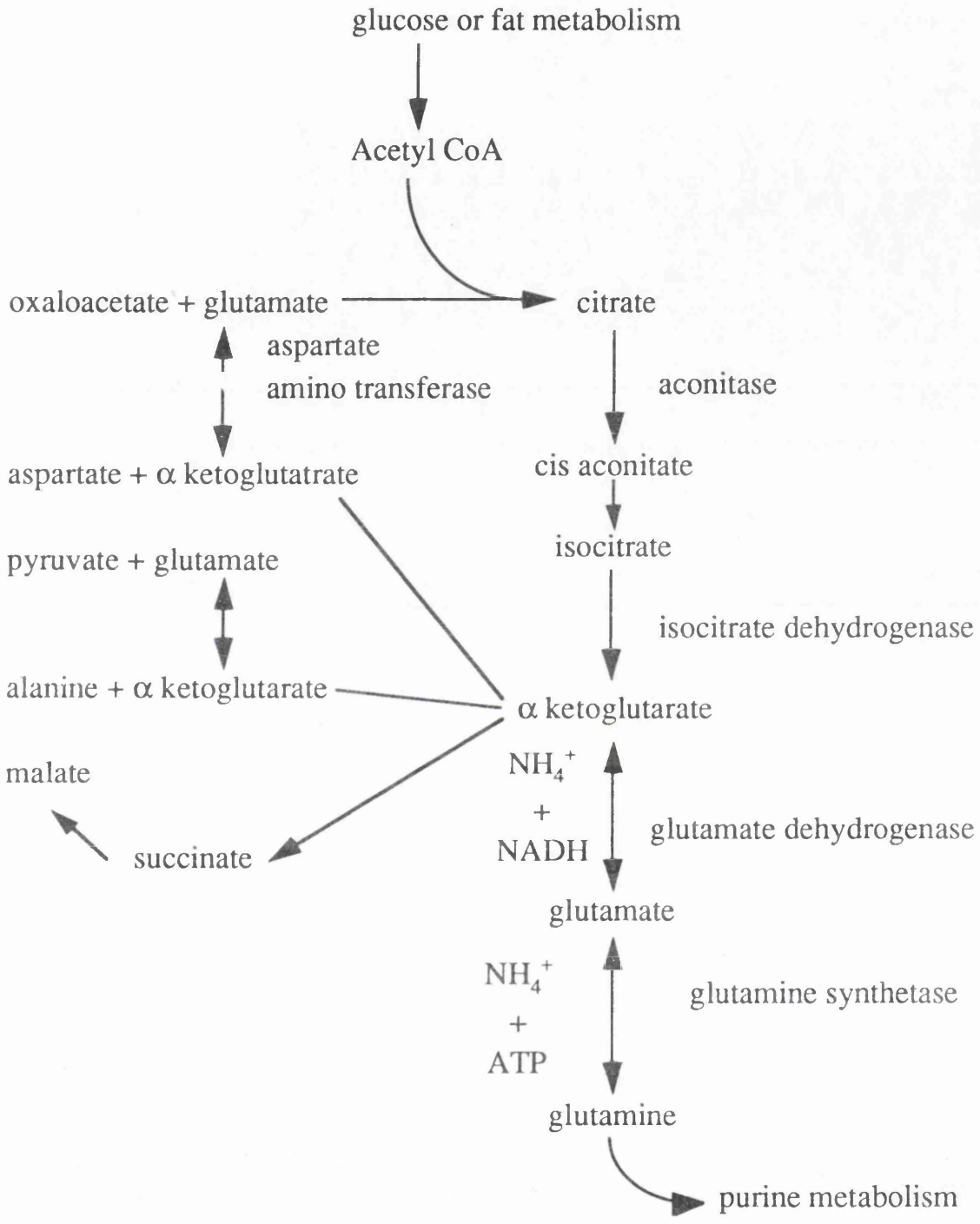
The survival of H pylori also decreased with increasing initial urea concentration in pH 6.0 citrate buffer again indicating that the suicidal destruction was linked to urease activity. This observation was consistent with the finding from the initial in vitro studies that the urease inhibitor hydroxyurea enhanced the survival of the organism in citrate buffer pH 6.0. The percentage recovery of the ammonium fell with increasing initial urea concentration. This indicated that the organism may have been unable to excrete the ammonium generated by urea hydrolysis as rapidly as it was produced. This failure to excrete ammonium may explain its rapid death.

