DUODENAL ULCER, HELICOBACTER PYLORI

and EPIDERMAL GROWTH FACTOR

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ABSTRACT

This thesis examines the role of epidermal growth factor (EGF) and *Helicobacter pylori* (*H pylori*) in the pathogenesis of duodenal ulcer (DU). EGF concentration was measured in 60 subjects, controls (n=20) and in patients with non-ulcer dyspepsia (NUD, n=20) and DU (n=20), in saliva and in gastric juice during basal conditions and after maximal histamine stimulation. EGF concentrations in gastric juice were similar during basal conditions in all three groups. Salivary concentrations were similar in the DU and control groups, but lower in the NUD group. After histamine stimulation EGF concentrations increased in gastric juice and saliva, DU subjects producing much more than the control group and patients with NUD.

H pylori status was determined by measuring urea and ammonia in the gastric juice (ammonia test). EGF concentration was not related to *H pylori* status. The ammonia test was compared with the ^{13}C-urea breath test in nine control subjects: there was complete concordance in the results.

The prevalence of *H pylori* in 100 healthy controls, 39%, was strongly age-dependent and there was a trend suggesting that smokers had less chance of having *H pylori* infection. Prevalence was significantly higher in men than in women, a finding not explained by socio-economic status, age, race or smoking habits.

The prevalence of *H pylori* in controls was compared with that in dyspeptic subjects previously studied here. In dyspeptics there was no influence of smoking or gender. The prevalence of *H pylori* in DU was significantly higher than controls in two age groups (36-45 and 56-65) .

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Using EGF as a marker of swallowed saliva, Whitfield's hypothesis that the aspirated gastric juice is composed of three distinctive components, primary gastric secretion (Vacid), duodenogastric reflux (Vreflux) and swallowed saliva (Vsal) has been validated.

This thesis does not confirm the roles of *H pylori*, or of lack of EGF, in causing DU, but the higher EGF in DU subjects may aid the healing of the ulcer.

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TO REHANA (Jano)

Whose support and encouragement was the most valuable of all.

CHAPTER ONE

HISTORICAL REVIEW

1.1 PEPTIC ULCER

Peptic ulcer occurs in duodenum, stomach, oesophagus, Meckel's diverticulum and at the site of an anastomosis (gastro-jejunal anastomosis). Peptic ulcer always occurs at the site where acid producing mucosa joins non-acid producing mucosa. It never occurs in the mucosa which produces acid; this suggests that there is some form of a defence mechanism which protects it from the effects of acid. Duodenal ulcer occurs within 3 centimetres of the first part of the duodenum. Gastric ulcer occurs in the antrum along the lesser curvature, the site of gastric ulcer becomes more proximal with the age because the junction between acid producing mucosa and non-acid producing mucosa regresses higher up. Peptic ulcer also occurs in the distal part of duodenum as well as in jejunum, in cases of Zollinger Ellison syndrome, where there is an increased production of gastrin by tumours of G-cells. The common finding in all types of peptic ulcers is the exposure to gastric acid. Peptic ulcer occurs in mucosa which is not usually exposed to the acid, for example lower end of oesophagus in cases of gastro-oesophageal reflux, at the site of gastro-jejunal anastomosis, in Meckel's diverticulum.

What is the reason for the development of an ulcer in any individual? The answer must be either that the development of an ulcer is the result of random exposure of the individual to ulcerogenic agents, dependent on social contacts and life style, or alternatively, widespread exposure of many individuals with ulcerogenic reaction only in a few 'sensitive' individuals. The bases of individual susceptibility to ulcerogens, if such there are, must be factors which permit or promote and, in addition, determine the site of action, of environmental ulcerogens. These factors are called aggressive agents. There are other factors which protect the

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mucosa from the effect of aggressive agents, and these factors are called endogenous protective factors. An ulcer results when there is a relative imbalance between the aggressive factors such as gastric acid and pepsin secretion and the defensive factors of normal mucosa such as mucus layer, prostaglandins, and epidermal growth factor.

There are other factors which can also determine the presence or absence of peptic ulcer, these are: biological factors such as blood group O, heredity, pharmacological factors such as smoking and NSAIDs, diet, micro-biological agents, such as *Helicobacter pylori*, and psycho-social factors and abnormalities of the immune system.

Despite of all the attention which has been paid to the secretion of gastric juice, little consideration has been given to the mechanism whereby gastric juice or environmental ulcerogens can produce focal ulcers rather than equally injure all areas of the duodenal mucosa. Clearly, duodenal ulcers are usually discrete and single, despite often widespread associated mucosal disease and apparently uniform exposure of the mucosa to environmental ulcerogens and to gastric juice. A number of suggestions have been put forward, including hypothetical 'mucosal boundaries'; adverse 'strain-inducing' relationships between the duodenal mucosa and the underlying musculature and distribution of the mucosal and submucosal blood vessels. In addition, it has been suggested that ulcers arise as a result of repeated focal trauma, directed towards selected areas of the duodenal mucosa. It has also been proposed that 'kinetic strain' is responsible for the ulcer which occurs where the pyloric muscle disappears in the duodenal wall (Wormsley 1990).

The role of gastric juice in ulcerogenesis seems to be confirmed by obvious therapeutic success resulting from the use of gastric secretion inhibitors. It is therefore necessary to ask: how do gastric secretory inhibitors and other antiulcer agents achieve their objective of healing ulcers? Clearly, inhibition of gastric juice by pharmacological agents or removal of gastric juice from the gastric and duodenal lumen by surgery results in healing of ulcers, and this suggests that gastric acid is an important factor in the pathogenesis of duodenal ulcer.

1.2 DUODENAL ULCER

A duodenal ulcer (DU) is a breach in the mucosa of the duodenum penetrating through the muscularis mucosa, and surrounded by inflammatory cells. The most common site of a duodenal ulcer is within the first 3cm of duodenum. Duodenal ulcer can be acute or chronic. Before 1850, peptic ulceration was poorly described, but then there were increasingly frequent descriptions of gastric ulceration (GU), particularly as a disease of younger women. DU was seldom mentioned before 1890. Exact timing is difficult, but descriptions of duodenal ulcer seem to have become common between 1890 and 1910 throughout Western Europe and North America.

During the past century, dramatic changes in the incidence of both gastric and duodenal ulcer have been observed throughout the world although these changes have not occurred at the same time or at the same rate in different geographic areas. The changes have been particularly well documented in the United Kingdom and Scandinavia, but it is likely that the same trends have occurred in most, if not all, of the European

population. Such rapid changes in the incidence must have been caused by changing exposure to environmental ulcerogens – infectious, chemical or "psycho-social" (Wormsley 1990). While there are marked geographic differences in the occurrence of ulcers, it is obvious that even in areas of high incidence, only a small proportion of individuals develop clinically apparent ulcer disease. In other words, some individuals react to environmental ulcerogens by developing overt ulcer disease and others do not.

Between 1900 and 1960, the prevalence of DU rose to several times that of GU, and GU became a disease of the elderly. Since 1960 two alternative views have been expressed: that ulcer incidence, particularly that of DU, has fallen or that no material change has occurred. In the United Kingdom, there is clear evidence that the ulcer perforation rate has fallen greatly in younger people, but no change or even an increase has been detected in the elderly, particularly in women (Misiewicz et al 1994).

For 80 years, the pathogenesis of ulcer disease was believed to reside in Schwartz's dictum of "no acid, no ulcer". Time, numerous environmental, genetic, and acid-pepsin, associations have been inculpated in the development of this disorder, yet its basic cause remains elusive. The point prevalence of peptic ulcer is between 1 and 2%, whereas the lifetime prevalence of DU is between 5 and 10%. Estimated rates of hospitalisation for the patients with DU continue to fall, but the community prevalence of this disorder is not declining. Smoking is a clear risk factor for DU, but the increase in perforation rate in elderly women corresponds not to smoking habits but rather to the use of nonsteroidal anti-inflammatory drugs (NSAID). Coffee may stimulate

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acid secretion, but decaffeinated coffee does not reduce either the acid stimulatory or dyspeptic effect of caffeine (Thomson 1990).

During the last two decades, trends have shown a significant decline in some Western countries such as the United Kingdom, and a significant rise in certain Asian countries such as Hong Kong and Singapore. There is a marked geographical variation in incidence, for example, it is five times more common in Hong Kong than in Sydney. The male to female ratio also varies; it is 1:1 in the United States of America, 9:1 in Africa, 4:1 in Hong Kong, 2:1 in the UK and 18:1 in India. The ratio of DU: GU is 1:2 in Japan, 32:1 in India, 4:1 in the UK, USA and in Hong Kong and 2:1 in Australia. The difference between Australia and the UK suggests that environmental rather than ethnic factors play a more important role (Lam 1989).

In Great Britain, duodenal ulceration appeared at the beginning of this century, and its prevalence has decreased over the last 50 years, especially in men. A similar pattern of decreasing incidence of duodenal ulcer in the first half of this century occurred in most of Northern and Western Europe, apart from France, and in both black and white populations of North America (Tovey 1994).

In the United Kingdom the incidence of duodenal ulcer and its complications have always been greater in the north than in the south, and in recent years there has been a further decrease in the south (Tovey 1994).

Jebril et al (1994) in their study from Scotland have examined the Scottish medical records between 1975 and 1990. They found a decrease in overall

admission of patients with DU from 157 to 98 per 100,000 population, a reduction of 38%, and / there was a reduction in the number of operations by 80% from 2955 to 582. There was a decline in perforation rate between 1975 and 1990 from 27 to 15 per 100,000 population. This study has also shown that there was an increase in the rate of duodenal ulcer perforation among women aged 65 years and above, from 21 per 100,000 in 1975 to 41 per 100,000 in 1990, a significant increase of 93%. They noticed a general trend in towards an increase in haemorrhage rate although this was significant only for men.

1.2.1 AETIOLOGY

The aetiology of duodenal ulceration is multifactorial with genetic factors including family history, sex, blood groups , gastric secretion of acid and pepsin; drugs, stress and sometimes the social class or occupation, probably also contribute towards a greater risk of developing duodenal ulcer. *Helicobacter pylori* infection has been suggested to be a factor in the pathogenesis of duodenal ulcer, although its role is still unclear. There is also increasing evidence of a relationship to diet, and the possibility that, in people predisposed to developing a duodenal ulcer, one of the precipitating factor may be exposure to a certain pattern of staple diet (Tovey 1974).

- Aetiological factors presumably include what are, at present, termed "aggressive " agents and lack of "protective" agents (Wormsley 1990). Duodenal ulcer is the result of disturbance of the balance between the aggressive factors and protective factors. The "protective" factors are the mucus layer, prostaglandin, and epidermal growth factor, whereas

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"aggressive" factors include smoking, *Helicobacter pylori* infection, NSAID, coffee, and spirits.

The role of *Helicobacter pylori* and epidermal growth factor in relation to duodenal ulcer are discussed later on in this chapter but the roles of the gastric acid, pepsin, mucus layer, prostaglandins, diet, smoking, NSAIDS, catecholamines, genetic factors, psychosocial factors and role of abnormalities of immune system in the pathogenesis of DU are discussed first.

1.2.1.1 <u>GASTRIC ACID AND DU</u>

The part played by acid in the development of duodenal ulcer has been recognised for many years and Schwartz's dictum of 1910, "no acid no ulcer" has held true. Baron (1963) stated that duodenal ulcers are very unlikely to develop with maximal acid secretion of less than 12mmol/hour but also suggested that excessive acid secretion above the normal range is found only in one third of patients with duodenal ulcer. Desai et al (1968) suggested that 45% of patients with duodenal ulcer had acid outputs significantly higher than control subjects. Hobsley et al (1975) correcting for pyloric losses found that a quarter to a third of patients with duodenal ulcer were hypersecretors.

In 1926 Galambos suggested that normal subjects produced no gastric secretion at rest, but that hypersecretors produced gastric juice even when they were not stimulated by food. Polland and Bloomfield (1931) showed that normal subjects produced a continuous though small basal secretion and they confirmed the hypersecretion of patients with duodenal ulcer, a

finding supported by others (Ihre 1947, Farmer et al 1951, Levin et al 1951). In 1943, based on Dragstedt's thesis that basal hypersecretion of gastric acid in duodenal ulcer patients was primarily due to vagal drive, vagotomy was advocated in the surgical management of duodenal ulcer. However, Hobsley and Faber (1977) showed that vagal drive could only be an aetiological factor in a small proportion of patients.

In a post-mortem study Cox (1952) found that the number of parietal cells counted in the stomachs of patients with duodenal ulcer was double the normal; about half the stomachs had parietal cell counts above the upper limit of normal. Hypersecretion has been thought to reflect the increased parietal cell mass, but Hobsley et al (1975) have suggested that this hypersecretion in patients with duodenal ulcer may be an acquired phenomenon: when the ulcer first develops the acid secretion is probably normal but increases in relation to the length of the disease.

Apart from an increased secretory capacity and a possibly increased drive acting on the parietal cells, there is a suggestion that there is increased sensitivity of parietal cells to various stimuli in duodenal ulcer patients. Thus the dose of pentagastrin required to produce half-maximal stimulation in duodenal ulcer subjects is only one-third of that required in normal subjects (Isenberg et al 1975). However, this phenomenon is not observed in every patient with duodenal ulcer. Roxburgh, Whitfield and Hobsley (1993) found no difference in parietal cell sensitivity to histamine between control subjects and patients with duodenal ulcer, or between smokers and non-smokers. They have suggested that parietal cell sensitivity plays no part in the hypersecretion in duodenal ulcer patients compared with controls: moreover, that there is no basal hyperstimulation (vagal drive) in patients with duodenal ulcer. Their counter suggestion

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was that patients with duodenal ulcer have a larger number of parietal cells than control subjects, and that the increased basal secretion in these patients was due solely to increased parietal cell mass rather than to an increased parietal cell sensitivity or increased basal drive.

Many patients with DU have normal acid secretion, their predisposition to ulcer disease may rest with defective mucosal resistance to acid-peptic environment of the duodenum. Postprandial duodenal pH may remain lower for more prolonged periods in DU patients than healthy subjects, although once again these distributions overlap (Malagelada et al 1977). This lower pH may reflect an increase in the delivered load of hydrogen ions as a result of gastric acid hypersecretion or reduced duodenal acid buffering. Basal and acid-stimulated duodenal bicarbonate secretion was found to be reduced in 11 out of 12 DU patients when compared with healthy subjects, with difference evident in the proximal but not in the distal duodenum (Isenberg et al 1987). Gastric bicarbonate secretion is similar in DU patients and in healthy subjects (Feldman & Barnett 1985). Quigley & Turnberg (1987) supported the presence of a "mucous bicarbonate barrier" in the human. They have suggested that the patients with DU cannot keep their mucosal surface as alkaline as can healthy subjects when exposed to luminal acid. When the juxtamucosal pH was measured using a microelectrode with endoscopic guidance, the level in the gastric fundus and the body and in the duodenum remained near neutral, under conditions of luminal acidification to a pH of 1.5. In contrast, pH values at the oesophagus and antrum fell sharply at the luminal pH values less than 3, with DU patients being unable to maintain as high a pH value as healthy subjects. Thus, DU patients may have an impaired mucosal bicarbonate barrier against luminal acid, which may be due to impaired duodenal, not gastric bicarbonate secretion.

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There is no difference in the mean acid output of various blood groups (ABO); there is a significant correlation between acid output and body weight and fat-free body mass (Novis et al 1973). This latter finding confirmed the results of Baron (1969) and Hassan & Hobsley (1971).

1.2.1.1.1 Basal secretion

The term "basal" is used by convention for the fasting interdigestive secretion even though the stomach of an untrained subject is not in a basal state because of nervous and hormonal activity. In 1915 Carlson, after careful study of basal interdigestive secretion stated that "we practically never fail to demonstrate a continuous gastric secretion of relatively low acidity even though the observations be continued for hours". In 1928 Chalfen found that normal subjects when asleep at night produced little or no free acid; however, Hellebrandt et al (1936) were the first to show that normal fasting subjects do secrete continuously by night as well as by day. Faber and Hobsley (1977) pointed out that basal secretion is small in volume and low in titratable acidity, and therefore it is particularly susceptible to the effects of pyloric losses and duodenogastric reflux. When they examined basal secretion on more than one occasion, extreme variability was found from one study to the next; they concluded that such variability was due to inherent instability of basal secretion. This inherent variability of basal secretion is amply supported by others (Levin et al 1948, Kirsmer et al 1956, Sun and Shay 1957, Gillespie et al 1972).

In 1926 Galambos showed that the volume and titratable acidity during basal collection were greater in patients with duodenal ulcer than in

normal subjects and this was confirmed by Polland and Bloomfield (1931). Dragstedt in 1956 stated "I believe that duodenal ulcers are due to hypersecretion of gastric juice in the empty stomach dependent upon excessive and abnormal secretory impulses in the vagus nerves". Most workers have reported greater basal gastric secretion in patients with duodenal ulcer (Ihre 1947, Farmer et al 1951, Baron 1963). Faber and Hobsley (1977), after eliminating errors due to pyloric losses and duodenogastric reflux showed that the basal mean gastric secretion in a group of duodenal ulcer patients was definitely greater than normal controls. In terms of individual subjects, however, only about a quarter of duodenal ulcer patients secreted more acid than the normal population.

Basal secretion has been shown to vary with age and sex. Kirsner et al (1956) found that basal secretion was greater in men than women, both in normal controls and in patients with duodenal ulcer. Levin et al (1951) showed that basal acid output declined with the age . Baron (1963) also found that basal secretion decreased with the age; there were no significant differences between the basal secretion of men and women except in women over the age of 30 years who secreted much less than men. However, Collen et al (1994) found no correlation between basal acid output and age in healthy controls, or in patients with gastro-oesophageal reflux disease, duodenal ulcer and non-ulcer dyspepsia; they also reported an inverse correlation between age and basal acid output in male patients with gastric ulcer and duodenal ulcer. The same authors have also found basal acid output significantly higher in patients with duodenal ulcer than patients with gastro-oesophageal reflux and controls.

1.2.1.1.2 Stimulated gastric secretion

In 1871 Leube was the first worker to stimulate and measure gastric secretion. He gave the patient a test meal of soup, beef steak and whole bread; later in the evening, he aspirated gastric residue and analysed it for its volume, acidity and pepsin concentration as well. Ewald and Boas (1886) developed a test breakfast of white bread with tea; they measured the gastric secretion either at one, two or three hours after this meal. Popielski (1920) found that histamine stimulated gastric secretion of acid in the dog; similar findings were observed by Carnot et al (1922) in man. Kay (1953) introduced the augmented histamine test. He found that in man an increase in acid output is produced by increasing the dose of histamine up to a dose of 0.04mg/kg, but above this dose any further increase produced no further increase in acid output. Card & Marks (1960) compared the size of the gastrectomy specimens with the maximal acid output before and after operation. Their results suggested that the maximal acid output is a correlate of the total number of parietal cells in the stomach.

There is no reasonable doubt that an association exists between secretion maximally stimulated either by histamine or by pentagastrin and duodenal ulcer (Polland 1933). Maximal gastric secretion, i.e. the output rate of acid that can be achieved using maximal concentration of a powerful secretagogue such as histamine or pentagstrin, is an important concept in gastric physiology because there is evidence that it represents the output when the whole of the parietal cell population has been stimulated; it is also called the parietal cell mass (Card & Marks 1960, Cheng 1977). However, there is considerable overlap between the ranges of maximal gastric secretion of normal subjects and duodenal ulcer patients, so that the maximal secretion of about three-quarters of duodenal

ulcer subjects lies below the lower boundary of the duodenal ulcer group (Kay 1953, Hobsley & Whitfield 1987a). Maximal gastric secretion depends on stature, expressed in various ways such as the lean body mass, height, weight, total body potassium, together with age (Hassan & Hobsley 1971, Hobsley & Whitfield 1987a, Baron 1969). The maximal gastric secretion is higher in smokers than in non-smokers, both in men and women. There is a positive correlation between the total numbers of cigarettes smoked over a period of years and maximal gastric secretion (Whitfield & Hobsley 1985, Hobsley & Whitfield 1987b).

1.2.1.2 PEPSIN

The activity of pepsin is closely linked to intragastric pH for two reasons: (1) pepsinogen is converted to the active protease by high concentrations of hydrochloric acid and (2) pepsin is inactivated when the intragastric pH is greater than 4. The secretion of pepsin usually parallels that of acid, and the role of peptic activity in DU remains to be established (Soll 1989). Nonetheless, pepsins may be important in DU pathogenesis (Joffe 1980). The activity of pepsin is pH-dependent, falling at intragastric pH greater than 4, and the enzymes become denatured at pH 6.5 to 7 (Piper & Fenton 1965). Generally, the physiologic or pharmacologic manoeuvres that reduce acid secretion also reduce pepsin secretion.

1.2.1.3 MUCUS LAYER

Claude Bernard in 1859 (Avery Jones 1987) commented that the mucus encloses the gastric juice as in a vase, as impermeable as though it were made of porcelain. It does indeed form a coherent protective layer

covering the lining of the gastrointestinal tract, and its integrity in health prevents both bacterial infection and biochemical damage.

Gastroduodenal mucus consists of large molecular weight glycoproteins; it is a water-insoluble gel which associates by hydrogen bonding to form an adherent viscoelastic lubricating gel over the surface epithelium (Allen 1978). Mucus is secreted by surface epithelium and forms a continuous covering approximately 180mm thick in humans, and it acts as a partial barrier to small molecules and an effective barrier to macromolecules and bacteria. Its secretion is increased by local irritants possibly by prostaglandin synthesis, and its secretion is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs).

1.2.1.4 PROSTAGLANDINS

These are derivatives of 20-carbon chain unsaturated fatty acids widely distributed in the body and in the gut and serving a vital protective function. They were first isolated in seminal fluid and hence the name. In the gut the prostaglandins are released locally in the mucosa and submucosa in response to any irritant stimulus, instantly accelerating cell replacement by opening up the microcirculation to bring cell nutrients and to remove toxic metabolites. It is a mechanism which enables the gastric mucosa to withstand even absolute alcohol and boiling water and to encounter a wide variety of chemical hazards, even without any help from simultaneous reduction of gastric acid secretion. Its action can be blocked by NSAIDs by preventing the enzyme cyclo-oxygenase from producing prostaglandins from their precursor arachidonic acid and hence NSAIDs's ability to promote peptic ulcer.

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Exogenous prostaglandins of the E and I series prevent gastric mucosal injury induced by stress (restraint or cold) and by noxious stimuli such as strong acids, bases, ethanol, steroids, NSAIDS, bile salts and hypertonic solutions (Endoh & Leung 1994). Prostaglandins exert their protective effect on gastric mucosa by the prevention of gastric mucosal barrier disruption, stimulation of mucus secretion, enhancement of gastric blood flow, stimulation of nonparietal cell alkaline secretion, stimulation of macromolecule synthesis, stimulation of cellular transport processes, stabilization of tissue lysosome and stimulation of surface-active phospholipids (Miller 1983).

1.2.1.5 <u>DIET</u>

Diet plays an important role in the pathogenesis of duodenal ulcer. In the West, at the end of 19th century and the beginning of this century there was a marked change in the milling of flour from the old stone-mills to the new steel roller mills and the production of fine white flour free from bran; this change was accompanied by a rise in incidence of duodenal ulcer. However, in recent years there has been a fall in the incidence of duodenal ulcer in southern areas of Britain which has coincided with a greater consumption of fresh fruit, vegetables, milk products, wholemeal bread and flour. There has also been an increased consumption of polysaturated fats and of essential fatty acids.

Cleave (1962) reported the disappearance of peptic ulcer in Japanese prisoner-of-war camps in Japan and in the Burma Road project when the polished rice diet was changed to unrefined coarse cereals or supplemented with fresh rice bran. Peptic ulcer was rare in the prison

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camps of the Dutch East Indies where the diet was red unmilled rice. Peptic ulcer also disappeared from the German troops when they had to live on unrefined food off the land during the siege of Stalingrad, and from German prisoners-of-war in Russian camps where they were given bread made from unrefined wheat, barley or maize (Cleave 1962).

1.2.1.5.i EFFECT ON ACIDITY

Diet with a high fibre content buffers acid by virtue of its protein content (Cleave 1962). It has been confirmed that unrefined carbohydrates do have a variable but marked buffering effect due to both their mineral and protein content, but that any shortfall in acidity is followed by increased acid output due to antral stimulation (Tovey 1974). Lennard-Jones et al (1968) found higher acid levels in response to white bread, likewise Pimparker and Donde (1977) also found a higher acid output following various unrefined pulses compared with refined cream of wheat. It seems unlikely that the effect of high fibre in the diet alone accounts for its protective effect; this is supported by the high incidence of duodenal ulcer in Ethiopia, despite a high intake of fibre in the diet. The incidence of ulcer is also high in Rwanda and Burundi where the diet consists largely of unrefined starch and staple foods.

1.2.1.5.ii INCREASE IN SALIVA OUTPUT

Malhotra and colleagues (1965, 1967a, 1967b, 1970) postulated that the increased mastication required to eat a north Indian chapatti diet, as compared with the sloppy south Indian rice diet, resulted in an increased output of saliva. This increased saliva not only protected development of ulcer by its buffering action, but also by its high concentration of epidermal growth factor which has a cytoprotective effect and promotes the healing

of duodenal ulcer. However, in Africa, and in some parts of India, where a sloppy diet is consumed that does not require mastication, there is no correlation between such a diet and the incidence of duodenal ulceration (Tovey 1994).

1.2.1.5.iii MUCOSAL IRRITATION

There is evidence that fresh rice bran oil or unmilled rice may have a protective effect against ulceration. It has been shown that during storage rice bran oil undergoes lipolysis and peroxidation, resulting in the production of ketoaldehydes which are ulcerogenic in an animal model (Jayaraj, Rees, Tovey, White 1986). These change are initiated by the lipase present in rice germ which is released during milling. This phenomenon may account partly for the high incidence of duodenal ulcer wherever refined or polished rice is the staple diet. It is commonly thought that consumption of spices is an important aetiological factor in the duodenal ulceration, but there is no evidence for this: the consumption of peppers is high in many low incidence areas such as north India, Indonesia, and Malaysia (Tovey 1994).

1.2.1.6 SMOKING

In 1927 Barnett was the first person to notice that duodenal ulcer seemed to be more common in habitual smokers than in non-smokers, although he did not believe that the association was meaningful. Harrison et al (1979) reviewed six major studies and found that the mean prevalence of duodenal ulcer in smokers compared with non-smokers was 1.9:1. A subsequent epidemiological survey showed that in men, 75% of the attributable risk of duodenal ulcer could be abolished by removing

(statistically) exposure to smoking and alcohol, alone or combination (Piper et al 1984). More recently, an endoscopic survey of 1200 outpatients found a prevalence ratio of 1.8:1 (Ainley et al 1986). Doctors often advise their patients with duodenal ulcer to stop smoking; there is epidemiological evidence of an association between cigarette smoking and duodenal ulcer (Friedman et al 1974), and some patients notice that smoking increases their symptoms.

There is evidence that stopping smoking increases the rate of healing of a duodenal ulcer (Korman et al 1981, Sonnenberg et al 1981). The incidence of duodenal ulcer in outpatients with dyspepsia is higher in smokers than in non-smokers. Whitfield & Hobsley (1985 & 1987) measured maximal gastric secretion after stimulation with histamine in pre-operative DU patients. They noticed that the smokers secreted significantly more gastric juice than non-smokers. They suggested that chronic smoking, at least in men, increases maximal gastric secretion and thereby plays an important role in the aetiology of DU. Roxburgh, Whitfield and Hobsley (1992) suggested that acute smoking decreases gastric secretion, leading to antral hypoacidity. This hypoacidity, induced by regular smoking, results in an increase in parietal cell mass via a feed-back mechanism mediated by gastrin, which explains the increase in the maximal gastric secretion in chronic smokers. Similarly Lindell et al (1993) also found a reduction in gastric secretion after acute smoking. The authors of this study have postulated that smoking increases the susceptibility to ulcer formation not only by increasing the stimulated gastric secretion but also possibly by the very high concentration of nicotine in gastric juice affecting the cytoprotective properties of the mucosa. It is also possible that nicotine may have a direct toxic effect on epithelial cells.

The prevalence of DU is higher in smokers than non-smokers (Edwards 1959, Whitfield & Hobsley 1987), and correlates directly with the number of cigarettes smoked (Whitfield & Hobsley 1985, Edward 1959, Ainley et al 1986). There are various mechanism by which smoking might cause the DU. Whitfield & Hobsley (1985 & 1987) suggested that chronic smoking increases gastric secretion, which leads to increased duodenal acidification. Smoking also increases motility and it may reduce the pancreatic secretion (Kaufman et al 1990). It has been suggested that cigarette smoking exerts its effect by decreasing mucosal resistance, inhibiting the ability of the mucosa to regenerate (Kaufman et al 1990). Sonnenberg (1982) suggested that nicotine reduces the mucosal blood flow. Cooper et al (1957) claimed that smoking reduces mucus production. Quimby et al (1986) suggested that endogenous prostaglandin production is reduced by smoking, which affects mucosal regeneration.

Ainsworth et al (1993) in their prospective study measured duodenal bicarbonate secretion in smokers and non-smokers; they reported a decrease in duodenal mucosal bicarbonate secretion in smokers. They suggested that reduced bicarbonate secretion in duodenal mucosa explains at least in part the role of cigarette smoking in the pathogenesis of duodenal ulcer disease.

Zhang et al (1994) in an animal study claimed that cigarette smoking produced a reduction in mesenteric blood flow; they speculated that the vasoconstrictive effect of smoking on mesenteric vessels may be involved in the potentiation of acid-induced duodenal mucosal injury. Kato et al (1992) suggested that cigarette smoking depresses prostaglandin synthesis, and thus reduces its protective effect against the development of duodenal ulcer.

1.2.1.7 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

It is not clear how NSAIDs produce ulcers, but the ulcers seems to be "nonspecific" and not necessarily related to the mucosal exposure to gastric juice since NSAID-induced ulcers occur throughout the intestine. However, in the duodenum it seems certain that NSAIDs produce chronic relapsing ulcer disease. It has been proposed that NSAID drugs are ulcerogenic because they interfere with the metabolism of prostaglandins in the alimentary mucosa (Soll et al 1989). For example, it seems that NSAIDs inhibit the cyclo-oxgenase system of gastric mucosa and therefore decrease the production of "mucosally protective" prostaglandins. Indeed, the deficit produced by NSAIDs can be corrected by supplying exogenous prostaglandin analogues. The injurious effects of NSAIDs may be partly systemic, as they can be reproduced by parenteral administration of these drugs, and also partly due to the local damaging effects of the preparations, which are lessened by enteric coating (Schoen et al 1989).

NSAIDs may injure the alimentary mucosa by directing the metabolism of prostaglandin precursors, such as arachidonic acid, into potentially dangerous pathways, such as the 5-lipoxygenase system, resulting in the production of leukotrienes and active oxygen radicals. In addition NSAIDs also result in the production of platelet aggregating factor, the latter being the most important endogenous ulcerogen and therefore the most important mechanism whereby NSAIDs damage the gastroduodenal mucosa (Wormsley 1990).

However, a number of studies have shown little correlation between the inhibition of prostaglandin production and the degree of NSAID-induced mucosal damage. Therefore direct toxic effects, attributable to nonionic diffusion of the drugs into the gastric mucosal cells may be injurious and permit additional damage from luminal factors such as gastric juice and bile salts (Schoen et al 1989, Whittle 1983).

1.2.1.8 CATECHOLAMINES AND DU

Endogenous dopamine and norepinephrine in the gastroduodenal tract may play an important role in the pathogenesis of peptic ulcer disease. Dopaminergic stimulation is known to reduce gastric acid secretion (Caldara et al 1978, Valenzula et al 1979). Kaise et al (1993) measured the concentration of catecholamines (dopamine and norepinephrine) in the gastroduodenal mucosa of 12 ulcer-free dyspeptics, 9 patients with active DU and 8 patients with inactive DU. They reported a reduction in the amount of dopamine and norepinepherine in patients with duodenal ulcer. They have suggested that alteration in the catecholaminergic system may be one of the pathogenic factors of duodenal ulcer, although the actual mechanism remains unclear.

1.2.1.9 GENETIC FACTOR

An increase in the prevalence of duodenal ulcer within families may imply the involvement of environmental or genetic factors or both. It has been known for nearly 40 years that duodenal ulceration follows a familial pattern, that within such families one type of ulceration (i.e. GU or DU) tends to prevail, and that the increased ulcer prevalence is observed both

across and within generations. Familial aggregation is distinct, however, with first degree relatives having a three fold increase in the prevalence of DU but not GU, and the relatives of GU patients having a threefold increase in the prevalence of GU but not DU. The concordance for peptic ulcer in monozygotic twins is less than 100%, so other factors must be operative; peptic ulcer is nonetheless more common in monozygotic than dizygotic wins. Although these patterns are consistent with the operation of inherited factors, they could also result from environmental influence (Doll & Kellock 1951).

The existence of a racial influence on susceptibility to peptic ulceration has long been postulated, but it has been difficult to decide whether observed differences in ulcer prevalence or mortality between, for example, coloured and white populations are due to differing inherent susceptibility, to varying exposure to environmental predisposing factors, or to greater or lesser chance of disease diagnosis (Misiewicz et al 1994).

1.2.1.10 PSYCHO-SOCIAL FACTOR

Much of the rapid increase in the incidence of duodenal ulcer disease has been attributed to noxious psycho-social influences. A dramatic increase in DU has been observed in South African Blacks during the past few decades. These changes have been attributed to urbanisation, with the imposition of an intolerable amount of urban stress. It has been noted that large numbers of Black miners, migrants from neighbouring countries, do not develop ulcers while town-dwelling Blacks do suffer from a high ulcer incidence. The high incidence of ulcers in second generation urban dwellers is considered to reflect the influence of upward mobility, so that

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ulcers affect especially individuals with amibition, drive and those with greater responsibility, without achieving greater authority. Subsequently, these individuals become less vulnerable, a result of 'adaptation' (Wormsley 1990). Supposing that psycho-social factors are involved in ulcerogenesis, why do only some individuals develop ulcer? It may be that these patients with ulcers are somehow susceptible to environmental stress.

1.2.1.11 IMMUNE SYSTEM ABNORMALITIES

It has been suggested that abnormalities of the immune system may both affect adversely the protective mucosal immune system and stimulate the aggressive secretion of gastric juice. It has been found that many ulcers occur in patients with allergic diseases such as allergic rhinitis, asthma and allergic dermatitis (Wormsley 1990) and in immunosuppressed patients such as those who have undergone organ transplantation (Steger et al 1990). An apparently different disturbance of the immune system is the finding of antibodies to the secretory component of secretory IgA in patients with DU. It seems possible that interference with the secretion of IgA may permit infection of the upper alimentary tract with viral or bacterial ulcerogens. It has even been suggested that the antibodies may act selectively on the H2-receptors and may induce resistance to the gastric inhibitory effects of the histamine H2 receptor antagonists (De Lazzari et al 1988).

1.3   GASTRIC SECRETION

The gastric mucosal epithelium includes cells that line the surface, cells that line the gastric pits and cells composing the gastric glands beneath the pits. Cells that line the surface and the pits are the same throughout the stomach; they are of columnar type, and they secrete mucus and bicarbonate.

In contrast to surface cells and the cells that line gastric pits, cells composing gastric glands differ from one region of the stomach to another. The glands of the body and the fundus have different secretory functions from those of the antrum. The glandular cells of the gastric antrum synthesise and release alimentary polypeptide hormones such as gastrin.

The gastric glands of body and fundus comprise mucus, parietal and chief cells. The parietal cells secrete hydrochloric acid and intrinsic factor, whereas chief cells secrete pepsinogen (I and II), which is activated into pepsin in the presence of acid. There are at least four endogenous substances which stimulate the parietal cell to secrete acid: calcium, histamine, gastrin and acetyl-choline. Histamine type 2, and gastrin and muscarinic cholinergic receptors are present on the basolateral membrane of the parietal cell.

1.3.1 GASTRIC JUICE

Pure gastric juice is a mixture of acid and alkaline components, or acid component modified by back diffusion. The hydrochloric acid is secreted by parietal cells which contribute the major part of gastric juice. It is

difficult to obtain pure gastric juice because of contamination with mucus, and with extragastric secretions consisting of duodenogastric reflux and swallowed saliva. Under maximal histamine stimulation in man, parietal secretion contains 170 mmol per litre of chloride, 145 mmol per litre of hydrogen, 17 mmol per litre of potassium and 6 mmol per litre of sodium. These concentrations, remembering that 1000ml plasma contains only about 930 ml of water, make it isotonic with plasma (Hobsley and Whitfield 1977).

The pepsinogen is secreted in inactive forms, pepsinogen I and II. These pepsinogens are secreted by the chief cells and the cells of the pyloric antrum and by Brunner's glands. These pepsinogens are activated by acid into active pepsins which break down the polypeptide links adjacent to the aromatic amino acids of ingested proteins.

The gastric mucus is produced by the surface epithelium in response to chemical and mechanical irritation of the stomach; it is also produced following sympathetic and parasympathetic stimulation. It protects the gastric mucosa against the action of acid and pepsin.

1.3.2 NATURE OF THE ACID

Reaumer (1752) was the first to discover that gastric juice is acid. He made birds swallow perforated metal tubes containing samples of food. On recovering these tubes, he found that the contents turned "blue paper" red, thus indicating acidity. The first attempt to determine the nature of the acid was made by MacQuart in 1786; in his opinion the gastric juice contained various salts of acids such as acetic acid, lactic acid and phosphoric acid. John Hunter as a result of his post-mortem work on

humans and fish suggested that digestion was due to "something secreted by the coats of the stomach which is thrown into its cavity and thereby animalfies the food or assimilates it to the nature of blood" (Hunter 1786). He also noted that there was acid in the stomach even though no vegetative matter had been ingested; perhaps this was the first report of basal acid secretion in humans.

Bence Jones (1819) found that in man during digestion urine becomes alkaline; he concluded that there is an increase in alkalinity of the blood. In 1803 Young also attempted to identify the acid and until 1824 it was thought that the acid in the gastric juice was phosphoric acid. William Prout (1824) was the first one to describe correctly the composition of human gastric juice as composed of the free acid and salts of muriatic acid (HCl). He also stated that in the human, contrary to the previous reports on animals, there was no acid fixed to ammonia. He introduced the terms of free acid, combined acid and total acid, and expressed the concentration of these as grains of HCl in one pint. It took nearly 30 years to settle the dispute about the nature of the acid in gastric juice and it was finally concluded in 1852 by Bidder and Schmidt in favour of hydrochloric acid, 2 years after the death of William Prout.

In 1898 Pavlov advanced the theory that the gastric hydrochloric acid is secreted from the mucosal glands at a high and constant concentration, independent of other conditions such as secretion rate, etc. This so called "primary acidity" undergoes secondarily a reduction to lower and variable values, owing to interference from various acidity regulating factors. His findings were later quantified by Hollander (1932) in his "Two Component Hypothesis" of gastric juice. In his hypothesis, Hollander stated that pure gastric juice is a mixture of the parietal secretion with an alkaline fluid

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which is principally mucus. The parietal secretion is essentially an isotonic solution of hydrochloric acid and contains no fixed base. The alkaline component is an isotonic solution, the main constituents of which are neutral chloride and bicarbonate. He also remarked that the concentration of chloride in gastric juice decreases much more slowly than the decrease in hydrogen ion concentration. He went on to say "The maximum acidity which can be maintained by gastric juice under normal condition is that of a solution of hydrochloric acid which contains no neutral chloride and is isotonic with mammalian blood".