The addition of 10 mmol/L ammonium to the citrate buffer solution did not reduce the survival of the organism which indicated that it was the production of ammonium by urease activity and the effects of the ammonium within the cell which proved harmful to the organism.

Citrate was also necessary for the bacterium's destruction as the organism survived well in phosphate and acetate buffers containing the same concentration of urea. The percentage recovery of ammonium was similar in acetate and phosphate buffers to that in citrate buffer. The contribution of citrate to the destruction of the organism cannot be explained by alteration of urease activity or ammonium excretion.

If ammonium produced by urease activity entered the cell then it could be used in the formation of glutamate or glutamine by glutamate dehydrogenase and glutamine synthetase respectively. Both of these reactions consume high energy intermediates but glutamate dehydrogenase also uses alpha ketoglutarate to form glutamate. Alpha ketoglutarate is a key intracellular metabolite which links carbon metabolism with nitrogen metabolism (Fig 9.4). It is possible that citrate when present in high concentrations could inhibit the activity of the enzyme isocitrate dehydrogenase (250). Isocitrate differs from citrate only by the transposition of the hydroxyl group from the central carbon atom of the molecule to one of the adjacent carbon atoms (Fig 9.5). Isocitrate dehydrogenase

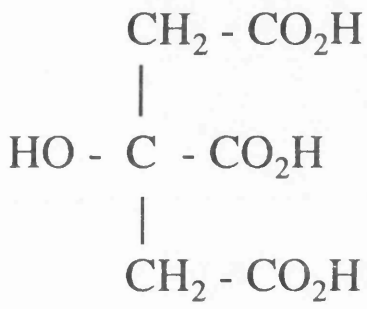
Fig. 9.4



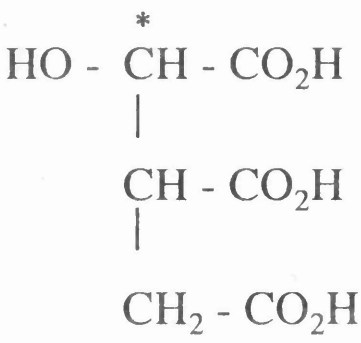
The key position of α ketoglutarate in the intracellular metabolism of ammonium and organic compounds containing nitrogen.