On the assumption that the primary secretion of stomach is pure hydrochloric acid, Teorell (1947) advanced the concept of back-diffusion to account for the presence of sodium ions. His finding suggested that H^+ was lost from the lumen twice as fast as Na^+ was gained from the gastric mucosa. The primary acidity was constant and the relationship between hydrogen ion concentration and volume was reciprocal, so that at low secretion rates the hydrogen ion concentration was low, which he suggested was due to a relatively high proportion of back-diffusion, but at higher secretion rates the hydrogen ion concentration remained constant and did not increase with the volume. This back diffusion theory was supported by Ihre (1947 & 1956). He found that the stimulated hydrogen ion concentration was no higher in patients with duodenal ulcer than in normal controls, and believed that primary secretion of hydrochloric acid was at 170meq/litre. However he accepted that some reduction in hydrogen ion concentration was due to an alkaline non-parietal secretion, which he believed was due to back diffusion.

James and Pickering (1949) suggested that the antral secretion was less acidic than the secretion of the body and the fundus of the stomach.

Hirschowitz (1961) found that the chloride concentration correlated with the osmolality, and during maximal histamine stimulated secretion, was compatible with either theory. However, in a series of secretion studies performed by two authors on themselves, Makhlouf et al (1966) showed that under the circumstance of continuous gastric aspiration their data did not fit the back diffusion theory. They found their data were best described by the two component hypothesis and that gastric secretion consist of an acid component of fixed composition and variable volume, and an alkaline component of fixed composition and volume. The composition of alkaline component was probably identical to intestinal fluid, and sodium output gave a precise index of this component.

Hobsley and Silen (1970) came up with same conclusion as Makhlouf et al (1966). Furthermore, Hobsley and Silen (1970) were able to postulate the probable composition of the alkaline component. Hobsley and Gardham (1970) found that the data could be explored in terms of a fixed secretion of alkaline component, which was altered by the addition of swallowed saliva. Assuming that no saliva had been swallowed by two subjects of Makhlouf et al (1966), they determined the theoretical relationship of various pairs of electrolytes on mixing saliva with pure alkaline juice. Lambling et al (1956) suggested that the alkaline component was of neither fixed volume nor composition.

In 1970 it was shown by Gardham and Hobsley that the variations of electrolyte concentrations in gastric aspirate from subjects with pernicious anaemia are probably due to the dilution of gastric juice by saliva; and the purest specimen of alkaline gastric juice closely resembled the alkaline component predicted by "two component" hypothesis. In 1974, this hypothesis was modified by Hobsley, who showed that pure gastric juice is

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altered by an alkaline contribution from the duodenogastric reflux. In 1978 Hobsley and Whitfield produced the evidence that the alkaline component in the gastric juice consists of duodenogastric reflux and swallowed saliva.

1.3.3 MEASUREMENT OF THE ACIDITY OF GASTRIC JUICE

Jaworski and Gluzinski (1886) tritrated the gastric juice with N/10 sodium hydroxide using litmus paper as indicator and expressed acid concentration as cc N/10 NaOH per cc of gastric juice (N is the concentration of a solution expressed in gram-equivalents of active material per litre). With the description of the pH scale for hydrogen ion concentration by Sørenson (1909) it became possible to titrate to a specific end point. In 1931 Hollander recommended a pH of 7 as the end point. With recent electronic pH meters it is possible to measure the acid accurately.

1.3.4 CORRECTION FOR PYLORIC LOSS

The pylorus, which is composed of a prominent muscle ring, marks the junction of stomach and duodenum. Electrical and radiographic studies show that the pylorus is a high pressure zone (Fisher and Cohen 1973). The antrum, pylorus and duodenum can function as a single unit in expelling the gastric contents into the duodenum (Heading 1984). When the flow is in opposite direction, the result will be a gain of duodenal contents in the stomach.

The vast majority of workers in gastric secretion ignore the loss of gastric

juice via pylorus. Levin et al (1948) suggested that loss via the pylorus

(pyloric loss) is too small to account for variation. Royle and Catchpole

(1967) used a double lumen naso-gastric tube, and infused edicol supra

blue down one lumen as a label of gastric juice and also positioned a glass

probe in the duodenum. They showed that during continuous histamine

infusion there were periods of diminished recovery of gastric juice; these

periods sometimes coincided with a fall in the intra-duodenal pH,

indicating pyloric losses, but at other times there was no such fall

suggesting that the diminished recovery was due to intragastric

sequestration. They also showed that the recovery of the marker dye

varied between 68% and 90% suggesting pyloric losses always occurred but

were variable. Hobsley and Silen (1969) investigated this further during

histamine-stimulated sub-maximal gastric secretion, using phenol red as a

marker. They found that the coefficient of variation of the samples on the

plateau was less if the volume was corrected for the pyloric losses. Hassan

et al (1969) used phenol red as marker in 51 subjects: they found that 20%

of the acid output was lost through the pylorus.

Phenol red had first been used as a marker of gastric juice by Gorham in

1923. For the calculation of pyloric losses to be valid, it is necessary that the

marker should be inert, and should not be absorbed from the stomach.

The first requirement had always been assumed until Panda et al (1974)

suggested that phenol red in animal experiments reduced back diffusion;

however the quantity even in the most extreme cases was no more than

1meq of acid per hour.

Penner et al (1938) stated that the recovery of phenol red was 100% when

gastric emptying was delayed by intra-duodenal fat, suggesting that no

absorption had occurred. On the other hand Cook and French (1968) stated that significant amounts of phenol red were absorbed in acid media, although it was not clear whether it was from stomach or duodenum or both. Panda et al (1974) doubted the inertness of PSP in contact with stomach mucosa, but their experimental model was canine and not necessarily applicable to human beings. Weiman, Whitfield and Hobsley (1988) have shown that estimated pyloric loss is physiological in the sense that it gives an index of the intrinsic emptying ability of the stomach. Recent work has shown that in any one subject pyloric loss is related to the maximal secretory capacity of that person and has a constant value (Roxburgh 1989), no matter what the current rate of secretion of gastric juice happens to be.

1.3.5 CORRECTION FOR DUODENOGASTRIC REFLUX

During gastric secretion studies many samples are contaminated with duodenogastric reflux, made obvious by frank bile staining. This reflux introduces errors in the measurement of acid secretion which some workers have sought to overcome by discarding obviously bile stained samples. There is no doubt however that duodenogastric reflux sometimes lacks bile, and to ignore it is to ignore a phenomenon which may be important. Du Plessis (1965) emphasised the importance of duodenogastric reflux, not only as an explanation of the observed hypoacidity and hypotonicity of gastric aspirate in patients with gastric ulceration, but also in the aetiology of the condition. In attempting to assess the role of duodenogastric reflux, Hobsley (1974) assumed that the reflux is isotonic and that the sodium concentration in the reflux is

143mmol/l. On the basis of these assumptions he derived a formula to quantify the reflux(V_R):

$$V_R = \{V_{cor} \times [Na^+] - 7\} / 143$$

V_{cor} is the volume of gastric juice corrected for pyloric loss, multiplied by the concentration of sodium $[Na^+]$ measured in the aspirate, subtracting 7, which is the concentration of sodium in mmol l^{-1} in primary gastric juice, and dividing it by 143, which is the concentration of sodium in mmol l^{-1} in duodenogastric reflux.

Based on the two-component hypothesis (Hollander 1932) Hobsley, Gardham and Hassan (1969) proposed to use sodium as a marker for the measurement of duodenogastric reflux. The two-component theory postulates that the acid component is of constant concentration but of variable volume and the alkaline component is of constant volume and with a composition approximately that of interstitial fluid.

In 1974 Faber, Russell , Royston et al recognised and measured the reflux of duodenal contents into the stomach, especially during the insulin test after a vagotomy and drainage procedure. They studied 37 patients with duodenal ulcer who were given bromsulphalein (BSP) or indocyanine (ICG), both these dyes being excreted into the bile. They found that aspirated juice showed highly significant positive correlations between the concentration of each dye and the concentration of duodenal reflux calculated from a formula based on the output of sodium ions.

Fiddian-Green et al (1979) used indocyanine green (ICG) intravenously to label the bile in order to measure the duodenogastric reflux; they found a

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large increase in volume of aspirated gastric juice and the recovery of sodium following the injection of secretin. The possible explanation of the increase in the aspirated volume was an increase in the duodenogastric reflux, suggested by the bile staining and increase in concentration of ICG in the aspirate.

^{99m}Tc-labelled iminodiacetic acid was used to radio-label the bile and the duodenogastric reflux was measured by external scintillation scan. Quantification was possible over the region of interest (stomach, duodenum, liver) but an accurate measurement of low intermittent reflux was not possible (Thomas et al 1984). However, sodium as a natural marker is better than an artificially labelled substance to study the duodenogastric reflux because it exists at the same concentration in all the possible contaminants, bile, pancreatic juice and succus entericus.

Duodenogastric reflux occurs in healthy controls (Johnson and Eyre-Brook 1984) but the amount of the refluxate is minimal. However, it is considered to be present to a pathological extent in patients with duodenal ulcer (Thomas et al 1984). It been suggested that increased duodenogastric reflux leads to antral gastritis, and suppression of antral somatostatin. This results in an increase in the gastrin output which in turn increases the parietal cell sensitivity. The net effect is a hypersecretory state, and an increase in acid delivery into the duodenum. This theory has been challenged by Wolverson et al 1984 and Muller-Lissner et al 1983, they have suggested that the amount of duodenogastric reflux is the same in patients with duodenal ulcer and in controls. In 1987 Frizis, Whitfield and Hobsley found that duodenogastric reflux was higher in the gastric ulcer group as compared to controls, the mean volume of duodenogastric reflux was greatest in the patients with a prepyloric gastric ulcer. The absolute

values of duodenogastric reflux were greater during basal conditions and tended to decrease during maximal histamine stimulation. At maximal stimulation duodenogastric reflux was significantly lower than under basal conditions in both controls and duodenal ulcer group, it was higher in patients with duodenal ulcer than controls during basal conditions, but there was no difference during maximal histamine stimulation (Roxburgh 1989).

1.3.6 CONTAMINATION FOR SWALLOWED SALIVA

The contamination of aspirated gastric juice by swallowed saliva is well recognised, but need to prevent for, or possibility of the prevention of this contamination has been controversial. Okada et al (1930) suggested that salivary contamination is under nervous control and not humoral control. Ihre et al (1938) reported that saliva was less during insulin hypoglycaemia but in 1956 he advised that salivary contamination should be prevented by means of continuous suction. Kirsner et al (1956) and Baron (1963) required their subjects to expectorate saliva, while Makhlouf et al (1966) prevented the swallowing of saliva by the insertion of dental plugs which were repeatedly changed. However, considerable doubt on the ability of these methods to prevent the swallowing of saliva was cast by Gardham and Hobsley (1970). They examined alkaline gastric secretion in patients in whom no attempt was made to prevent the swallowing of saliva, and compared their results to those obtained in a similar study by Lambling et al (1956), who did attempt to prevent their subjects swallowing saliva. No difference could be found between either group, and the variability of the alkaline gastric secretion by Lambling et al (1956) in their subjects could be explained by the intermittent contamination of

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gastric juice by saliva. Gardham and Hobsley (1970) concluded that attempts to prevent the swallowing of saliva were always unsatisfactory, and might result in the production of even more saliva.

In 1980 Boulos, Whitfield, Dave, Faber and Hobsley used thiocyanate as a marker in saliva and in gastric juice; they found that when histamine was given, despite the increase in the rate of gastric secretion, there was no matched fall in the concentration of thiocyanate in the gastric juice. Moreover, in one third of gastric juice specimens the thiocyanate concentration was greater than in the simultaneous sample of saliva. In other words, and contrary to what had been previously claimed, thiocyanate is not only present in the saliva but also in gastric juice.

1.4 EPIDERMAL GROWTH FACTOR

1.4.1. Historical background

The first observation of epidermal growth factor (EGF) was by Rita Levi-Montalcini who noted that certain mouse tumours, when implanted into chick embryos, released a factor that stimulated the growth of specific embryonic neurons (Cohen 1986). Then Cohen (1962), during the course of purifying nerve growth factor in male mouse submaxillary glands, found that injection of a crude preparation of submaxillary glands into newborn mice resulted in unexpected "side effects" not related to the known activities of nerve growth factor. These effects included precocious eyelid opening (6-7 days, compared with 12-14 days for controls) and precocious tooth eruption (5-6 days compared with 8-10 days for controls).

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These observations were followed by several studies by Cohen (1965a,b), in which he found that the agent produced proliferation of epidermis and hence he named this substance epidermal growth factor (EGF). By 1970 Cohen found that EGF causes various metabolic alterations (enhancement of polysome formation, induction of ornithine decarboxylase) that accompany the growth-stimulating effects of EGF on epidermal cells. It was also found that the tubular cells of the submandibular gland, which in the mouse exhibit sexual dimorphism, are the major site of synthesis of EGF in this species. The synthesis was markedly enhanced by administration of testosterone, especially in female mice.

In 1975 Gregory isolated and purified a polypeptide from human urine called urogastrone (Gregory, 1975; Gregory & Willshire, 1975). Comparing the primary structure of human EGF with β-urogastrone, of 53 amino acid residues 37 were found to be common to both molecules, and three disulphide bonds were found in the same relative positions, suggesting that human EGF and β-urogastrone are similar (Gregory 1977).

1.4.2. Biochemistry of EGF

EGF belongs to a family of growth factors known as polypeptide growth factors. The word "growth factor" is a misnomer as these polypeptide growth factors do not always stimulate the growth of their target cells but can also inhibit their growth, for example EGF stimulates epidermal proliferation in the gastrointestinal tract but inhibits gastric secretion (Gregory et al, 1975), Interleukin-1 inhibits the growth of breast cancer cells (Onozaki et al, 1985), but can also stimulates their growth (Moses et al,

1985). Tumour necrosis factor (TNF) stimulates the growth of fibroblast and epithelial cells, but inhibits tumour proliferation (Lillien & Claude, 1985). These growth factors are involved in a wide variety of actions including embryogenesis, growth, regeneration, repair and neoplasia.

The chemical and physical properties of EGF were studied by Taylor et al (1972); he showed that EGF consists of a single polypeptide chain of 53 amino acids and is devoid of alanine, phenylalanine and lysine residues. It is heat stable, non-dialyzable, and resistant to trypsin, chymotrypsin and pepsin digestion (Marti et al, 1989, Taylor & Cohen, 1974).

Human EGF exist in two sizes. A small molecular weight EGF (molecular weight $\sim$ 5700-6000 Da) is predominantly found in all body fluids and consists of 53 amino acid residues, 37 of which are analogous to those of mouse EGF and having three disulphide bonds in the identical place (Gregory 1975). The second form, a large molecular weight human EGF (molecular weight $\sim$ 30 kDa) is found in urine (Hirarta & Orth 1979a). Hirarta and Orth (1979b) suggested that the high molecular weight form of human EGF could be converted to the low molecular weight form by incubation with mouse arginine esterase. They have also suggested that the high molecular weight form of human EGF found in urine may represent a precursor molecule which can be cleaved by specific endoproteolytic activity to the fully active small molecular weight human EGF. The EGF-like factors such TGF α (transforming growth factor alpha) can compete with EGF for their joint receptor sites, but they cannot compete with EGF for the anti-EGF antibodies in radioimmunoassays. Thus these factors are antigenically different from EGF (Todaro et al, 1981).

In man EGF is found in the pituitary, thyroid, parathyroid, adrenal, submandibular, gastric and pyloric glands, Brunner's glands in the duodenum, jejunum, pancreas, renal medulla, reproductive organs like ovary and endometrial glands of the uterus, bone marrow, sweat glands, lactating breast, and body fluids like saliva, gastric juice, pancreatic juice, breast milk, urine, seminal fluid and plasma (Elder et al, 1978; Kasselburgh et al, 1985; Read 1987; Mori et al, 1989). EGF is found in urine in a concentration of approximately 10-100ng/ml, in milk 80-120ng/ml, in plasma 0.138-2ng/ml, saliva 1.5-12ng/ml, bile 0.3-5ng/ml, gastric juice 0.3ng/ml, tears 0.7-2.2ng/ml, prostatic fluid 28ng/ml, seminal fluid 22-115ng/ml, amniotic fluid 0.06-0.94ng/ml, and serum 2-4ng/ml (Starkey & Orth 1977, Gregory et al 1977, Barka et al 1978, Marti et al 1989). Human EGF and mouse EGF cross-react antigenically and show similar biological activities (Cohen et al 1975).

1.4.3 EGF receptors

Specific, saturable receptors have been demonstrated using ^{125}I-labelled human EGF and a variety of cultured cells including corneal cells, human fibroblasts, lens cells, human glial cells, human epidermoid carcinoma, human vascular endothelial cells. Indirect evidence suggests that the receptor for EGF is a glycoprotein found on the plasma membrane (Carpenter 1981). The interaction of EGF with its receptors was first investigated on human fibroblasts in culture. The EGF receptor contains three different structural elements: an extracellular EGF-binding domain of 621 amino acids, a transmembrane domain and an intracellular or cytoplasmic domain.

EGF receptors are found in gastric glands, small intestinal mucosa and liver. The receptors are also found in different carcinoma cell lines of gastrointestinal origin, including human colon, rectal adenocarcinoma, pancreatic carcinoma and oesophageal carcinoma. It has been suggested that the number of EGF receptors might be correlated with the growth and tumorigenicity of human carcinoma cells. Although some evidence indicates that receptors correlate with tumorigenicity, other evidence suggests that a high receptor number might be responsible for growth inhibition by EGF in *vitro* (Marti et al 1989).

The binding of EGF to its cell surface receptor is the first step in a cascade of events that culminates in mitosis for many target cells. EGF, after binding to its receptor, activates its tyrosine-specific protein kinase activity, which results in the phosphorylation of several substrates including the EGF receptor itself (Marti et al 1989)

1.4.4 Cellular changes

In vitro experiments have shown that the interaction of EGF with its receptor on the cell membrane results in an increase in the uptake of uridine by fibroblasts (Rozengurt et al, 1978), and increases the transport of sugar when added to quiescent fibroblasts (Barnes and Colowick 1976). It increases the cation fluxes in fibroblasts when added to the culture, especially with regard to K^+; this action is dependent on the amount of K^+ in the medium and can be blocked by ouabain, suggesting that EGF acts on the Na^+/K^+ pump (Rozengurt & Helppel 1975) . There is increase in transport of the polyamine putrescine upon the addition of EGF to fibroblasts (DiPasquale et al 1978).

EGF also acts in the cytoplasm by increasing glycolytic activity and enhancing production of lactic acid (Diamond et al 1978). The ability of EGF to stimulate lactate production is decreased by omission of Ca^+ from the medium which shows that Ca^+ may act as second messenger for EGF. EGF also stimulates the synthesis of DNA.

1.4.5. EGF and Gastrointestinal mucosa

The mucosa of the gastrointestinal tract is one of the most rapidly proliferating tissue in the body. Proliferation and growth are balanced by the loss through the exfoliation of the surface cells so that under normal conditions the cell population is maintained at a dynamic steady state. Because of the rapid turnover of the mucosal cell population, any alteration in the processes that regulate the growth is likely to produce functional changes and may lead to either atrophy or ulceration. The growth of the mucosa is affected by non-gastrointestinal hormones such as growth hormone and thyroxin, and various gut hormonal peptides, particularly epidermal growth factor (EGF), gastrin, and somatostatin. The mucosa is capable of producing a variety of biologically active substances such as arachidonic acid and biogenic amines. The metabolites of arachidonic acid have been implicated in the protection of gastrointestinal mucosal cells from damage by various irritants and ulcerogens (Konturek 1990).