Fig. 9.5

CITRIC ACID



ISOCITRIC ACID



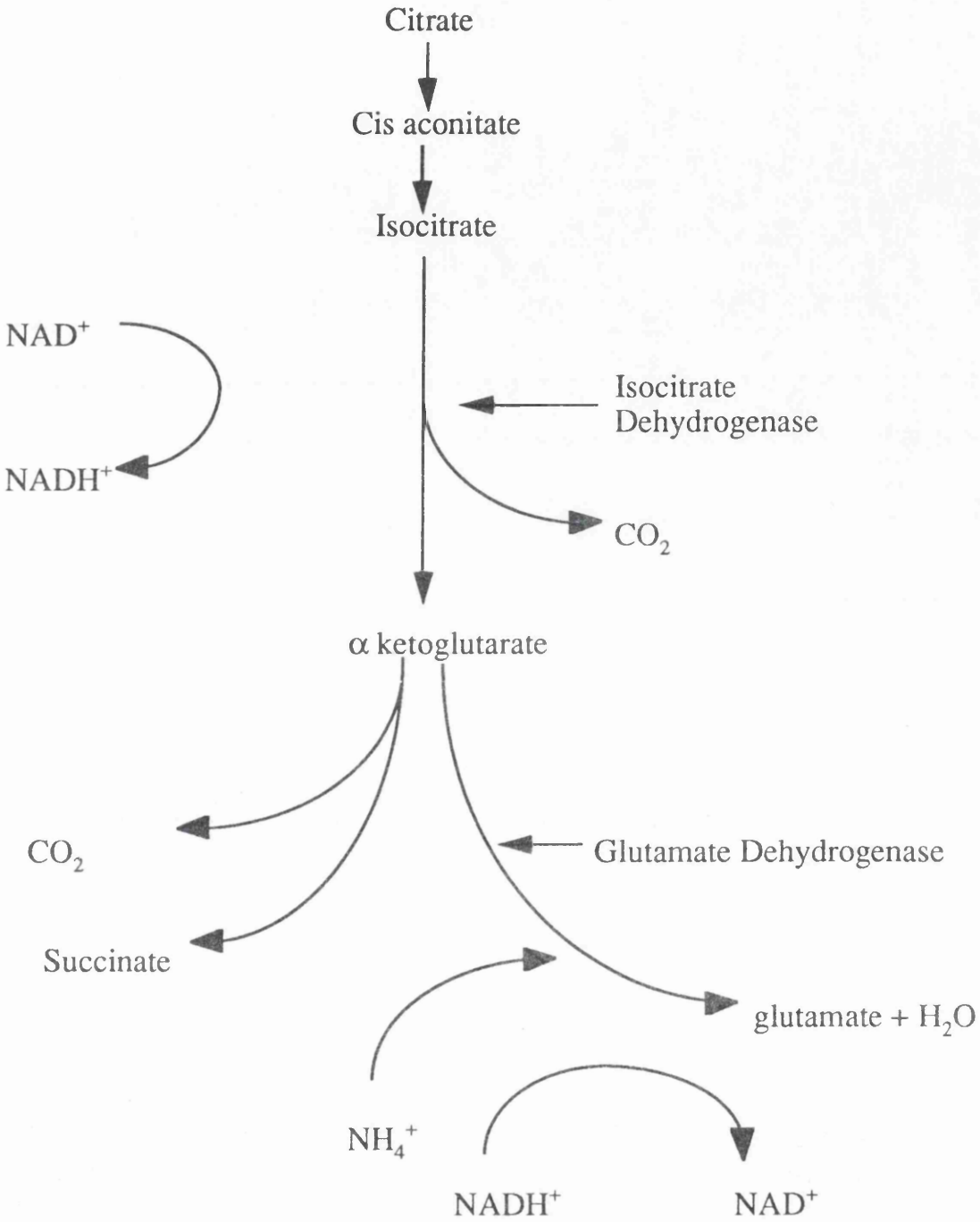
The structure of citric acid and isocitric acid.
* indicates chiral centre, which results in D and L stereoisomers.

catalyses one of the key steps involved in the generation of alpha ketoglutarate from citrate (Fig 9.6). The simultaneous inhibition of synthesis of alpha ketoglutarate by citrate and stimulation of its utilisation by urease activity could result in the complete depletion of this compound in the bacterium with intracellular accumulation of ammonium and a rise in intracellular pH.

The survival of H pylori in the isocitrate buffer pH 6.0 with 50 mmol/L urea was greater than in citrate buffer pH 6.0 containing the same concentration of urea. This was consistent with the hypothesis that the depletion of intracellular alpha ketoglutarate resulted in the destruction of the organism. The absence of any difference in recovery of ammonium or urea consumption over the 5 minutes of the experiment between the isocitrate and citrate buffers was consistent with protection of the organism by the production of more intracellular alpha ketoglutarate which reduced intracellular ammonium concentrations.

The protective effect of 0.2 mol/L isocitrate buffer was less effective than the destructive effect of citric acid. This may be due to other metabolic factors such as a greater rate of production of ammonium by the organism's abundant urease activity than the rate of production of alpha ketoglutarate by isocitrate dehydrogenase. In addition the isocitric acid used was a racaemic mixture and only the L stereoisomer would be biologically active

Fig. 9.6



The metabolism of isocitrate.

(Fig 9.5). As a result a 0.2 mol/L solution of isocitric acid could be less biochemically effective than the same concentration of the citrate buffer.

The hypothesis that intracellular depletion of alpha ketoglutarate leads to the intracellular accumulation of ammonium and thereby results in the bacterium's destruction was also supported by the observation that pre-incubation of the organism with alpha ketoglutarate protected it from the lethal effects of citrate buffer at pH 6.0 containing urea.