The idea that EGF may have a role in healing gastroduodenal ulceration is by no mean new. In 1930 Sandweiss observed that pregnancy has a beneficial effect on the symptoms of duodenal ulcer. Stimulated by this

observation, and in an effort to determine whether there is an endocrinological relationship to peptic ulcer, Sandweiss et al (1945) tested the effect of hormones found abundantly during pregnancy (the oestrogenic and anterior pituitary-like hormones) on experimental ulcers in Mann-Williamson dogs. In 1938 the same workers found that an extract of urine from pregnant women has a prophylactic and therapeutic effect on experimental Mann-Williamson ulcers in dogs. Chief factors involved in the chronicity of the Mann-Williamson ulcer appear to be: (a) a nutritional disturbance as a result of shunting bile and pancreatic and duodenal juices into the ileum; (b) mechanical stress due to either spasm of the jejunum or forcible ejection of gastric contents against the jejunal mucosa; (c) the specific susceptibility of the jejunal mucosa and (d) the irritant action of unneutralized gastric contents on the unprotected jejunal mucosa.

In 1939 the same workers reported similar results with an extract of urine from non-pregnant women and pointed out that the beneficial effect was not due to the inhibition of gastric secretion. In 1943 Sandweiss named the antiulcer factor anthelone (Greek; anti = against, helcos = ulcer) and defined it as an antiulcer factor having a prophylactic, therapeutic and immunizing effect against Mann-Williamson ulcers without depressing gastric secretion (Sandweiss 1945). It has also been shown that pregnant rats with induced chronic ulceration showed a marked increase in healing rate compared to virgin animals (Konturek et al 1991), hence suggesting that EGF might have a role in healing of ulcers. Warzecha et al (1989) have reported that pregnancy causes an increase in the speed of gastric and duodenal ulcer healing; they have suggested that this effect was due to hyperplastic changes in the gastro-duodenal mucosa. The hyperplastic mucosa was the result of the increase in the levels of endogenous EGF.

The authors of this study have suggested that during pregnancy gastric and duodenal mucosa has an increased ability to regenerate; this ability results from hyperplasia which takes place during pregnancy, which is an effect of an increase in the levels of endogenous EGF.

The ability of EGF to stimulate the healing of epithelial wounds has been demonstrated in experimental studies on animals: the topical application of EGF accelerated the rate of epithelial regeneration of partial-thickness wounds and second degree burns (Brown et al 1986, Nanney 1987, Brown et al 1988). The corneal epithelium of rabbits was experimentally wounded and EGF was topically applied to one-half the wounded animals. The application of EGF to these wounds resulted in a marked hyperplasia of corneal epithelium and slight decrease in the time required for the wound closure (Carpenter 1981). It was also noticed that during regeneration of the epithelia in animals treated with EGF, the epithelium increased from its normal thickness of 4-6 layers to 10-15 layers after six days and then returned to the normal thickness by 4-6 days. Topical application of EGF to control corneas (non-wounded) had no effects. Brown et al (1989) in their prospective double-blind randomised trial in patients found that EGF accelerates the rate of healing of partial-thickness skin wounds. The mechanism by which exogenous EGF promotes wound healing is not known with certainty, but it may stimulate the division of keratinocytes and dermal fibroblasts both of which have been shown to express receptors for EGF (Nanney et al 1984, Coffey et al 1987). It is also possible that exogenous EGF stimulates healing indirectly by enhancing the production of other growth factors such as transforming growth factor alpha.

The ability of EGF to promote healing of gastroduodenal ulcer was examined in several studies. Konturek et al (1981) in their animal study

have shown that when EGF was given topically, it prevented formation of aspirin-induced gastric ulcers without affecting prostaglandin generation but with a significant rise in DNA synthesis in oxyntic mucosa. Hiraishi et al (1984) have shown that EGF stimulates the synthesis of prostaglandins in monolayer culture of gastric mucosal cells and they have claimed that EGF may contribute to the maintenance of the integrity of the gastric mucosa. The increased susceptibility of gastric mucosa in animals after sialoadenectomy, when mucosa is exposed to bile salts, a high concentration of HCl, ethanol or stress indicates that saliva is an important source of EGF in these animal and that it plays a crucial role in maintaining the mucosal lining (Konturek et al 1991b)

Konturek et al (1988) in their other animal study examined the healing of acetic acid-induced gastric and duodenal ulcers. They found that after the extirpation of salivary glands, the healing of such ulcers was delayed. This was accompanied by a significant decrease in DNA and RNA in the gastric and duodenal mucosa. Repeated administration of EGF either subcutanously or orally accelerated the healing of gastroduodenal ulcers. They suggested that EGF plays an important role in the ulcer healing due to its mitogenic action. Hui et al (1993) have suggested that EGF protects against gastric mucosal injury in rats by increasing the gastric blood flow.

Additional evidence for the importance of saliva in healing of wounds comes from another investigation in which artificial wounds produced in the neck of mice caged individually healed more slowly than those in mice caged in groups where communal licking takes place. Desalivation retarded the healing whereas EGF topically applied to these wounds greatly enhanced healing. These observations emphasise the important physiological role of saliva and EGF in wound healing (Konturek 1990).

Hansson et al (1990) in an animal experiment, produced ulcers in the ventral portion of the mucosa of the gastric corpus of anaesthetized rats by applying acetic acid for 60 seconds, and compared the concentration of EGF in chief cells with similar observations in sham-operated rats in which saline was applied and in unoperated controls. In both sham-operated and controls, there were few chief cells containing EGF, but there was a considerable increase in EGF-laden cells around the margin of ulcers. This study suggested that EGF is produced locally in the gastric mucosa in response to presence of the ulcer.

The putative role of EGF in the healing of gastrointestinal ulceration in humans has been investigated in various studies. When EGF/urogastrone is given intravenously in normal human volunteers it reduces the gastric acid, pepsin and intrinsic factor. Urogastrone inhibited acid and intrinsic factor secretion whether stimulated by pentagastrin, histamine or insulin, but it had a less marked effect on gastric pepsin output. Elder et al 1975 reported mean reductions in pentagastrin-stimulated gastric acid output by 60%,85% and 90% respectively after intravenous infusion of 0.125, 0.25 and 0.5mg of urogastrone kg^{-1} h^{-1}. Acid volume remained below pre-urogastrone infusion for 60-90 minutes after the end of infusion of urogastrone, but the inhibitory effect of urogastrone infusion on histamine-stimulated gastric acid output was less marked. The inhibition of histamine-stimulated acid output was up to 40% of pre-infusion levels, as compared to 60-90% reduction of pentagastrin-stimulated acid output. This study has also demonstrated that there was a reduction of pepsin concentration within 15 minutes of the start of urogastrone infusion, but it increased sharply 30 minutes after the end of

the infusion. The intrinsic factor output decreased markedly within 15-30 minutes after the urogastrone infusion when histamine was the stimulus.

However, EGF given orally does not reduce gastric acid secretion (Oslen et al. 1986). Intraduodenal administration of EGF significantly increased the thickness of mucus in the duodenal mucosa but not in Brunner's glands, and it stimulated alkaline secretion (Yoshikawa 1993). Li et al (1993) have found that there is an increase in salivary EGF secretion following mechanical and chemical stressors, mimicking the natural events occurring during the passage of food and gastro-oesophageal reflux and suggesting that EGF protects against mucosal damage. Rourk et al (1994) have demonstrated that there is an impairment of salivary EGF secretion which they suggest facilitates the production of oesophagitis and delays the healing of mucosal injury in cases of gastro-oesophageal reflux.

Ohmura et al (1987), and Maccini et al (1990) reported that the salivary concentration of EGF is significantly lower in gastric or duodenal ulcer patients than in healthy subjects. They suggested that a lower EGF in these patients reduces the defensive mechanism responsible for the protection of gastroduodenal mucosa from injury by physiochemical agents, thus contributing to ulcer development. On the contrary Itoh et al (1988 & 1993) have reported that EGF concentration in saliva was much higher in peptic ulcer patients than healthy controls and they have also suggested that this rise in EGF concentration in saliva was a response to the presence of an ulcer, facilitating ulcer healing. They believed that beta-urogastrone, human EGF, might prove to be an effective drug in the clinical treatment of gastric ulcers.

Jones et al (1992) measured the output of salivary EGF and found that it is diminished in smokers, and in those patients with rheumatoid arthritis taking non-steroidal anti-inflammatory drugs, a group most likely to develop gastric ulceration. Hirasawa et al (1991) measured EGF in saliva in controls and in patients with duodenal ulcer and gastric ulcer. They found a higher salivary EGF output in patients with duodenal ulcer and gastric ulcer than in controls and suggested that salivary EGF may promote the healing of ulcer and prevent its recurrence. However, Maccini et al (1990) have shown no difference in salivary EGF concentration between smokers and non-smokers. Calabrò et al (1990) in their study measured EGF in gastric juice samples in patients with duodenal ulcer, with gastric ulcer and in controls. They found that the concentration of EGF in patients with ulcer was lower than in controls. They suggested a possible role for EGF deficiency in the pathogenesis of peptic ulcer disease. They found no influence of sex and age on the concentration of EGF in gastric juice. Zandomeneghi et al (1991) in their study obtained duodenal biopsies from normal subjects and patients with duodenal ulcer, and the biopsies were cultured in *vitro*. They found a fall in EGF secretion from tissue culture of ulcer patients. Moreover they also found that the EGF-producing cells around the lesion in ulcer patients seemed to be hyperactive, and suggested that this hyperfunction of EGF-producing cells might contribute to the *in vivo* repair of tissue damage.

Wright et al (1990a) have shown that ulceration anywhere in the human gastrointestinal tract induces the development of a novel cell lineage from the gastrointestinal stem cells. This lineage initially appears as a bud from the base of intestinal crypts, adjacent to the ulcer, and grows locally as a tubule, ramifying to form a new small gland, and ultimately emerging onto the mucosal surface. This lineage produces neutral mucin, shows an

unique lectin-binding profile and immunophenotype, is nonproliferative and contains and secretes abundant immunoreactive EGF/URO. They propose that all gastrointestinal stem cells can produce this cell lineage after mucosal ulceration, secreting EGF/URO to stimulate cell proliferation, regeneration and ulcer healing.

This novel lineage has been appropriately called ulcer-associated cell lineage (UACL). UACL are seen at sites of chronic ulceration, particularly in ulceration caused by Crohn's disease and peptic ulceration in the stomach, duodenum and distal oesophagus (Wright et al 1990b and Roberts et al 1993). This distinctive lineage was until recently known as pseudopyloric metaplasia because of morphological resemblance to the gastric epithelial cells. UACL also shares many features with pyloric and Brunner's glands, namely the presence of a neat acinar structure in their deep portions lined by cuboidal/low columnar palely-staining cells containing neutral mucin.

These animal experiments and human studies have shown that EGF plays a very important role in wound healing, and there is suggestive evidence that EGF plays a significant role in the healing of gastroduodenal ulcers.

1.5　HELICOBACTER PYLORI

1.5.1.　Historical background

(Pre-1985 references in this section are quoted by Rathbone & Heatley 1992, but were not available to me).

In 1893 Bizzozero demonstrated bacterial colonisation of mammalian stomachs with spiral organisms. Studying the microscopic appearance of the gastrointestinal epithelium of various animals, he noted the presence of spirochaetes in the gastric glands and parietal cells. In 1896 Salomon confirmed the presence of spiral organisms in dogs, cats and rats, but said they were absent from human, ape, pig and monkey tissues. However, Balfour demonstrated spirochaetes in gastric and intestinal ulcers in dogs and monkeys. Human gastric spirochaetes were first reported in necrotic material at the surface of ulcerating carcinomas and in gastric secretion by Krientiz and Luger. Little attention was paid to these organisms at that time (Rathbone & Heatley 1992).

In 1888 Letulla found that oral or parenteral administration of staphylococcus pyogenes to guinea-pigs resulted in gastric ulcer; the implication of this finding was that infection may be an occasional or accessory cause of ulceration. In 1939 Doenges published an histological study of 242 autopsy stomachs, using haemotoxylin and eosin staining, showing that spirochaetes were present in 43% of the cases. The organisms occurred predominantly in the glandular lumen but were also seen in the parietal cells (Rathbone & Heatley 1992).

In 1924 Luck and Seth described the presence of considerable urease activity in the stomach, and in 1959 Leiber and Lefevre suggested that this urease might be bacterial in origin, and demonstrated that the hypoacidity found in many of these subjects could be reversed with antibiotic therapy. In 1975 Palmer demonstrated bacteria on the luminal surface of epithelial cells of gastric ulcer patients; they were deep in the layer of mucus, the latter being scanty compared to the mucus in normal stomach.

In 1983 Warren noticed that the majority of endoscopic biopsies from patients with chronic gastritis and peptic ulceration were colonised with curved campylobacter-like organisms, which did not stain well with haemotoxylin and eosin. A prospective study was initiated and attempts were made to culture the organism from the gastric biopsies, using non-selective media for 48 hours. No growth was observed until the 35th biopsy, which was incubated during an Easter holiday and in consequence was only examined after 5 days incubation. A heavy growth of campylobacter organisms was found in non-selective media. When other biopsies were also incubated for 3-4 days a similar growth of campylobacter-like organisms was seen. This organism was named *Campylobacter pylori (C pylori)*.

Many of the biochemical and ultrastructural characteristics of *C pylori* turned out to be different from the genus campylobacter, and for this reason Goodwin and his colleagues 1989 proposed a new genus with the name of *Helicobacter pylori (H pylori)*.

1.5.2. Morphology of *H pylori*

H pylori is a spiral or curved, gram-negative rod, 2.5 to 4.0μm long and 0.5-1.0μm wide. It is a unipolar, multiflagellate with bluntly rounded ends. Occasionally this organism may contain bacteriophages. It varies in shape between V-shaped, U-shaped and straightened forms. The organism has the cell wall characteristic of gram-negative organisms; in some preparations the outer membrane-like wall component can be seen exuding from the body of the bacterium (Goodwin & Worsley 1993).

When cultured on solid media, true spiral forms may be few or absent. The cell wall is covered with ring-like subunits 12 to 15 nm in diameter. Four to six flagella are attached to one pole, each 2.5 μm long and about 30 nm in thickness, with a membranous terminal bulb, which is an extension of the flagellar sheath.

Prolonged culture gives rise to the emergence of coccoidal forms, which also appear after exposure to oxygen. It is assumed that coccoidal forms are indicative of a dormant state and assist the survival of the organism. The coccoidal forms of *H pylori* originate when the outer envelope appears to separate and ceases to keep pace with cell growth, leading to folding of the inner cytoplasmic rod.

1.5.3. Virulence of *H pylori*

H pylori infection causes chronic active gastritis, and has been implicated in the pathogenesis of gastric and duodenal ulcer, gastric lymphoma and gastric adenocarcinoma. It has been suggested that this variation in disease

pattern could be due to differential bacterial and host factors resulting in different responses. Goodwin et al (1989) proposed that *H pylori* contributes to gastroduodenal injury by impairing the local mucosal defence; however, Levi et al (1989a &b) suggested that *H pylori* increases the antral gastrin secretion which leads to increased gastric acidity, ultimately resulting in gastroduodenal injury.

The success of *H pylori* as a gastric pathogen is dependent on virulence factors. Virulence factors are those that allow *H pylori* to survive in the hostile environment of gastric acid. These factors include the shape of the organism and its motility, the enzymes it produces, and its ability to adhere to gastric mucosal cells and mucus. *H pylori* shows significant motility in a highly viscous solution of methylcellulose, which is due to its spiral shape and the presence of flagella. The active motility is thought to promote rapid passage of *H pylori* through the acid milieu of the gastric lumen and its penetration of the gastric mucus layer prior to reaching the neutral environment immediately overlying the gastric epithelium ((Dunn 1993).

H pylori produces various enzymes, *urease, mucinase, catalase, lipase* and *phospholipase* enzymes. *Urease* is the important one because it hydrolyses urea and is said to produce a cloud of ammonia which surrounds the organism and forms a micro-environment in the midst of the highly acidic surroundings, thus overcoming the first barrier against the micro-organism. Also the ammonia produced by urease may disturb the ionic integrity of the gastric mucus, allowing back-diffusion of H^+ towards the gastric mucosa and resulting in tissue damage. *Mucinase* results in degradation of gastric mucus, thereby disrupting the normal barrier function, facilitating back-diffusion of H^+ ions and leading to

injury to gastric epithelial cells. The reduction in mucus viscosity might also facilitate penetration of *H pylori* towards the gastric mucosa and release of essential nutrients for *H pylori* survival and growth (Dunn 1993). *Catalase* is yet another enzyme which is produced by *H pylori*: it protects the organism against the toxic effects of reactive oxygen metabolites formed in the neutrophil from hydrogen peroxide (H_2O_2) as a result of the oxidative burst. *Catalase* hydrolyses H_2O_2 to H_2O and O_2 and thus inhibits formation of reactive oxygen metabolites that kill the bacteria (Dunn 1993). *H pylori* also produce lipase and phospholipase which contribute to breakdown of the gastric mucus. Recently Salmela et al (1994) have suggested that *H pylori* contains an alcohol dehydrogenase enzyme which is capable of producing acetaldehyde from ethanol, which results in local mucosal injury and contributes to the pathogenesis of gastroduodenal disease associated with this organism.

1.5.4. Distribution and Isolation of *H pylori*

The organism lives closely attached to the gastric epithelial cells, beneath a protective layer of mucus, in an environment where the pH is virtually neutral. *H pylori* has been visualised histologically in association with metastatic gastric-type epithelium in the duodenum (Wyatt et al 1987), oesophagus (Paull & Yardley 1988), and Meckel's diverticulum (De Cothi et al 1989, Morris et al 1989), and also in rectum (Pambianco et al 1988), dental plaque (Shames et al 1989) and saliva (Ferguson et al 1993).

The gastric tissue associated with *H pylori* infection is invariably associated with infiltration of the lamina propria with mononuclear cells. An antibody response to *H pylori* infection is common; however, the

organism is able to evade elimination because of its location in the gastric mucus.

The specimens taken for isolation of *H pylori* should be set up for culture as quickly as possible. The gastric biopsies should be maintained in either broth or saline to prevent the organism from drying out and also to protect them from the effects of atmospheric oxygen. A variety of basal media with agar and added blood (5-10% v/v sheep or horse) have been used for successful culture of *H pylori* .. The basal media used include: blood agar no.2, Columbia agar base, brain heart infusion agar, Mueller-Hinton agar, Wilkins-Chalgren agar and isosensitest agar (Rathbone and Heatley 1992). For primary isolation, antibacterial and antifungal agents are added to the media to inhibit the contaminant. *H pylori* has never been recovered from blood cultures or sites outside the gastrointestinal tract.

1.5.5. Diagnosis of *H pylori* infection

Both invasive and non- invasive methods are used for the diagnosis of *H pylori*. For invasive methods a biopsy specimen is obtained by endoscopy, and used for the rapid urease test, culture and histology, or gastric juice is collected for the measurement of urea and ammonia concentration. The non- invasive methods include serology, which identifies the presence of antibodies against *H pylori* antigens, and the urea breath tests.

1.5.5 .1. Histology

This provides the most reliable method of detection of *H pylori*. The organism is identifiable in tissue section by virtue of its characteristic

morphology, position, diffuse distribution and high population density. The organism appears curved or S-shaped and lies in the mucus layer but close to the epithelial cell surface. The organisms colonise the surface and foveolar regions of the gastric mucosa of antral, body and cardiac type, but occur mostly in the antrum. They do not colonise the metaplastic intestinal epithelium in the stomach, but do colonise the gastric mucosa present in oesophagus or duodenum and the heterotopic gastric epithelium in Meckel's diverticulum.

The ability to detect *H pylori* depends upon the type of stain used and the number of samples. The characteristic histologic appearance of *H pylori* is a small 3X0.5μm spiral rod located adjacent to the gastric mucosa. None of the stains used is specific for *H pylori* (Brown et al 1993). Routine haematoxylin and eosin staining may demonstrate *H pylori*; however, the sensitivity of this technique depends to a considerable extent on the experience of the observer.

The Warthin-Starry silver stain used by Warren and Marshall in their original work makes the organism appear larger in the sections, but the disadvantage of this method is that it is technically demanding, expensive and time consuming.

Of the routine histochemical stains, none is specific for *H pylori*, but they demonstrate the characteristic appearance of the organism which allows *H pylori* to recognised. The bacteria recognised should be described as "Helicobacter- like organism". Recently specific anti-*H pylori antibodies* have become available enabling positive identification of *H pylori* in tissue section.

Another factor which influences the ability to detect of *H pylori* is its uneven distribution in the gastric mucosa. Although the antrum appears to be more uniformly involved, a single biopsy from this area may lead to false negative results. Two biopsies within 5cm of the pylorus should be taken to improve the chance of a correct diagnosis (Brown et al 1993). The sensitivity and specificity of histological detection of *H pylori* depends on the expertise of the pathologist.

A gold standard for determining *H pylori* status has proved difficult to define, but careful histological examination of two biopsies appears to come closest to achieving this. The advantage of a histological diagnosis is that this method is cheap, provides specific information on the relationship between *H pylori* and the mucosal inflammation and allows retrospective diagnosis.

Histological diagnosis is accurate in conditions where no other bacteria are likely to be encountered in the stomach. In patients with achlorhydria or with fungating tumours there is a risk that other bacteria of similar morphology may be mistaken for *H pylori* , and in these circumstances specific identification of *H pylori* by the use of antibodies would be desirable.

1.5.5 .2 <u>Culture</u>

The identification of *H pylori* in biopsy material by culture is arguably more precise than histology but it is more difficult. The important observation was made by Marshal and Warren that incubating for 96 hours or more increases the chance of growth of *H pylori*. Growth is also enhanced by a moist microaerophilic atmosphere at 37^{0} C. Blood agar and

chocolate agar are commonly used for culture. Once the bacterium is cultured, the identification of *H pylori* is based on colony morphology and positive urease, catalase and oxidase reactions (Brown et al 1993).

As with histology, the problem of uneven distribution of the organism may contribute to falsely negative cultures; however, this would seem to be less of a problem, because even a single bacterium can give rise to a positive culture. Other reasons for unsuccessful culture include the recent use of antibiotics, ingestion of topical anaesthetic during endoscopy and contamination of biopsy forceps with other organisms or with glutaraldehyde, the antiseptic agent needed for sterilising endoscopy instruments. An important application for culture in selected settings is the determination of the antibiotic susceptibility profile of treatment-resistant organisms (Brown et al 1993).

1.5.5 .3 <u>Serology</u>

Most patients colonized with *H pylori* show a systemic antibody response. The antibodies are consistent with prolonged chronic infection, IgG and IgA are usually seen, rarely the IgM and only in cases of acute infection. The response of the host to those bacterial antigens to which it is exposed, thereby producing antibodies, depends on both host and bacterial factors. There is very little known of how these antibodies spill into the general circulation. The antibodies can be detected by a variety of methods, but the commonly used method is Enzyme-Linked Immunosorbent Assay (ELISA), because it is simple, cheap, rapid and reproducible. Other methods for detecting these antibodies are the complement fixation test (CFT), haemagglutination, bacterial agglutination and immunofluorescence.

Serology is a relatively non-invasive method of diagnosing *H pylori* infection, it is useful in epidemiological studies, and also in regard to *H pylori*-related gastroduodenal disease in children. Antibodies against *H pylori* persist in the circulation for up to six months after the treatment; re-infection results in a rapid rise of antibody titre.

1.5.5 .4 Urease test

An important characteristic of *H pylori* that forms the basis of several diagnostic tests is the ability of the organism to produce urease (Langenberg et al 1984). Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. This reaction increases the pH of the surrounding medium, a change which can be detected with a pH indicator. Hence the presence of urease is signalled by a colour change, for example the normal yellow-brown colour of phenol red at pH 6.8 changes to pink at pH 8.4. The first urease test described for the detection of *H pylori* employed Christensen's 25 urea broth, a standard microbiological reagent used to identify urease producers (McNulty & Wise 1985). A number of variations on this theme have been developed, ranging from a simple homemade solution of urea, water and phenol red to the commercially produced CLO test (Campylobacter Like Organism), which consists of Christensen's agar and an indicator mounted on a slide (Thillainayagam et al 1991). Positive tests are usually evident on the same day, often within minutes of addition of the tissue to the medium. The sensitivity of this test is 93% and specificity 98% (Brown et al 1993). This test is cheaper and quicker and results of *H pylori* status can be obtained for a fraction of the cost of histology or culture without need for any special handling of the specimen. Like histology and culture, the urease test is dependent on the

density of the bacteria and is therefore sensitive when performed on antral tissue. The need for endoscopy is a shortcoming of this method; however, on the basis of its excellent sensitivity and low cost compared with those of histology and culture, urease testing should be considered the endoscopic method of choice to diagnose H pylori and a result can be obtained before the patient leaves the endoscopy department. The disadvantages of this test are that some positives will be missed, and it is not sensitive in picking out those patients who have received antimicrobial or bismuth treatment.

1.5.5 .5 Urea breath test

Another type of test based on H pylori 's efficient hydrolysis of urea is the labelled carbon breath test. In this test, urea labelled with a carbon isotope is administered orally. In infected individuals, the urea is metabolized to ammonia and labelled bicarbonate, and the latter is excreted in the breath as labelled carbon dioxide, which can be quantified. There are two breath tests which have been used, the ^{13}C-urea and the ^{14}C-urea breath test. The ^{13}C-urea breath test has the advantage of being nonradioactive, but its measurement requires a gas isotope ratio mass spectrometer, which is not widely available. The ^{14}C-urea breath test can be easily quantified using a gamma-scintillation counter, but the disadvantage is the theoretical risk of radioactivity.

1.5.5.5.i ^{13}C-Urea breath test:

In this urease test the carbon moiety of urea is labelled with the ^{13}C isotope. The carbon-labelled urea is rapidly hydrolysed in the presence of urease enzyme of H pylori into ^{13}CO$_2$ and ammonia. The ^{13}CO$_2$ is then absorbed through the gut mucosa to be excreted in the exhaled breath. If H

pylori is not present, hydrolysis does not occur and labelled $^{13}CO_2$ is not produced (Rathbone et al 1992).

The ^{13}C-urea breath test is non-invasive; in this test the subject is given a high fat "test" meal the purpose of which is to slow down gastric emptying, thus prolonging the time the ^{13}C-urea remains within the stomach. Following the test meal, pre-dose breath samples are collected in duplicate; the subject is then given the ^{13}C-urea capsule dissolved in water to drink, and post-dose breath samples collected in duplicate 30 minutes after drinking a ^{13}C-urea capsule. These breath samples are analysed by mass spectrometry. If the level of $^{13}CO_2$ in post-dose is more than 5% greater than that of the pre-dose samples then the test is considered positive, i.e. the subject has *H pylori* in his/her stomach (Logan et al 1991 and 1992).

1.5.5.5.ii   Mass spectrometry

This is a method by which ^{13}C enrichment is measured in breath samples. These breath samples are stored in capped gas containers, and are sampled directly by a simple needle probe. Container contents are flushed onto a gas chromatograph which purifies CO_2 from N_2 and O_2. Water is removed by a chemical trap. The pure CO_2 flows directly to an on-line stable isotope analyser for the measurement of its $^{13}C/^{12}C$ ratio. The $^{13}C/^{12}C$ ratio is usually expressed as parts per thousand (per ml), and the results are expressed as excess of $^{13}CO_2$ excretion per ml (difference between pre-dose and post-dose).

1.5.5.5.iii   Factors affecting the pattern of ^{13}C excretion

There are three phases of $^{13}CO_2$ excretion: the initial exponential phase, peak excretion and steady state before $^{13}CO_2$ excretion returns to basal

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levels. The overall shape of the excretion depends on conditions adopted for the test, such as the test meal, dose of the isotope, load of *H pylori* and the host response. Host factors which influence this test are rate of gastric emptying, intragastric pH and extent of colonization. Factors relating to *H pylori* which can affect the $^{13}CO_2$ excretion are the urease activity, virulence factors and location within the mucosa.

1.5.5.6. Urea and ammonia concentration in gastric juice

This test is based on the same principle as that of the urea breath test and the urease test. The high urease activity of *H pylori* splits the urea present in gastric juice into ammonia and carbon dioxide, later excreted through lung. As a result of this reaction, there is a reduction in urea and increase in ammonia concentration in gastric juice. Diagnosis of *H pylori* can be made by measuring the gastric juice for urea and ammonia concentrations, and urea:ammonia ratio (Kim et al 1989, Neithercut et al 1991 & 1993). It has been suggested the urea:ammonia ratio clearly separates those who are infected with *H pylori* from those who are not (Neithercut et al 1991), though these same workers found no correlation between the urease activity as assessed by the urea breath test (^{14}C-urea breath test) and the ratio of urea:ammonium concentration in gastric juice. It has been suggested that the ^{14}C-urea breath test measures the response of the urease enzyme to a urea load, whereas the urea:ammonia ratio is a measure of basal *H pylori* urease activity.

1.5.6 Prevalence of *H pylori*

Human infection with *H pylori* can originate from another person, from animals or from the environment. Food is unlikely to be the source of *H pylori* infection in humans. A seroepidemiologic comparison of Asian meat eaters with Asian vegetarians showed no difference between the two groups (Webberley et al 1992); this study suggests animals are unlikely the source of *H pylori* infection. However, another investigation implicated consumption of salty food as increasing the risk of *H pylori* infection (Tsugane et al 1994). This study also suggested that *H pylori* could be a marker of salty food intake or an intermediate risk factor in the aetiological sequence between salty food intake and gastric cancer. Hopkin et al (1993) reported that *H pylori* is transmitted by consumption of uncooked vegetables, suggesting that contamination of irrigation water by raw sewage and subsequent contamination of vegetables that are eaten, is the key factor in transmission of *H pylori* infection. Husson et al (1991) studied prevalence of *H pylori* in abattoir workers by measuring IgG; they reported statistically higher IgG titres in men and women who had worked in an abattoir for 1-2 years as compared to controls. Thus they postulate the possible transmission of *H pylori* infection from animal to man.

Person-to-person transmission of *H pylori* is suggested by the clustering of infections found in families, institutions and occupational groups having close person-to-person contact. Wilhoite et al (1993) in their study using serodiagnosis found that 39% of nurses were positive for *H pylori* as compared to 26% among blood donors, and this difference was carried through different age groups. However, this mode of transmission of *H pylori* infection was not supported by Malaty et al (1992), who in their seroepidemiological study reported no increase in rate of infection among

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dental workers. It is well known that *H pylori* is found in the dental plaque (Shames et al 1989, Majmudar et al 1990, Krajden et al 1989, Desai et al 1991). The prevalence of *H pylori* among children living in institutes was 38% (Vincent et al 1994); further analysis of the characteristics of infection in children showed the predominant role of overcrowded living conditions, and the period of time cohabiting explained the unexpectedly high prevalence of *H pylori* infection in children living in otherwise good sanitary conditions. In another study (Webb et al 1994) the prevalence of *H pylori* in adults was related to their living and socio-economic condition during childhood. The method used in this study was enzyme-linked immunosorbent assay (ELISA); they found that prevalence increases with age (22/74, 29.7% in those under 30 years age, as compared to 29/46, 63% in those in the age group of 55-65 years). After the data were adjusted for the age and occupation, subjects from large families, whose childhood homes were overcrowded or who regularly shared a bed in childhood, were significantly more likely to be seropositive. This study has suggested that person-to-person contact during childhood is an important factor for the prevalence of *H pylori* .

Prevalence of *H pylori* among asymptomatic controls in several studies using various methods of diagnosis has been examined. Vaira et al (1994) in their study on a population of 1010 asymptomatic blood donors, found 128 seropositive for *H pylori* . All these subjects subsequently had endoscopy: 121 of the 128 were found to be positive on histology and urease test/culture. All 121 were positive for chronic active gastritis, 25 subjects had peptic ulcer, 21 had erosive duodenitis, two gastric cancer and the remaining 73 showed no evidence of gastroduodenal disease. This study has suggested that *H pylori* infection is of relevance to subclinical disease among an asymptomatic population.

Many studies have shown that the prevalence of *H pylori* increases with age in asymptomatic subjects. In 316 randomly selected subjects in the age group 18-72 Van-Zanten et al (1994) have shown that the prevalence of *H pylori* increases from 21% in the third decade to 50% in the eighth decade. In this Canadian study there was a crude annual increase of 1.6% in the prevalence of *H pylori*. However, Katelaris et al (1993) suggested that the prevalence of *H pylori* infection does not increase with age; they found the prevalence lower in those older than 40 years than those under the age of 40 years. This difference persisted after the adjustment of socio-economic status.

It has been suggested by various studies that the prevalence of *H pylori* is greater among the black population than the white. Graham et al (1991a) reported that the *H pylori* prevalence among an asymptomatic population in the Houston metropolitan area was 52%. Like other studies they also confirmed that the rate of infection increases with age and showed *H pylori* infection was commoner among blacks (70%) than whites (34%), with this difference remaining after adjustment for age, gender, educational levels, income, and use of tobacco or alcohol. Sather et al (1994) in their study on asymptomatic blood donors found the prevalence of *H pylori* was 93% in blacks and 83% in American Indians, whereas it was 42% among whites. Interestingly enough they found no difference in the prevalence of *H pylori* between rural and urban populations. Also there was an increase in hepatitis A virus infection among those who were *H pylori* positive, suggesting that there may be a similar mode of transmission. Similar observations were made by Smoak et al (1994). They found that 44% of blacks were positive for *H pylori*, as compared to 38% and 14% of Hispanics and whites respectively. Like other studies they

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also found an increase in the prevalence of *H pylori* with age, 24% in the age group 17-18 years, 43% in the age group 24-26. In another study Blecker et al (1993) found a difference between Caucasian and non-Caucasian children born in same community, there being a higher prevalence of *H pylori* among non-Caucasians.

The prevalence of *H pylori* varies between different nations. In one major study by the Eurogast group (Forman 1993), in which the prevalence was determined by serum assay, 3000 asymptomatic subjects in two age groups, 25-34 and 55-64, from 17 geographically different population in Europe, North America, North Africa and from Japan were described. This study showed that the prevalence was higher among the older age group (62.4%) as compared to the younger (34.9%). They found no difference in the prevalence of infection between men and women. Subjects with higher education had considerably lower levels of infections (34.1%) as compared to those who had education up to secondary level (46.9%) or those with primary education (61.1%). This study showed no difference of infection between smokers and non-smokers, or between consumers and non-consumers of alcohol.

There is conflicting evidence regarding the prevalence of *H pylori* in smokers. The Eurogast study group (Forman 1993) found no difference in the prevalence of *H pylori* between smokers and non-smokers, whereas Bateson (1993) found an increase in *H pylori* prevalence among smokers. However, Lindell et al (1991) in their study which included 106 patients referred for endoscopy found a lower prevalence of *H pylori* among smokers (18%) than in non-smokers (45%), and this difference was statistically highly significant. They have suggested that the lower

prevalence of *H pylori* in smokers might be a direct toxic effect of nicotine on colonization with *H pylori* .

The use of long-term nonsteroidal anti-inflammatory drugs (NSAID) has been investigated by Graham et al (1991b); they found that NSAID drug-induced mucosal injury, either haemorrhage or erosions, was more frequent in those without *H pylori* infection than with infection (61% vs 32% for haemorrhages and 57% vs 34% for erosions for those without and with *H pylori* infection). They found no difference in the presence of symptoms of dyspepsia in those with and without *H pylori* infection. The authors suggested that the NSAID-induced damage to the gastrointestinal mucosa does not increase the susceptibility to *H pylori* infection. The majority of ulcers in chronic NSAID users were associated with *H pylori* infection. It remains unclear whether most NSAID-associated duodenal ulcers are *H pylori* -associated ulcers, or NSAID-exacerbated *H pylori*-associated ulcers. The known age-related increase in *H pylori* infection coupled with the relatively older population characteristic of patients with chronic arthritis ensured that, by chance, a large number will both receive NSAID and have *H pylori* infection. In another study (Laine et al 1992), reported a decrease in gastritis and *H pylori* prevalence in NSAID-associated gastric ulcer compared to non-NSAID-associated gastric ulcer. Goggen et al (1993) suggested that *H pylori* infection increases the dyspeptic symptoms in patients receiving NSAID, though there was no difference in the extent of NSAID-induced gastric damage between *H pylori* +ve and -ve subjects.

The acquisition of *H pylori* does not appear to have any relationship to seasons. However, Moshkowitz et al (1994) reported that the frequency of *H pylori* infection in dyspeptic patients in Israel is significantly increased

during the winter months and decreased in the summer. The most likely explanation of this seasonal rise is the increased overcrowding indoors in cold weather, providing increased opportunity for the disease transmission, or it could be an effect of cold on the host's resistance. It is also possible that a combination of changes in environmental conditions that occur during winter months may create favourable circumstances for both the survival and transmission of *H pylori*.

All these studies have shown that prevalence of *H pylori* in asymptomatic subjects can be as high as 100% (Majmudar et al 1990), and is higher among developing countries than in developed countries. In developing countries, people become infected much earlier in life than in developed countries: in northern parts of China 36% under the age of 3 years are infected with *H pylori*. Some studies have suggested *H pylori* infection is related to lower socio-economic status and in particular when there is overcrowding. Rate of infection increases with age both in developing and developed countries. The rate of acquisition of the infection is remarkably similar in countries such as China where prevalence is high and countries like Australia where rate of *H pylori* infection is low. In most countries the rate of infection is about 1% per year (Lee 1994). Most infections are passed from parents to their children. Mitchell et al (1993) reported that 76% of families of infected children were *H pylori* positive, as compared to only 15% of families of non-infected children, a highly significant difference. Without a significant animal or environmental reservoir for the *H pylori*, person-to-person contact appears to be the most likely mode of transmission. Exactly how the organism spreads from one person to another is not clear.

1.5.6 .1 Prevalence of *H pylori* in Duodenal ulcer

H pylori is now implicated in the pathogenesis of duodenal ulcer. *H pylori* may infect the duodenal mucosa in patients who have areas of gastric epithelium in the duodenum, resulting in inflammation of the duodenal mucosa. Alternatively the presence of *H pylori*-gastritis, which causes an alteration of gastric physiology with elevation of gastrin, results in an increase in the gastric secretion that thereby leads to DU.

In some studies the prevalence of *H pylori* in DU has been reported as 90-100% (Rauws et al 1988, Diomande et al 1991, Rohrbach et al 1993, Tytgat et al 1993, Sandikci et al 1993, Huang & Chen 1993, Lin et al 1993) suggesting that *H pylori* causes DU. However, Koch's postulate 1, which says that the organism must be found only in subjects with the disease is not fulfilled, as one finds *H pylori* in subjects with gastric ulcer (Li et al 1991), non-ulcer dyspepsia (Veldhuyzen et al 1994) and even in 40-100% of asymptomatic subjects (Koch et al 1990, Majmudar et al 1990, Aska et al 1992, Dehesa et al 1991). Although many authorities are convinced that *H pylori* is an important casual factor in duodenal ulcer formation, there is no direct evidence in humans that *H pylori* infection precedes the development of duodenal ulcer; duodenal ulcer has been absent in human volunteers and animals infected with *H pylori* (Barthel et al 1988, Gregson et al 1989, Dooley et al 1989, Krakowa et 1987, Lee et al 1990). Therefore, Koch's postulates are not fulfilled for a casual link between *H pylori* and duodenal ulcer.