The almost complete recovery of ammonium produced when Jackbean urease enzyme was added to citrate buffer with 50 mmol/L urea demonstrated that the ammonium lost from the experimental system was not blown off during the experiment or lost on the plastic containers, or glassware or during storage. The ammonium not recovered must have accumulated within the bacterium which was removed by filtration prior to urea and ammonium analysis.

Failure to detect urease activity in the filtered samples also indicated that any loss of urea prior to analysis did not occur as a result of residual urease activity not removed by filtration.

The location of the urease enzyme is thought to be on the exterior surface of the organism's periplasmic membrane (201, 202). Uncontrolled production of ammonium by the urease activity at localised sites could lead to the rapid accumulation of high concentrations of ammonium

just outside the cell. Intracellular ammonia concentrations are low and it is therefore possible that ammonia produced by urease activity could diffuse down the concentration gradient into the cell almost as rapidly as it was produced. Once the ammonia had been protonated within the cell the resultant ammonium ions would be unable to re-cross the cell membrane and diffuse out of the cell as easily. This would explain why high concentrations of extracellular ammonium ions which were not able to cross the cell membrane easily were not harmful to the organism and also how utilisation of small quantities of urea at low pH could enhance the survival of the bacterium through the neutralisation of intracellular acid.

H pylori is difficult to eradicate with conventional treatment. Even triple therapy with tripotassium dicitrato bisthmuthate, metronidazole, plus either amoxycillin or tetracycline for 2-4 weeks fails to eradicate the infection in 10-20% of patients (218). The development of antibiotic resistance is also proving a problem (219). These in vitro observations may indicate the biochemical basis of a potential new approach to killing the organism in vivo. The in vivo urea experiments have already demonstrated that ammonium production by the organism can be stimulated in the human stomach (243), thereby raising the possibility that the in vitro conditions could be reproduced in patients.

In conclusion, H pylori rapidly dies in citrate buffer pH 6.0 containing urea. This is due to the metabolic consequences of the rapid intracellular accumulation of ammonium.

CHAPTER 10

FINAL DISCUSSION AND CONCLUSIONS

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10.1 The detection of Helicobacter pylori infection

Infection of the gastric antral mucosa by Helicobacter pylori results in low gastric juice urea concentrations and raised gastric juice ammonium concentrations. Standard enzymatic and chemical analytical methods may be used to measure these changes in concentration. The ratio of gastric juice urea to ammonium concentrations is characteristically less than 0.8 when the infection is present and greater than 0.9 when infection is absent. Calculation of this ratio may be used to detect infection with H pylori as reliably as using the ^{14}C-urea breath test. The ratio was unaffected by high plasma and gastric juice urea concentrations found in renal failure and was also not altered by the administration of H_2 receptor antagonists such as ranitidine. This test has the further advantage that delay in analysis should not affect the discriminating ability of the ratio as any urease activity in a stored sample could only cause a further reduction of urea concentration and resulting increase in ammonium concentration, which would only ensure that the ratio remained within the range found in H pylori positive individuals. An additional advantage of this test for the

detection of infection is that it may be possible to analyse the gastric juice samples in the endoscopy suite using some of the currently available bedside biochemistry analysers. The Kodak Ektachem system (Kodak, Herts, UK) which could be suitable for endoscopy suite measurement of ammonium concentrations, has already been used successfully to measure gastric juice ammonium concentrations (251).

The disadvantage of the test was that fasting gastric juice samples needed to be collected.

When simple and reliable methods of eradicating the infection are developed then the importance of establishing the presence of infection for the clinical management of patients with duodenal ulcers will increase. The measurement of the urea/ammonium ratio in gastric juice could prove a useful test as it is unlikely that arrangements for ^{14}C-urea breath tests would be readily available for the investigation of patients attending District General Hospitals. Histological examination of biopsies and the culture of the organism will be widely available but are more labour intensive, and less easily automated than biochemical analysis. In addition, once a widely usable effective treatment for the organism becomes available the number of patients requiring determination of H pylori status may exceed the capacity of the standard histological and microbiological techniques. The only other easily automated and relatively simple method of investigating infection is by

serology which has not currently gained wide acceptance and has proved to be unreliable in older age groups (80-85).