Evidence against a causal role for *H pylori* in DU is also provided by the high prevalence of *H pylori* in some areas which is not matched with an increased incidence of peptic ulcer. Holcombe et al (1992a & b), examined

the population of northern Nigeria which has a very low incidence of peptic ulcer (32/1000), and found that the majority of the population were positive for *H pylori* antibodies (85%). This apparent contradiction emphasises the fact that *H pylori* is only one of a number of possible factors predisposing to peptic ulceration. For example the staple diet in northern Nigeria may protect from peptic ulceration.

Various studies from the Far East have suggested a low prevalence of *H pylori* in patients with DU. Uyub et al (1994) found a low prevalence of *H pylori* in DU subjects (53.1%), and also showed that the *H pylori* infection was lower in Malays (27.8%) as compared to non-Malays people (85.7%). Hsu et al (1992) in their study examined the prevalence of *H pylori* in 63 endoscopically proven DU with 34 patients of non-ulcer dyspepsia (NUD, those patients with no history of ulcer and no ulcer documented by endoscopy). They found 76% of DU were positive for *H pylori* as compared to 27% in NUD. Similarly Kachintorn et al (1992) found *H pylori* infection in 66% of DU subjects. Indians of Fiji appears to have a rate of DU at least twice than that seen in ethnic Fijians (Parshu 1975), but Beg et al (1988) found that *H pylori* was present with equal frequency in both races.

The lower rates of *H pylori* infection in these subjects could be due to population differences in resistance to the organism, or to different strains of the organism. Although *H pylori* has been demonstrated in the antral mucosal tissue in 100% of patients with DU (Tytgat 1993), the organism is also present in greater than 60% of people over the 65, the majority of whom do not develop DU, thus putting the casual role of *H pylori* in DU in question. By contrast, there is no doubt about the role of *H pylori* in

chronic active gastritis, a condition which also increases with the age (Rabeneck & Ransohoff 1991).

1.5.6 .2 Pathogenesis of *H pylori* in Duodenal ulcer

Infection of the gastric antrum with *H pylori* is strongly associated with chronic active gastritis. It has been suggested that the infection causes duodenal ulcer. The mechanism by which it causes duodenal ulcer remains unclear, although various hypotheses have been proposed. The important one which has been suggested by many studies is the gastrin link hypothesis. Gastrin is a polypeptide secreted by antral G cells that stimulates parietal cells to secrete acid and, to a lesser extent, chief cells to secrete pepsin. Gastrin secretion is at least as important as vagal stimulation in the control of gastric secretion (Wolfe et al 1988). Levi et al (1989a & b) initially reported that the individuals with DU and antral *H pylori* infection had significantly higher basal and meal-stimulated plasma gastrin concentration and higher peak, but not basal, acid output than individuals with DU who were not infected with *H pylori*. Eradication of the infection decreased meal-stimulated gastrin levels, but not basal gastrin, basal acid output or peak acid output levels. These investigators have proposed that *H pylori* in the gastric antrum increases antral gastrin release (gastrin-link hypothesis). In this hypothesis, ammonia produced by hydrolysis of urea catalysed by urease from *H pylori* increases the pH of the mucus layer overlying the gastric epithelium. This interferes with the normal feedback inhibition of gastrin by luminal acid, resulting in a raised gastrin secretion. This increased secretion of gastrin increases gastric acid secretion either directly by stimulating parietal cells

or indirectly by exerting a trophic effect on parietal cell mass, or both. The resultant increase in acid secretion promotes DU formation.

Many but not all studies have demonstrated that basal and meal-stimulated gastrin levels are elevated in *H pylori*-infected individuals with DU; however, significant differences in basal and peak acid output have not been demonstrated consistently in individuals with *H pylori* infection. Eradication of *H pylori* characteristically decreases basal and meal-stimulated gastrin secretion without affecting basal or peak acid output (Graham et al 1990, Graham et al 1991c, Levi et 1989a). In addition, augmenting *H pylori* ammonia production by infusing urea into the stomach of *H pylori*-infected individuals with DU significantly increases intragastric ammonia levels without affecting the plasma gastrin concentration (Chittajallu et al 1991). These results suggests that mucosal ammonia produced by *H pylori* urease is not the critical factor involved in the regulation of gastrin levels (Graham et al 1990). The inability to detect any changes in gastric acid secretion despite decreasing gastrin concentration after eradication of *H pylori* seems to cast doubt on the gastrin-link hypothesis. In another study Chittajallu et al (1992) have suggested that the increased level of gastrin in patients infected with *H pylori* is a compensatory response to the reduced parietal cell sensitivity to gastrin; consequently a higher concentration of gastrin is required to maintain the same levels of acid secretion. This study also suggests that there is no increase in basal acid output or the acid output in response to pentagastrin in these *H pylori*-infected DU patients. However, Levi at al (1989a, b) and McColl (1989) have claimed increased gastric secretion.

Chandrakumaran et al (1994) found that *H pylori* infection was associated with a decreased gastric secretion in both DU and NUD subjects. The effect

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was only clear cut when gastric secretion was corrected for pyloric loss, duodenogastric reflux, and stature. Failure to make such corrections probably accounts for the discrepancies in the gastric secretion results reported by other workers. Calam (1993) has also shown that chronic *H pylori* infection does not increase maximally stimulated acid output (MAO) which reflects the parietal cell mass; on the contrary, *H pylori* may eventually decrease MAO by initiating gastric atrophy. Peterson et al (1993) found that *H pylori* infection in controls is associated with a significant reduction of basal acid output, but in DU patients it is associated with an increase in gastrin secretion and a rise in both basal and stimulated acid secretion. They have claimed that hypergastrinaemia in DU is largely related to *H pylori* infection, but the increase in acid output was not.

Moss et al (1992) have suggested that in *H pylori* infection there is a reduction of somatostatin secretion. Somatostatin is secreted by the D cells of gastric mucosa in the proximity of the G cells which secrete gastrin. Somatostatin has an inhibitory effect on G cells. The authors of this study have suggested that *H pylori* destroys the D cell, reducing the somatostatin and thereby increasing the gastrin secretion. This study also showed that after eradication of *H pylori* infection there was an increase in somatostatin-producing cells. Although this is the most interesting theory regarding the pathogenesis of DU by *H pylori*, there are several questions unanswered in this study. Firstly, the dramatic increase in somatostatin after the treatment of *H pylori* infection was found only in half the patients, while in the others it increased only slightly or not at all. Secondly, if a decrease in somatostatin is indeed responsible for the increase of gastrin secretion in *H pylori* infection, then one might have expected gastrin cells to have decreased after the eradication of *H pylori* infection, but the authors found no significant difference in the density of

gastrin cells before or after the eradication of H pylori infection. Lastly, in this study there were no H pylori-infected controls, and so this somatostatin link which causes hypergastrinaemia remains elusive (McHenry et al 1993).

Histamine has been long recognised as a potent stimulus for gastric secretion. Infusion of histamine in *vivo* and in *vitro* results in high acid production; however, only after the introduction of H2-receptor antagonists did the role of histamine in regulating gastric secretion become unquestionable. Queiroz et al (1993) have measured the histamine concentration in oxyntic mucosa in patient infected with H pylori, they reported a decrease in histamine contents which increased after the eradication of H pylori. They claimed that H pylori infection promotes a high release of histamine from gastric mucosa which may be responsible for the high acid gastric secretion observed in patients with DU.

Other mechanisms by which H pylori may cause DU are :1). The disruption of the mucus barrier by production of ammonia, cytotoxin and/ or phospholipase. 2). Enhancement of aggressive factors such as acid, platelet activating factor and pepsin. 3). Activation of monocytes and macrophages with release of tumour necrosis factor, interleukin-1, and reactive oxygen metabolites. 4). Production of antibodies directed against the organism that reacts with gastric tissue (autoantibodies). 5). Enhanced release of gastrin (McHenry et al 1993)

1.5.6 .3 *H pylori* and gastric cancer

Recent studies have suggested that H pylori plays an important role in the pathogenesis of gastric cancer. Infection at an early age, especially among

individuals in developing countries, produces a destructive lesion of the mucinous surface of the epithelium which probably enables other aggressive luminal factors to cause further mucosal damage. As a consequence, active chronic gastritis appears. This gastritis gradually changes to atrophic gastritis. When atrophic gastritis becomes severe and extensive, hypochlorhydria ensues. Hypochlorhydria favours the appearance of bacterial growth, nitrites and nitroso compounds in the gastric lumen. These compounds, because of their mutagenic carcinogenic properties, probably induce a premalignant gastric lesion (Recavarren et al. 1991). Burstein et al (1991) in their study have compared the prevalences of peptic ulcer (duodenal and gastric ulcer) and gastric cancer in 1796 dyspeptic Peruvian patients with those reported in 2883 similar patients from developed countries. They found a lower prevalence of peptic ulcer and a higher prevalence of gastric cancer in the Peruvian patients. They suggested that the reason for this difference was probably the high prevalence of H pylori associated with chronic atrophic gastritis and hypochlorhydria in Peruvian patients, factors which predispose to gastric cancer. In another study the correlation between H pylori infection and the risk of gastric cancer was examined by Corea et al (1990). They obtained serum samples for H pylori antibody assay from two populations with contrasting gastric cancer risks. The prevalence of H pylori infection was up to 93% in the adult population of Pasto Colombia which has highest risk of gastric cancer, whereas in a low risk area like the Colombian city of Cali, the prevalence of H pylori was 63%. Similar observations were also made by Lin et al (1993) who found the prevalence of H pylori was 62.9% among patients with gastric adenocarcinoma as compared to 54.4% in controls. They suggested that H pylori infection in early life may be a contributory factor in gastric carcinogenesis. However, in a study from Japan where the prevalence of gastric cancer is highest, Takahashi et al.

(1993) found no difference between the prevalence of *H pylori* antibodies in patients with gastric carcinoma and those with chronic gastritis, implying that *H pylori* is not a direct causal agent in the pathogenesis of gastric carcinoma. Kuipers et al (1993) also found no difference in prevalence of *H pylori* antibodies in patients with gastric cancer and in controls matched for age and sex. Farinati et al (1993) evaluated the endoscopic prevalence of *H pylori* in a population of patients with gastric precancerous conditions and lesions by studying endoscopic biopsies from 252 patients and recording the presence and the degree of *H pylori* infection. Like other workers he found the rate of *H pylori* infection increased with age, but became significantly lower with the progression of gastric mucosal damage. *H pylori* infection among gastric cancer patients was 36%. They suggested that *H pylori* infection correlates inversely with the presence of gastric precancerous changes and cancer.

H pylori infection does not influence the site of gastric cancer, or its histological type (diffuse or intestinal). There is no significant association between *H pylori* infection and the presence of intestinal metaplasia, age, sex or the type of gastric cancer in their study. The only significant finding in their study was the association of *H pylori* infection and gastritis (Clarkson & West 1993). However, Buruk et al (1993) suggested that there is a possible association between the intestinal type of gastric cancer and *H pylori* infection. Reed et al (1993) reported that *H pylori* infection is closely associated with a differentiated-type of gastric cancer but not with undifferentiated type. Estevens et al (1993) studied the prevalence of *H pylori* antibodies in 80 gastric cancer patients and 80 controls matched by age and sex. They found no significant difference between the two groups. Nomura et al (1991), Hansson et al (1993) and Parsonnet (1993) found no association between tumours located at the gastric cardia and *H pylori*

infection, suggesting that *H pylori* has no aetiological role in the pathogenesis of these tumours. They proposed that *H pylori* plays a role in more distal cancer and suggested that the organism is an independent risk factor in gastric carcinoma.

There are several possible mechanisms by which *H pylori* infection may be involved in gastric carcinogenesis. *H pylori* adversely affects the chemical and physical properties of the mucus layer (Sarosick et al 1991), making the mucosa susceptible to carcinogenic factors. Acute infection of *H pylori* results in prolonged inhibition of naturally occurring ascorbic acid (Sobala et al 1991), thereby permitting the formation of N-nitroso compounds which are carcinogenic. It may be that *H pylori* acts as a promoter in the progression from normal to metaplastic epithelium, possibly by inducing a hyperproliferative state in the inflamed gastric mucosa (Sipponen et al 1992). Finally, the chronic inflammation per se may expose the mucosal cells to the oxidative stress of free radicals generated by the inflammatory cells (Hansson et al 1993).

There are several issues to be resolved, however, before the association between *H pylori* and gastric cancer can be accepted as causal. There are several populations such as in China (Forman et al 1990) and Africa (Megraud et al 1989) where *H pylori* infection is prevalent at young ages but gastric cancer is uncommon. There is a lack of specificity, as *H pylori* infection seems to be a risk factor of both diffuse and intestinal types of adenocarcinoma (Sipponen et al 1992, Forman et al 1991, Parsonnet et al 1991, Nomura et al 1991), as well as one type of gastric lymphoma (Isaacson 1994). Again *H pylori* has been suggested as a important causal factor in duodenal ulcer, but the negative association between DU and the later

development of gastric cancer is perplexing. Thus, the role of *H pylori* in the multifactorial genesis of gastric cancer needs to be explored further.

1.5.6 .4 *H pylori* and Non-ulcer dyspepsia

Non-ulcer dyspepsia (NUD), also termed functional dyspepsia, is chronic or recurrent abdominal pain or discomfort centred in the upper abdomen and lasting for more than 1 month with symptoms for 25% of the time (Lambert 1993). In subjects who have no other organic disease likely to explain the symptoms, on the basis of clinical, biochemical, endoscopic, or radiological investigation, the term idiopathic or essential NUD has been used. Dyspepsia with no endoscopic abnormality may occur in association with a number of other gastrointestinal diseases, including irritable bowel syndrome, gastrointestinal reflux, biliary disease and drug use, particularly aspirin and non-steroidal anti-inflammatory drugs.

The pathophysiology of NUD is probably heterogeneous. Differences in the pathophysiology and responses to treatment may thus exist between different countries and ethnic groups. *H pylori* colonizes the gastric mucosa as well as gastric metaplastic mucosa in the proximal duodenum. The role of *H pylori* and gastroduodenitis in the pathogenesis of NUD is unclear. Evidence to support the concept that *H pylori* has a role in NUD includes: 1). There are structural, functional, and pathological changes in the mucosa of infected individuals (Lambert 1993). 2). The prevalence of *H pylori* in NUD is higher than in a representative control population (Lambert 1993) and 3). finally it has also been suggested that there is improvement in the symptoms after the eradication of *H pylori* (Talley 1991).

H pylori is found in 43% to 70% of subjects with NUD (see table 1.1, p.91). The wide variation of *H pylori* frequency in NUD subjects reflects both differences in the criteria to diagnose NUD and in the populations evaluated. Few studies have compared *H pylori* prevalence in NUD subjects with an age, sex, and ethnically matched representative population. In developed countries from which data are available, the prevalence of *H pylori* infection in NUD subjects is higher than that in control subjects (Rokkas et al 1987, Pettross et al 1988, Rauws et al 1988, Lambert et al 1989, Strauss et al 1990).

Several studies have evaluated the effect of *H pylori* suppression and eradication on dyspeptic symptoms (Gilman et al 1987, Rauws et al 1988, Lambert et al. 1989, Kang et al 1990, Goh et al 1991, Patchett et al 1991). In most studies, improvement of symptoms has been observed, although the type of symptom reduced has been variable and the various results are often conflicting. There are many problems with these studies, including the diagnostic criteria for NUD, short term follow up, lack of adequate control groups particularly *H pylori* negative subjects, and inability to carry out truly blind studies of treatment in which bismuth is given because its side effects are often obvious. Moreover, a high placebo response in *H pylori* positive and negative subjects further compounds the problems. Despite the conflicting evidence, most studies evaluating *H pylori* clearance and eradication, particularly the long term studies, have observed clinical improvement in most subjects (Lambert 1993). However, Pieramico et al (1993) reported a decrease in postprandial antral motility in patients with NUD, but found no difference between *H pylori* +ve or -ve NUD subjects. In their view, it is unlikely that *H pylori* infection is the primary cause of the pathophysiology of NUD.

Table 1.1

H pylori infection in NUD subjects and population controls

Study	Country	NUD *Hp+ve* (%)	Controls *Hp +ve* (%)
Pettross et al	USA	43	13
Rauws et al	Netherland	70	20
Lambert et al	Australia	61	36
Rokkas et al	England	45	13
Strauss et al	USA	60	25

No one doubts that *H pylori* causes chronic active gastritis and that this form of gastritis is common in patients with non-ulcer dyspepsia; however, the little evidence that supports the hypothesis that *H pylori* is aetiologically linked to non-ulcer dyspepsia is highly questionable and conflicting. There seems no doubt that *H pylori* alone is not sufficient to cause symptoms, because dyspepsia can occur in the absence of infection and infection can occur in the absence of symptoms. Although it has been advocated that all patients with non-ulcer dyspepsia undergo routine gastric biopsy and then be considered for the treatment of their *H pylori* infection, this is not yet acceptable practice, based on current evidence (Tally 1993).

1.5.6 .5 *H pylori* and Oesophagitis

H pylori has also been found in the oesophagus. Walker et al (1989) isolated *H pylori* from the oesophagus in a diverse group of patients (27%) but they failed to find the organism in the oesophageal mucosa of normal controls. *H pylori* was only infrequently associated with endoscopic and histological oesophagitis. In Barrett's oesophagus the prevalence of *H pylori* was 23.8% (Kogan et al 1992). Interestingly, they found no *H pylori* in oesophageal biopsies from patients with reflux oesophagitis without Barrett's oesophagitis. The actual role of *H pylori* in Barrett's oesophagus is unknown. These features suggest that *H pylori* is of limited importance in the oesophagus and that its role is either that of commensal, or more likely contaminant (Walker et 1989). Whether or not *H pylori* is pathogenic in the oesophagus, the question arises where does it come from? The likely sources are the mouth and stomach. Interestingly Shames et al (1989) have identified *H pylori* in dental plaque, and the

same strain in the stomach of these patients. Dental plaque specimens were always taken prior to the gastric biopsy, to exclude the possibility of contamination of the tooth surface with *H pylori* during the withdrawal of the endoscope. Whether dental plaque represents a common or rare ecological niche for this organism has yet to be determined. Similar finding were also noted by Yang (1993) in whose study 29 subjects underwent dental plaque sampling and gastric biopsy at the same time. There were 21 subjects who were *H pylori* +ve by the gastric biopsies, and 8 of these were also *H pylori* +ve on dental plaque samples. Dual therapy was given to two patients who were *H pylori* positive in both the gastric mucosa and dental plaque. One month after the treatment, *H pylori* had been eradicated from the gastric mucosa but persisted in dental plaque. This study suggests that conventional treatment may not eradicate *H pylori* present in the dental plaque. Even in patients in whom *H pylori* had not been detected from the dental plaque, it has been found in the saliva; Ferguson et al (1993) have grown *H pylori* from salivary samples, collected prior to endoscopy from patients with upper abdominal symptoms. These studies do not rule out the mouth as a potential source of *H pylori* in the oesophagus.

In another study Agnholt et al (1991) investigated the rate of *H pylori* infection in the oesophagus and its role in oesophagitis. Forty six patients were included in this study; biopsies were taken from stomach and oesophagus. Interestingly they employed two method for the biopsies. In method 1, the biopsy specimens were initially obtained from the stomach followed by biopsy from oesophagus; in method 2 the procedure was reversed. A significantly greater frequency of *H pylori* in the oesophagus was found in patients biopsied in accordance with method 1 than those biopsied according to method 2. They found no difference between

patients with and without oesophagitis. They have suggested that *H pylori* is not associated with oesophagitis, the occurrence of the organism in the oesophagus is probably due to gastroesophageal reflux or due to contamination during endoscopy. Similarly Cheng et al (1989) reported that the oesophagitis is not associated with increased prevalence of either gastric or oesophageal *H pylori* infection. Borhan-Manesh et al (1993) found that prevalence of *H pylori* infection of heterotopic gastric mucosa (HGM) at the upper oesophagus, forms a part of the *H pylori* gastritis, and an independent colonization of HGM patches without gastric infection does not occur.

1.5.6 .6 *H pylori and gastritis*

H pylori is the most common cause of *chronic active gastritis*, also called *non-autoimmune gastritis*, or *H pylori-associated gastritis* or *non-erosive non-specific gastritis* . It affects mainly the antral part of stomach. It is called non-erosive because it has no erosive or haemorraghic lesions of the type often seen in gastritis in patients taking nonsteroidal anti-inflammatory drugs (NSAID). The term non-specific is used because the histology does not predict the cause or associated clinical condition. The patients with this form of gastritis may or may not have peptic ulcer. *Specific* gastritis refers to a type with various distinctive histological and sometimes endoscopic features, including eosinophilic infiltrates and granulomas. In non-erosive non-specific gastritis, the gastritis affects the mucosa of two major zones of the stomach, the antral and the fundic, or indeed both. Four major patterns of non-erosive non-specific gastritis have been described: antral gland type, fundic gland type, pangastritis and multifocal atrophic gastritis. The most common is the antral gland type,

which is also referred to as *type B gastritis*. It is diffuse in the antrum and is the type associated with *H pylori*. In *H pylori* gastritis, the fundal gland mucosa is also inflamed, but less severely. Fundic gland gastritis alone, which is also referred to as type A gastritis, is associated with pernicious anaemia. *Multifocal atrophic gastritis* has a different distribution and occurs in populations with a higher incidence of gastric adenocarcinoma. It begins at the incisura and fans out for a variable distance in a patchy fashion into both antrum and body (Correa 1988).

In general there is a very poor correlation between endoscopic appearance and the presence or severity of nonerosive non-specific gastritis. Because there is less involvement of fundic glands, the biopsies must be taken within 1-2cm of the pylorus to make a correct diagnosis of nonerosive non-specific gastritis.

The inflammatory cells in *H pylori-associated* gastritis are a mixture of mononuclear cells and neutrophils; because of this combination it is called *chronic active gastritis*. The inflammatory response seen in children is different from that seen in adults; there are areas of fine nodularity in the antrum which are due to lymphonodular hyperplasia. Such nodularity is uncommon in adults, but when seen in them it is referred to as *follicular gastritis*. It is not known whether this form of gastritis is associated with *H pylori* infection. *H pylori* organisms are commonly found in the lesion, but in the minority of cases in which it is absent, sampling error is the most likely explanation. The follicular type of gastritis is associated with areas of metaplasia: inflamed gastric epithelium is replaced by an intestinal type of epithelium, which may contain goblet cells, intestinal absorptive epithelium or even Paneth cells and endocrine cells (Robert et al 1993). However, Zerbib et al (1993) in their prospective study, which included 445

patients with a mean age of 36.4 (18-86), detected 14.2% with follicular gastritis; they also found that this type of gastritis was significantly associated with *H pylori* infection: 49/138 infected versus 14/307 non-infected patients.

CHAPTER TWO

PLAN OF STUDY

PLAN OF THE STUDY

The first aim of this thesis was to find out the prevalence of *Helicobacter pylori* in healthy controls, and to compare it with a previous study from this Department which measured prevalence in several groups of dyspeptic subjects (Chandrakumaran 1991).

The second aim of this thesis was to find out whether epidermal growth factor has a role in the pathogenesis of duodenal ulcer. Calabrò et al 1990 recorded a smaller EGF concentration in the gastric juice of patients with peptic ulcer than in normal subjects; they therefore suggested that the deficiency of EGF plays a role in the pathogenesis of peptic ulcer. Maccini et al 1990 found a smaller EGF concentration in salivary secretion in patients with duodenal ulcer. Jones et al 1992 reported that smoking reduces the secretion of EGF in saliva and suggested that was the reason for the observed link between DU and smoking.

This study was designed to measure the EGF concentrations in saliva and in gastric juice during the basal state and after histamine stimulation, in controls, in patients with DU, and in patients with non-ulcer dyspepsia (those dyspeptics who displayed no abnormality on endoscopy).

The third aim of this thesis was to find out whether there is any relationship between infection with *H pylori* and the EGF concentration in the gastric juice. Such a relationship might arise in at least two different ways. If *H Pylori* causes DU then *H pylori* +ve patients without a DU may be expected to have high concentration of EGF in gastric juice. Alternatively, if the presence of DU stimulates gastric EGF production in an ineffectual attempt at healing, and if DU is caused by *H pylori*, then *H*

pylori +ve patients with DU should have a higher EGF concentration than those without any ulcer.

The final aim of this thesis was to measure the salivary contamination of gastric juice by using EGF as a marker. It is known from previous studies from this Department that the electrolyte concentrations of gastric aspirate can be interpreted in terms of the aspirate being a mixture of a primary juice (VG) and duodenogastric reflux (VR). However, it is well recognised that this method of calculation ignores swallowed saliva. Whitfield (personal communication) had further speculated that the calculation of VG and VR could be modified to take into account and indeed quantify swallowed saliva. These calculations have not been published, due to the lack of a suitable marker for the swallowed saliva in the gastric juice. Boulos et al (1980) tried to use salivary thiocyanate to measure the contamination of gastric juice by swallowed saliva. They measured thiocyanate in saliva and in gastric juice in 22 duodenal ulcer patients undergoing routine insulin and histamine secretion studies. They found that despite the increase in the rate of gastric secretion this was not matched by a corresponding fall in the concentration of thiocyanate in the gastric aspirate. Moreover, in one-third of the gastric juice samples the thiocyanate concentration was greater than that in simultaneous saliva samples.

The aim of this study was to test whether endogenous EGF in basal and/or histamine-stimulated gastric juice was a suitable marker for the swallowed saliva in gastric juice. EGF is present in both saliva and gastric juice but Konturek et al (1991) recorded higher concentrations of EGF in saliva than in gastric juice, this suggested the possibility of using EGF as a marker of swallowed saliva.

2.1 Selection of Individual Studies

For the first part of my study I included one hundred control volunteers to find out the prevalence of *H pylori*. These volunteers were carefully selected after getting a detailed history: those with a past history of peptic ulcer disease, those who had been on antibiotics during the previous six months and those working in an endoscopy unit were excluded. The volunteers were nurses, doctors, medical secretaries, medical students, members of the paramedical staff and hospital porters. Some volunteers were also recruited from the surgical outpatient clinic, especially follow-up patients and also those preoperative patients waiting for a simple surgical procedure like repair of hernia or ligation of varicose veins.

For the second part of my study, dealing with the second, third and fourth of my aims, I measured epidermal growth factor in saliva and gastric juice during basal condition and during stimulation with histamine. In total there were sixty subjects. Twenty of these subjects were healthy controls, selected with the same careful exclusion criteria as those in the first part of my investigation and were obtained from the same sources. The remaining forty subjects were selected from the endoscopy unit in University College Hospital. These subjects had been referred for diagnostic endoscopy because of the complaint of dyspepsia. In twenty of these a duodenal ulcer had been found at endoscopy. The remaining twenty were those dyspeptics in whom endoscopy had shown entirely normal finding: they were called non-ulcer dyspepsia patients (NUD).

Both these studies were approved by the Clinical Investigations Panel of The Middlesex Hospital, one of the Research Ethics Committees of UCL Hospitals.

CHAPTER THREE

METHODS

3.1 METHODS OF GASTRIC SECRETION TEST

Gastric secretion studies were performed by a standard technique that has been developed in this Department and the details were described by Whitfield and Hobsley (1979). The procedure followed eliminates observer bias in the selection of the plateaux, and uses techniques for reducing collection errors by the use of two markers; one artificial, phenol red (PSP) and the other natural (Na^+).

3.1.1 PATIENT SELECTION

A total of 60 subjects were included for the gastric secretion test. Twenty were healthy volunteers; they were carefully selected after taking a detailed history, and those with a previous history of duodenal ulcer were excluded. Forty subjects were recruited from those undergoing upper gastrointestinal endoscopy in University College Hospital for the symptoms of dyspepsia; 20 of these patients had duodenal ulcer (DU), the remaining patients had normal endoscopic appearances and they were labelled as non-ulcer dyspepsia (NUD).

3.1.2 EQUIPMENT

All equipment involved in the gastric secretion test satisfied the Health and Safety standards. Since 1966 over 1600 gastric secretion studies have been performed in this department and there were no mishaps during the test including the histamine infusion. The equipment was regularly checked for any faults.

3.1.2.1 NASOGASTRIC TUBE

This was a double-lumen nasogastric tube; the narrower lumen was for the instillation of phenol red and the larger lumen for the aspiration of gastric contents. The tube was especially made (Portex Ltd) for the purpose of the gastric secretion test. This tube has a smooth contour and its passage was more easily tolerated by the subjects. The tube was end-weighted and marked at 10cm intervals from the tip. The aspiration channel has a standard 6mm sleeve connector.

3.1..2.2 SUCTION PUMP

A technique of continuous mechanical suction with intermittent blowback was used. The pump has been especially designed for the gastric secretion tests (Sycopel Scientific Ltd) and generated suction pressures of up to 100mm of Hg. It has a safety valve that automatically cuts off the pump when the pressure rises above this level. The pump is pre-set so that it clears the tube of any blockage by blowing back at regular intervals (3 minutes) for a fixed period of 30 seconds; this blow-back pressure was adjusted not to exceed 160 mm of Hg. The nasogastric tube was connected to another tube that emptied into the collecting flask. The inlet was connected at the neck of the flask, the outlet was at the base on the opposite side. A side port was connected by fine tubing to the mercury manometers, allowing continual pressure readings. The outlet tubing was connected to a soft tubing which ran through the roller pump mechanism and so to the final collecting flask.

3.1.2.3 INFUSION PUMPS

These were used for the infusion of phenol red, histamine phosphate and promethazine hydrochloride (Phenergan - May & Baker Ltd). Three

pumps of same design (Model 352, Sage Instruments) were used throughout the test; they could be set for varying infusion rates. A standard 50 cc syringe (BD Plastipak) which achieved an infusion of 10.6ml h^{-1} was used in all the syringe pumps. The calibration of the pumps was checked at regular intervals.

3.1.2.4 TIMER

The gastric juice was collected in 10 minute aliquots. To ensure that the investigator received advanced warning that a collection period was ending and changed over between samples at the same time, a specially designed timer was used. This electrical device was connected to two coloured lamps and a buzzer, which were activated in a standard sequence.

3.1.3 PHENOL RED (Phenolsulphonphthalein-PSP)

The phenol red was made up as a stock solution in the following manner. Six grams of phenol red were dissolved in a litre of distilled water and allowed to settle for one month. This allowed the optical properties of the phenol red to stabilise (Whitfield and Hobsley 1979). At the start of each test 50 ml was drawn up into the syringe through a CVP manometer line (Portex Ltd) secured to the syringe by a Luer-lock. The whole assembly was freed of air bubbles and fitted into the syringe pump. The pump was run at the maximum speed until its arm was flush against the piston of the syringe. Then the pump was switched down to its normal rate (10.6ml h^{-1}) and allowed to run for several minutes until PSP appeared at the other end of the manometer tube. A 10-minute aliquot of PSP was collected into a standard 10 ml collecting flask. The infusion pump was stopped and was now ready for the start of the test. The flask was then

capped and labelled *Pre-test PSP standard* and with the name of the subject and the date of the test. Another 10 minute aliquot of PSP was collected at the end of the test and labelled *Post-test PSP standard.*

3.1.4 SUBJECT PREPARATION

The subject was requested to have nothing to eat or drink for at least 8 hours before the test; smoking and alcohol were proscribed for at least 12 hour before the test. Those subjects who were on an H2 antagonist (ranitidine or cimetidine) or proton-pump inhibitors were asked to stop taking these medications 72 hours before the test. The subjects was asked to come in to the Department at 0830 hours, and arrangements were made for someone to come and collect him/her after the test. The weight and height were measured, a full history was obtained including any previous operation, age, smoking habits along with the duration of smoking and number of cigarettes smoked per day, and these data were recorded on the standard form. The test was explained to the subject and consent was obtained. Only one gastric secretion test was performed on a single day.

3.1.5 PASSAGE OF THE NASOGASTRIC TUBE

The subject was asked to sit in an upright position, and local anaesthetic (lignocaine 1%) was sprayed on to the nose and naso-pharynx. A few minutes were allowed for the anaesthetic to take effect. Then the subject was allowed to swallow a small amount of water to moisten the throat and the nasogastric tube passed through the nostril. The subject was then was given small amount of water to drink and   the tube was then passed

further in while the subject was encouraged to swallow. Coughing suggested laryngeal irritation: tipping the head forward overcame this problem by directing the tube into the pharynx. Once the tube had passed into the oesophagus the subject was allowed to drink more water to facilitate the passage of the tube. Adjustments were made to the tube until aspiration was achieved. Aspiration of gastric contents confirmed that the tube was in the stomach. The tube was passed in up to its furthest mark: if resistance was met this implied that the tube had been passed in too far and was coiling upon itself. Once the tube was so positioned the stomach was emptied of the overnight secretion and swallowed water.

3.1.6 THE WATER RECOVERY TEST

When initial aspiration was complete and the stomach was assumed to be empty, the subject was allowed to drink 20 ml of water and aspiration was immediately attempted. If the water recovered was between 16-20ml then the tube was said to in the most dependent position (Hassan and Hobsley 1970). The tube was then withdrawn by 2.5 cm at a time and aspiration was repeated until aspiration was unsuccessful. Then the tube was advanced by 2.5 cm and secured by micropore to the nose of the subject. This was Hassan and Hobsley's "ideal position".

3.1.7 POSITION OF THE SUBJECT

Once the nasogastric tube was secured the subject lay in the semi-recumbent position, with the knees and hips flexed at 45°. The subjects were allowed to choose any variant of this position in which they felt

comfortable; Hassan and Hobsley (1970) showed that the exact position made no difference to recovery.

3.1.8 INTRAVENOUS ACCESS

A large bore cannula was used; the size was not only necessary for the intravenous infusion of histamine/promethazine hydrochloride infusion, but for venous access in case of emergency. A 16-gauge cannula was inserted under local anaesthesia into a large forearm vein or antecubital vein. This was connected by three-way tap to a slow infusion of 0.9% saline. The cannula was secured with micropore.

3.1.9 PREPARATION OF THE INTRAVENOUS INFUSION

Maximal stimulation was achieved with histamine acid phosphate infused intravenously at the rate of $13\mu mol\ kg^{-1}h^{-1}$ ($0.04mg\ kg^{-1}h^{-1}$). This dose used was based on previous work (Lawrie et al. 1964) on the infusion test. The period of maximal stimulation was intended for one and half hours; to avoid problems in case of delay a dose sufficient for two hours was prepared. The dose of histamine was calculated ($0.04\ mg\ kg^{-1}$ hour^{-1}) and this was made up to 21.1ml with 0.9% saline in the 50ml syringe. The dose of anti-histamine (H1 receptor) used was 25 mg promethazine (Phenergan) and this was also made up to 21.1ml with 0.9% saline in a 50 ml syringe. A separate CVP manometer line was attached to each syringe and a 25 G needle fixed to the other end. This was to provide connection to the distal part of the three-way tap. The syringes were labelled accordingly and placed in the double syringe pump and secured in position. The pump was run at the maximum speed until its arm was

flush against the piston of the syringe. The pump was then switched down to its normal rate (10.6ml h^{-1}) and allowed to run for several minutes until the fluid appeared at the other end of the manometer line. The pump was then turned off and the free ends of manometer tube were connected to the three-way tap, which was connected to the venous access.

3.1.10 GASTRIC SECRETION TEST

The gastric secretion included a basal period and a histamine period, each lasting for an hour and a half. After the pre-standard PSP was collected, the free end of the tube was connected to the narrower lumen of the nasogastric tube, which is meant for the instillation of PSP, and the infusion pump was started. The gastric juice collected during the first 20 minutes was discarded to overcome the possible error of loss through adsorption (Hobsley & Silen 1969) and to allow for thorough mixing of PSP with gastric juice.

3.1.10A COLLECTION OF GASTRIC JUICE SAMPLES

The samples were collected in consecutive 10-minute intervals as signalled by the timer. The volume of secretion was measured; if the volume was less than 10ml it was returned to the collecting flask and pooled together with the subsequent 10 minute-collection(s) until the volume exceeded 10 ml. This pooling procedure was permitted only within each group; i.e. either basal or maximal histamine period. The volumes of the sample were recorded in the data sheet and the sample was filtered (Whatman's No 1 paper) and at least 8ml of the filtered specimen was saved in an air-tight bottle. Every half an hour about 2ml of

unfiltered gastric secretion was saved in a polystyrene tube for analysis of epidermal growth factor. Each of these bottles was labelled carefully (name of the subject, date, and specimen number). The pump bearing the histamine and anti-histamine was switched on a minute before the end of the collection of the last basal period sample. At the end of the test all bottles were sealed and stored at 4^oC to wait for the biochemical analysis of gastric secretion, except that the gastric secretion samples for epidermal growth factor analysis were frozen immediately after collection at -20^oC. The PSP instillation tube was disconnected from the nasogastric tube and the post-test standard PSP was collected over the next 10 minutes.

3.1.10B COLLECTION OF SALIVA

During both the basal and maximal histamine periods, half hourly saliva samples were collected by expectoration, and transferred into bottles. Both the saliva sample and the contemporaneous gastric juice samples were frozen immediately for the analysis of epidermal growth factor (EGF) by radioimmunoassay at a later date.

3.1.11 CARE OF PATIENT AFTER THE TEST

The subject was given coffee and biscuits after the test and made to stay in the laboratory until the effect of phenergan (Promethazine hydrochloride) had worn off. Once I was satisfied that the effect of phenergan had worn off the subject was allowed to go home with a relative or friend and advised not to drive a car or operate machinery for 24 hours, allowing time for the antihistamine to be metabolised.

3.1.12 ANALYSIS OF GASTRIC SECRETION SAMPLES

Usually all samples were analysed within a few days. The samples were removed from the refrigerator and allowed to warm up to the room temperature. From the sample, chloride, potassium, hydrogen and sodium in ionic forms and PSP were analysed. Titratable acidity was measured for the acid output, sodium was measured for the calculation of duodenogastric reflux, and the other two ions in order to check that the sums of cations and anions balanced. If the anion/cation difference was more than 4 mmol l^{-1} then analysis of the sample was repeated

3.1.12A PSP CONCENTRATION

The concentration of PSP was measured using a spectrophotometer (Corning Spectrophotometer Model 256) and readings at 558nm and 410nm were taken. The filtered aspirate was diluted 1 in 200, and passed through tubing to a cell in the spectrophotometer. The diluent contained a trace of ammonium hydroxide, to make the sample alkaline to develop the colour (pink). The pre- and post-test PSP standards were diluted with distilled water to 10ml, and measured in the same way as the aspirate samples. The readings for both standards (pre and post) at both wavelengths (558 and 410) were averaged before calculating the final reading for the PSP standard.