10.2 Ammonium production by Helicobacter pylori and its effect on gastrin release in vivo

The in vivo studies demonstrated that Helicobacter pylori urease activity in subjects with chronic infection and duodenal ulcer disease is usually limited by substrate availability (243). Ammonium production could be stimulated by the infusion of urea containing solutions and gastric juice urea concentrations also rose rapidly in response to inhibition of urease activity by acetohydroxamic acid.

While ammonium production by the organism's urease activity could be altered these studies also showed that raised plasma gastrin concentrations in individuals with the infection were not due to the organism's urease activity. Stimulation of urease activity by urea infusion, and inhibition of urease activity with acetohydroxamic acid did not alter the basal plasma gastrin concentration or the meal stimulated gastrin response either before or after eradication of the organism.

Prolonged alkalinisation of the gastric antrum by the infusion of citrate buffer pH 7.0 did cause an increase in plasma gastrin concentration and meal stimulated gastrin response but a significant difference between H pylori positive and negative subjects was not demonstrated (245).

As direct alkalinisation of the gastric antrum caused increased gastrin release while stimulation and inhibition of urease activity did not alter plasma gastrin concentration it is unlikely that the organism's urease activity created a significant alkaline microenvironment in the neighbourhood of the gastric epithelial cells in the antrum. Other explanations for the purpose of the abundant urease activity of this micro-organism were therefore examined.

10.3 The effect of pH and urea on survival of Helicobacter pylori in vitro

Urease activity was shown to enhance the in vitro survival of Helicobacter pylori at pH 3.0. The addition of the urease inhibitor hydroxyurea abolished the protective effect of urea at low pH confirming that enhanced survival was due to urease activity. Although urease activity enhanced survival at low pH only small quantities of ammonium were produced which were insufficient to alter the pH of the buffer. This suggested that intracellular buffering of pH may be of more importance than the creation of an alkaline microenvironment for the survival of the micro-organism. Urease activity may therefore be of importance in the survival of the organism during its initial passage through the gastric juice at the time the infection is acquired.

While urea enhanced survival of the organism in citrate buffer at pH of 3.0 it was also associated with

the rapid destruction of the organism at pH 5.0 and 6.0. H pylori could be protected from the destructive effects of incubation in citrate buffer pH 6.0 with urea by the addition of the urease inhibitor hydroxyurea. In addition, when the initial urea concentration in the citrate buffer at pH 6.0 was increased from 1 to 50 mmol/L an almost dose dependent reduction in survival occurred. These observations demonstrated that urease activity caused the in vitro destruction of the organism at pH 6.0.

Citrate buffer itself was required for the destruction of the organism through its urease activity. The organism survived lethal urea concentrations in phosphate and acetate buffers at pH 6.0.

The survival of the organism in citrate buffer pH 6.0 containing 10 mmol/L ammonium chloride, the reduction in recovery of ammonium as the initial buffer urea concentration was increased and the increased survival of the organism at pH 7.0 which was associated with increased recovery of ammonium all indicated that intracellular metabolism of ammonium produced by urease activity was required for the destruction of H pylori.

It is unlikely that the low recovery of ammonium in the in vitro studies was due to loss from the experimental system. Prolonging the incubation of the organism in citrate buffer pH 6.0 up to 2 h produced almost complete recovery of ammonium.

These observations confirmed that a significant amount of ammonium produced by urease activity was retained by the organism during the 5 min incubation studies.

Confirmation of the role of intracellular ammonium metabolism in the destruction of the organism was demonstrated by enhanced survival of H pylori in 0.2 mmol/L isocitrate buffer with 50 mmol/L urea compared with citrate buffer with 50 mmol/L urea. Isocitric acid differs from citrate by only the position of one hydroxyl group (Fig 9.5). Isocitric acid is also direct precursor of alpha ketoglutarate which is used to incorporate ammonium into glutamic acid and other aminoacids required for intracellular metabolic processes (Fig 9.4). The survival of H pylori when pre-incubated with alpha ketoglutarate was also enhanced. The improved survival of H pylori when the substrates required for intracellular metabolism of ammonium were supplied therefore demonstrated that the intracellular metabolism of ammonium was the key to the destruction of the organism by its urease activity.

10.4 Helicobacter pylori urease activity

Urease activity is expressed by many micro-organisms (192). It is usually a means of scavenging nitrogen from urea within the organism's environment. Ureaplasma urealyticum urease activity has been linked to proton

transport and could be used to produce high energy intermediates although the organism does not appear to be critically dependent on the production of high energy intermediates from proton transport linked to ammonium accumulation (192).

The Michaelis constant (Km) of the urease enzymes produced by other bacteria varies (192). It ranges from 0.12 mmol/L for Spirula maxima to 10.5-71 mmol/L for proteus species and up to 130 mmol/L for Bacillus pasteurii. The Km of an enzyme is the concentration of substrate at which the enzyme will operate at half maximum velocity. The Km of an enzyme can also be regarded as a measure of the affinity of the enzyme for the substrate. Enzymes with low Km values will have a high affinity and bind substrate readily at low concentrations while high Km values will require higher substrate concentrations for effective operation of the enzyme. High urea concentrations in the surrounding medium will therefore tend to select for enzymes with high Km values. Proteus mirabilis which infects the urinary tract exhibits a range of Km values from 13-60 mM (252,253). The concentrations of urea found in the urine 0.4-0.5 mol/L would allow the proteus urease enzymes to operate at or close to maximum velocity (192). The Km of H pylori urease is estimated to be between 0.2 and 0.7 mmol/L (193,197,254). At the concentrations of urea found in gastric juice from uninfected individuals (1.0-3.7 mmol/L) the enzyme would not operate at maximum velocity. At the concentrations of urea (0.5-2.9 mmol/L) found in infected individuals it

would be impossible for the enzyme to operate at maximum velocity. Some bacteria such as Alcaligenes entrophus and Klebsiella pneumoniae, use an energy dependent carrier mediated uptake mechanism to concentrate urea within the cell to increase the amounts available for hydrolysis by urease activity (255-257). It is unlikely that H pylori possesses a mechanism to concentrate urea within the cell as the enzyme appears to be located on the external periplasmic membrane surface (201,202). The abundant expression of urease activity by H pylori is therefore probably due to the fact that the enzyme's activity is limited by availability of substrate under normal conditions in the stomach.

10.5 Urease activity and ammonium metabolism

Ammonia diffuses across membranes easily when it is not protonated. Bacteria tend to leak ammonia which may be produced within the cell as a result of deaminase activity. In the gastric environment any ammonium from within Helicobacter pylori which diffused out of the cell would be rapidly protonated by the gastric acid and lost to the organism for metabolic purposes. Some bacteria are able to conserve ammonium or scavenge it from the immediate environment through energy dependent ammonium pumps which maintain the intracellular ammonium concentration higher than that of the surrounding medium (258). It has been shown that H pylori possesses deaminase activity and in the absence of urease activity will leak ammonium in vitro into the culture media (259).

This was associated with loss of viability of the organism and a rise in pH of the medium.

The abundant expression of urease activity by H pylori will result in the production of large amounts of ammonia close to the surface of the periplasmic membrane. Some ammonia will diffuse from the surface, become protonated and cause the observed increase in gastric juice ammonium concentration. As the organism has abundant urease enzyme activity there may be sufficient ammonia produced at the periplasmic membrane to allow the diffusion of ammonia down a concentration gradient into the cell before it was protonated by the acidity of the gastric juice. This would allow the organism to utilise ammonium produced from urea by glutamate dehydrogenase activity or glutamine synthetase activity. This theory could explain the paradoxically protective effect of urease activity at low pH and the lethal effects of urease activity at pH 6.0.

At low pH the residual urease activity could result in intracellular accumulation of sufficient ammonium to neutralise the effect of increasing intracellular acidity. At pH 6.0 reduced survival may be due to a reduced intracellular pH when compared with that in pH 7.0 buffer. The pKa of the ammonia-ammonium equilibrium is 9.13 (260). This means that in a buffer system at pH 7.4 more than 98% of ammonia present would be in the form of ammonium ions and less than 2% as ammonia. In a buffer

system at pH 6.0 almost 100% of ammonia would be in the form of ammonium ions. At physiological pH H pylori leaks ammonia as the unprotonated 2% diffuses across the cell membrane (259). At a lower intracellular pH the rate of loss of ammonia by diffusion would be reduced with the reduction in amount of free ammonia. H pylori would therefore tend to accumulate ammonium ions which might swamp its metabolic processes and favour the depletion of intracellular alpha ketoglutarate. In addition, at pH 6.0 sufficient urease activity remains to ensure that large amounts of ammonia could be produced when high concentrations of urea were present.

A reduced intracellular pH might also reduce the activity of the enzymes required to produce alpha ketoglutarate. Citrate also inhibits isocitrate dehydrogenase and thereby further impedes the replenishment of alpha ketoglutarate, thereby enhancing the accumulation of ammonia (Fig 9.4). Conversely the supply of alpha ketoglutarate and isocitrate required for the metabolism of ammonium resulted in enhanced survival of the organism. The rapid destruction of H pylori during the in vitro experiments was therefore due to rapid intracellular accumulation of ammonium ions and depletion of alpha ketoglutarate.

10.6 Urease mediated destruction of Helicobacter pylori and the treatment of duodenal ulcers

Omeprazole (Astra, Bucks, UK) irreversibly inhibits the gastric proton pump and results in a near neutral gastric pH. This might approximate to the near neutral in vitro conditions in which the organism was destroyed. Some individuals have been reported to have had Helicobacter pylori eradicated when treated with omeprazole (204-207). Claims of eradication of H pylori by administration of omeprazole are not well substantiated as other studies have not shown this effect (208,209). Bacterial overgrowth of the stomach during treatment with omeprazole has been suggested as the reason for the reported differences in the effect of omeprazole. Another possible explanation for the contradictory reports of an anti-H pylori effect for omeprazole may be differences in gastric juice urea concentrations in the subjects studied. If omeprazole, by raising gastric juice pH caused urease mediated destruction of the organism then a high gastric juice urea concentration would be essential. If this is the case then the administration of urea to subjects during a course of omeprazole might prove effective in eradicating the organism.

A lower prevalence of H pylori infection has also been found in individuals with chronic renal failure which is associated with raised gastric juice urea concentrations (5.4-20.8 mmol/L) (247,248). The higher

gastric juice urea concentrations in patients with chronic renal failure would enable the organism's urease enzyme to operate at or close to maximum velocity. Higher gastric juice ammonium concentrations would also favour increased diffusion of the ammonium produced into the organism. These effects might explain the reduced prevalence of infection with H pylori in individuals with chronic renal failure.

At present triple therapy to eradicate H pylori is not 100% effective and requires a prolonged course of treatment (218). Additionally there has been an increase in resistance of H pylori to metronidazole. Widespread treatment of this common infection would result in increasing antibiotic resistance.

Any new therapeutic measures which increase the effectiveness of current treatment will prove useful in the treatment of this common infection which is one of the most important acquired factors in the development of duodenal ulcer disease.

If an effective treatment for H pylori was available then the benefits could be considerable. Not only is the infection present in up to 100% of individuals with duodenal ulcer but there is a considerable reserve of asymptomatic individuals in the population some of whom may go on to develop peptic ulcers. Infection with H pylori has also been implicated in the development of gastric cancer and any treatment which could result in a reduction in the incidence of this malignancy which is

difficult to treat conventionally would prove to be of significant benefit.

10.7 Final Conclusion

The production of ammonium by the organism's urease activity is not the cause of the raised plasma gastrin concentrations found in individuals with duodenal ulcer disease. The urease enzyme of *H pylori* is used to scavenge nitrogen from the urea which diffuses from the bloodstream into the gastric juice. The protection of the organism by urease activity at low pH is probably due to the neutralisation of intracellular acidity by ammonia produced by urease activity.

The organism's urease activity can be harnessed to detect antral infection by the measurement of the urea/ammonium ratio in gastric juice. Urease activity has also been harnessed to cause the in vitro metabolic destruction of the organism. If the conditions required for urease mediated destruction can be achieved in vivo then this would represent a significant advance in the treatment of peptic ulcer disease.

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Scottish Society for Experimental Medicine.

May, 1990 — "Suicidal destruction by the urease activity of *Helicobacter pylori*"
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