3.1.12B CHLORIDE IONS

The concentration of chloride ions was measured using a chloride titrator CMT10 (Radiometer Copenhagen). Twenty microlitres (20μl) of the

aspirate was pipetted into a beaker of buffer, and titrated to electrical neutrality and a direct reading obtained in mmol l^{-1}.

3.1.12C SODIUM and POTASSIUM IONS

These concentrations were measured by flame photometry (FLM3 Radiometer Copenhagen), using a 1 in 200 dilution of the aspirate, the results being read directly in mmol l^{-1}. Since the same diluent (ammonium hydroxide) was used for PSP readings it was possible to semi-automate the readings of phenol red, sodium ions and potassium ions.

3.1.12D HYDROGEN IONS

Titratable acidity was measured with a pH meter and automatic burette (PHM82 Standard pH meter, TTT 80 Tritrator, Autoburette ABU 80, Radiometer Copenhagen), using 1ml of sample titrated against 0.1N NaOH to pH 7.0.

3.1.12E COMPUTER ANALYSIS

The patient's data and measured readings were fed into an IBM computer. The data were analysed using a specially written FORTRAN programme (Whitfield 1984). The results were stored on a hard disc and on floppy disc in duplicates. A hard copy was also printed.

3.1.13 CALCULATION FOR PYLORIC LOSS

The probable contaminants were blood and bile and they have small absorbance at 558 nm and peak at 410nm. Crawford and Hobsley (1968) investigated the effect of added blood and bile on the absorbance at 558 and 410nm of gastric juice samples containing phenol red. They found a linear regression of the form:

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$$PSP_{corr} = PSP_{558} - (0.135 \times PSP_{410}) + 0.004$$

Using this value the aspirated volumes were corrected for pyloric loss.
The absorbance reading of the PSP standard in the spectrophotometer, is
the value for the 10 minutes volume of infused PSP diluted to 10 ml
(PSP_{tot}).

The absorbance reading of the gastric sample (PSP_{asp}) is the two
hundredfold dilution in an unknown amount quantity of gastric juice.
Multiplication of PSP_{tot} by 20 gives PSP_{adj}, the PSP_{tot} expressed in the
same units as the sample reading.

If the total volume of the stomach content (V_{tot}) were known, the
adjusted standard reading (PSP_{adj}), when divided by that total volume
would give the sample reading PSP_{asp}, i.e.

$$PSP_{adj} / V_{tot} = PSP_{asp}$$

Rearranging, $V_{tot} = PSP_{adj} / PSP_{asp}$

Division of the adjusted volume by the sample reading gives the total
volume of gastric juice in the stomach during the collection period.
The total volume (V_{tot}) of gastric content i.e. the volume of stomach
contents in which the instilled phenol red has been diluted, was calculated
as follow:

$$V_{tot} = [PSP_{adj} / PSP_{asp}] \times V_{asp}$$

The volume of infused PSP per minute (1.7ml) was subtracted from V_{tot} to
give the corrected volume (V_{cor}), this being the volume of gastric secretion

corrected for incomplete aspiration and pyloric loss. The volume of aspirated juice (V_{obs}) was divided by V_{tot} to give the fractional recovery of gastric juice and hence of PSP. This same fraction of 1.7 ml was deducted from the aspirated volume (V_{obs}) and the difference between V_{cor} and V_{obs} was the pyloric loss.

3.1.14   CORRECTION FOR ELECTROLYTE CONCENTRATION

Electrolyte concentration had been reduced by the diluting effect of infused PSP. Correction for this effect was made by multiplying the observed concentration by the fraction V_{tot}/V_{obs}, which at low volume (<20ml/10 min) of the secretion can be appreciable.

3.1.15   CALCULATION FOR ACID OUTPUT

Hydrogen ion output was calculated from the product of the corrected hydrogen ion concentration (as titratable acidity) and V_{obs} multiplied by 0.006 to give the output in mmol h^{-1}.

V_{obs} is in mls/10 minutes

H^+ concentration is in mmol/L

in order to obtain $[H^+]$ / hour:

$$\frac{V_{obs}}{mls} \times \frac{[H^+ \ mmol]}{1000mls} \times 6$$

or H^+ mmol $\times$ 0.006

3.1.16 CALCULATION FOR DUODENOGASTRIC REFLUX

No correction has previously been possible for the swallowed saliva. The calculation of duodenogastric reflux relies on the disparity between the sodium concentrations in gastric and duodenal juice, and the analysis requires that the effect of the swallowed saliva is negligible.

a).   Vcor = VG + VR

Vcor= volume of gastric juice corrected for pyloric loss

VG =volume of primary gastric acid

VR= volume of duodenogastric acid

The concentration of sodium in the reflux was assumed to be the same as in the extracellular space, i.e. 143mmol l^{-1}. The electrolyte composition, all expressed in mmol l^{-1}, (Whitfield & Hobsley 1979) of primary gastric juice is as follows:

$[Cl^-] = 170$, $[H^+] = 145$, $[Na^+] = 7$, $[K^+] = 17$

The volume of duodenogastric reflux (VR) can be calculated by the following formula based on the sodium output:

b).   Vcor X $[Na^+]_{cor}$ = VG X $[Na^+]_{VG}$ + VR X $[Na^+]_{VR}$

The $[Na^+]_{cor}$ is the concentration of sodium in the aspirated juice, and the $[Na^+]_{VG}$ is the sodium in primary gastric juice, and the $[Na^+]_{VR}$ is assumed to be 143 mmol l^{-1}. Rearranging the equation b we get:

Vcor X $[Na^+]_{cor}$ = (Vcor -VR) X7+ VR X 143

or

$$VR = \{Vcor \times [Na^+]_{cor} -7]\}/143$$

Once VR was calculated , this was then subtracted from Vcor (volume of gastric juice corrected for pyloric loss) to give VG, which was volume of gastric juice corrected for pyloric loss and duodenogastric reflux.

3.1.17 SELECTION OF PLATEAUX

This was done by the FORTRAN computer programme (Whitfield and Hobsley 1979), selecting a plateau of maximal length using the latest possible samples in the maximal secretion period. The PSP recovery of each sample had to be within 15% of the mean of proposed plateau. No plateau could start from the first period nor could it last for less than 20 minutes. From the plateau the mean values of acid output, volume of gastric secretion, pyloric loss and duodenogastric reflux were calculated for each period.

METHOD 2

3.2 ^{13}C-Urea breath test

Each subject was starved for 12 hours before the test. Fully informed consent was obtained, after explaining the procedure and the reasons for the test. Age, sex, height, weight and history of smoking habits were recorded. Kits for the ^{13}C-urea breath test method for determining whether the patient was infected with *H pylori* were purchased from the Bureau of Stable Isotope Analysis (BSIA Ltd Brentford, Middlesex, England).

A ^{13}C-urea breath test kit consists of:

1. Test meal. A mixture of 50 mls Ensure and 50 mls Calogen. Calogen is a peanut oil emulsion, and Ensure is a liquid nutrient which consists of 16% proteins, 32% fat and 54% carbohydrate. The purpose of the test meal was to slow the emptying of stomach, so as to allow enough time for the urease enzyme to act on the ^{13}C-urea molecule.

2. ^{13}C-urea capsule.

3. Exentainer Tube (10ml polystyrene tube)

4. Sampling straw

5. Disposable pair of forceps

I followed the protocol suggested by BSIA for the ^{13}C-urea test.

Subjects were given 100 ml of test meal which consisted of 50 ml Ensure and 50 ml Calogen. Five minutes after the meal a pre-dose breath sample was collected in triplicate by asking the subject to blow into Exetainer tubes using an ordinary straw until condensation appeared in the tube. The straw was then removed and a screw cap applied immediately to the tube. Five minutes after the pre-dose sample, the 100 mg capsule of ^{13}C-urea was opened and the two halves of the capsule were dropped into 50 ml of tap water in a glass which was then gently swirled. The two half- capsules were then removed with forceps. The subject was asked to drink this ^{13}C-urea solution, and then to lie down for 2 minutes on each side to aid distribution within the stomach. Thirty minutes after ingesting the ^{13}C-urea solution, a post-dose breath sample was collected in the same way as the pre-dose breath samples. These tubes were labelled with the name and identification number of the subject and the date, mentioning whether these samples were pre- or post-dose. Two pairs each of samples pre- and post-dose were sent to BSIA (Bureau of Stable Isotope Analysis, Brentford, Middlesex, England) on the day of the test in the first class post and one set

of pre- and post-dose samples were kept as insurance against loss or damage. The submitted samples were analysed by BSIA by means of mass spectrometry. The results were posted to me by BSIA within a week in a sealed envelopes. All the results were gathered and the envelopes opened only after the completion of the urea breath test in all 100 subjects.

If the ^{13}C enrichment of the post-dose breath sample was more than 5% greater than the pre-dose sample, the result was considered positive; if less than 5%, the result was considered to be negative.

METHOD 3

3.4 Radioimmunoassay of EGF in Gastric juice and Saliva

Principle of radioimmunoassay:

Radioimmunoassay is a competitive immunoassay in which the antigen labelled with an isotope competes with the unlabelled antigen to bind to antibodies. It is well known that antigens react with (i.e. bind to) their corresponding antibodies.

$$[Ag] + [Ab] \longrightarrow [Ag\ Ab]$$
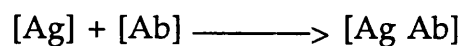
If by means of a radioactive isotope, the antigen taking part in such a reaction is labelled $[Ag^*]$, it can be subsequently identified when combined with its corresponding antibody.

$$[Ag^*] + [Ab] \longrightarrow [Ag^*Ab]$$
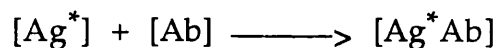
Furthermore, if unlabelled antigen is added to isotope-labelled antigen the two will compete for available antibody on equal terms, antigen for antigen. Should there be an excess of, say, labelled over the unlabelled, the labelled will stand a better chance binding to the antibody. One can estimate how much labelled antigen has managed to bind on to the antibody by separating bound from free and counting the β- or γ-emission (according to which particular isotope has been used).

Total count (TC) measures the total radio-activity of labelled antigens.
Non-Specific Binding NSB A sample is often prepared which contains no antibody that specifically binds the labelled antigen; instead the antigen will bind unspecifically to the walls of the tube. This sample can be used as a blank (NSB).
Maximum binding (Zero, or B0) A sample where there is no competition between labelled and unlabelled antigen. The concentration of unlabelled antigens is zero and the maximum amount of labelled antigen will be bound. This sample is less than total activity because normally there is an excess of antigen.

I purchased nine radio-immunoassay Kits from Amersham plc (Amersham, Buckinghamshire, England), and followed the protocol suggested by them for the assay. The radio-immunoassay was carried out at the Medical Research Laboratories of the School of Postgraduate Medicine at the University of Keele in Stoke-on-Trent. Most of the assays were performed by myself, but the first ten assays I did in the presence of Elizabeth Elderton, chief technician in that laboratory. The gastric juice and saliva samples were thawed slowly at room temperature for one hour before proceeding to radio-immunoassay.

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3.3.2 The EGF assay kit consists of:

1.   **Tracer:** Contains ~92kBq, 2.5μCi (3-[^{125}I] iodotyrosyl) epidermal growth factor (human recombinant) (freeze dried or lyophilized).

2.   **Antiserum:** This was raised in rabbit against EGF. This antibody is specific to human EGF (100% crossreactivity), but it also cross-reacts to mouse EGF (88% crossreactivity); it did not cross-react with any other human peptide such as gastrin or pepsin.

3.   **Buffer for Amerlex-M separation:** This contained phosphate buffer concentrate (pH 7.2) for Amerlex-M separation (Amerlex-M is the generic name of the product).

4.   **Amerlex-M:** It contained 220ml of Amerlex-M donkey anti-rabbit reagent code RPN510.

3.3.3 PROTOCOL OF RADIOIMMUNOASSAY
(As suggested by Amersham)

3.3.3. 1. PREPARATION OF REAGENTS

A.   Tracer: The contents of the tracer vial were transferred with washing to a container with 25ml of assay buffer.

B.   Antiserum: The contents of the antiserum vial were dissolved in 2.0ml of the assay buffer and transferred with washing into a container with a further 48ml of buffer added into it and mixed well.

C.   Assay buffer: The Amerlex-M assay buffer concentrate was diluted and used in all operations. The bottle containing Amerlex-M buffer concentrate was heated up to 40° C until the gel-like material melted (care

was taken not to exceed 60° C). The contents of the bottle with the washing were transferred to a 250ml measuring cylinder, diluted to 250ml with distilled water, and mixed well.

3.3.3.2. SEPARATION REAGENT

Amerlex-M antibody was ready for use after mixing it well.

3.3.3.3. PREPARATION OF STANDARDS

EGF (human) was prepared from a stock concentration of 1000ng/ml, and a 0.1ml aliquot of stock solution was taken. This 0.1ml aliquot containing 1000ng/ml of EGF was made up to 1.0ml with assay buffer prior to use, giving a solution with a concentration of 10ng hEGF/100μl which was used as the top standard. In order to obtain a suitable curve (spline curve) these standards were serially diluted within the range 0.08-10ng/100μl.

3.3.3.4. PREPARATION OF STANDARD CURVES

i). Polystyrene tubes were labelled in duplicates for total count (TC), non-specific binding (NSB), zero binding (BO), standards in the range of 0.08, 0.16, 0.312, 0.625, 1.25. 2.5, 5.0 and 10.0ng/100 μl, and for the unknown samples, which were saliva and gastric juice samples both in basal and histamine periods.

ii). Buffer, standard, unknowns, and tracer were added to these tubes in the amounts shown in the Table 3.1 (p.121); all volumes are in μl (microlitre).

iii). All tubes were vortex mixed, and incubated for three hours at room temperature (15-30°C).

Table 3.1

Arrangement of tubes for EGF radioimmunoassay

Tube	TC	NSB	BO	Standards	Unknown
Buffer	-	300	100	-	-
Standard	-	-	-	100	-
unknown	-	-	-	-	100
Antiserum	-	-	200	200	200
Tracer	100	100	100	100	100

3.3.3.5. PHASE OF SEPARATION

The bottle containing Amerlex-M second antibody reagent was gently shaken to ensure a homogeneous suspension. Then 500μl of Amerlex-M was added to all tubes except TC, which was stoppered and set aside. All the tubes were vortex mixed and incubated for 10 minutes at room temperature. To separate the antibody-bound fraction all these tubes except TC were centrifuged for 10 minutes at 1500g (MSE Coolspin, Fisons Instruments, Crawley, Sussex, England). After centrifugation these tubes were placed on a decantation rack, and the supernatant liquid was poured off by placing these tubes on a pad of absorbent paper, keeping the tubes inverted for 5 minutes to allow them to drain. After 5 minutes the rims of these tubes were blotted, and their radioactivities determined by using gamma scintillation (Clinigamma 1272-002, LKB- Wallace, Milton Keynes, England). This gamma counter was linked with a BBC computer which had a spline-function programme and results were analysed using a spline interpolation curve: these results were printed out.

3.3.3.6 SPLINE INTERPOLATION CURVE

The spline-function interpolation standard curve is constructed from curved segments that join togather to form a smooth, complete curve through standard points. Only one assumption is needed in the spline-function construction of the standard curve: apart from being a smooth continuous curve, the curve should be|straightest curve consistent with passing through the points; i.e., there should be no oscillation in the response curve. The spline-function construction is like a mathematical "flexible ruler"; instead of a flexible ruler, a computer is used to construct a

smooth curve. Once function is computed, it is possible to calculate the concentration of unknown with great precision (Rawlins & Yrjönen 1978).

In preparing the spline-function programme for routine on-line use with the LKB–Wallace Rack-gamma, especial attention has been paid to producing a programme that functions not only automatically without the requirement of skilled human intervention, but also reliably and reproducibly to produce consistent results. In contrast to most "one-equation functions" for the whole curve, the spline function curve tries to find the ideal equation for each part of the curve. The different segments are then coupled to each other in a smooth and continuous way (Rawlins & Yrjönen 1978). It uses the difference between sample-replicates as limits inside which the curve must fit, and uses smoothing to remove the effect of large differences between sample-replicates.

METHOD 4

3.4. Method of ammonia and urea assays in gastric juice

3.4.1. Principle:

Helicobacter pylori possesses a very high urease activity, which splits urea into ammonia and carbon dioxide, resulting in lowering of the urea and increasing the ammonia concentration in the gastric juice in infected people.

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + NH_3 + CO_2$$
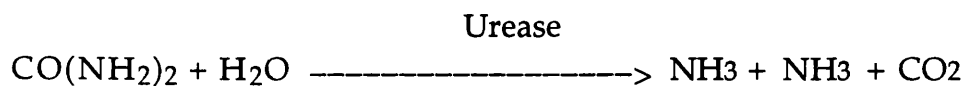
The gastric juice samples were collected from 60 subjects, and were measured for urea and ammonia concentration.

I purchased all the reagents for the ammonia and urea assay from SIGMA (Sigma Diagnostics, Poole, Dorset, UK). These assays were carried out in the Department of Microbiology, University College London Medical School, and I followed the protocol for the analysis as suggested by SIGMA.

3.4.2 The ammonia assay kit consist of:

1. Ammonia control solution. This contains an ammonium sulphate solution, with an ammonia concentration of 2mg/ml or 118mmol/L.

2. Ammonia assay solution, used as specified by the manufacturer, which contains 2-oxoglutarate 3.4mmol/L and dihydronicotinamide adenine dinucleotide phosphate (NADPH) 0.23mmol/L.

3. L-Glutamate dehydrogenase solution (1200 U/ml in 50% glycerol with a phosphate buffer to pH 7.4).

3.4.3 The urea assay consists of:

1. BUN acid reagent, which contains ferric chloride in phosphoric and sulphuric acid.

2. BUN colour reagent, which contains diacetyl monoxime, 0.18% [w/v] and thiosemicarbazide.

3. Urea Nitrogen Standard Solution, which contains urea at 30 mg/dl with benzoic acid as preservative. Catalog No: 535-30

4. Urea Nitrogen Standard Solution which contains urea at 150mg/dl with benzoic acid as preservative. Catalog No: 535-150.

3.4.5 Principle of ammonia assay

A mixture of oxoglutarate and dihydronicotinamide adenine dinucleotide phosphate (NADPH) is added to the unknown solution. If the latter contains any ammonia, a reaction occurs in which NADPH is consumed. However, this reaction only occurs when the enzyme, glutamate dehydrogenase (GLDH) is added. During this reaction the NADPH gives up hydrogen ions to become NADP; these hydrogen ions are taken up by 2-oxoglutarate which is converted into glutamate. Due to the disappearance of NADPH, there is a decrease in the absorbance of light at 340 nm wave length detected by a spectrophotometer; this decrease is proportional to the ammonia concentration in the test solution. The absorptivity of NADPH is 6.22 at 340 nm.

$$\text{2-oxoglutarate} + NH_3 + NADPH \xrightarrow{\text{GLDH}} \text{Glutamate} + NADP$$
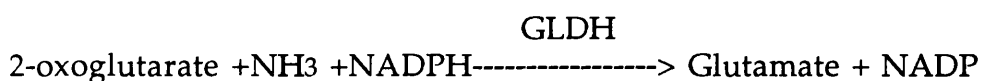
3.4.6 Protocol for ammonia assay

The cuvettes were set for BLANK, Control and for the gastric juice samples and the volumes were added as shown in the Table 3.2 (p.126). All these cuvettes were covered with parafilm, and mixed by gently inverting them. The cuvettes were allowed to equilibrate for 3 minutes at room temperature. The INITIAL absorbance of each cuvette was read at 340nm on the spectrophotometer (Ultraphane II, LKB, Milton Keynes, UK). Ten microlitres (10μl) of glutamine dehydrogenase was added to every cuvette. Each cuvette was gently mixed and after 5 minutes the FINAL absorbance was read at 340nm on the spectrophotometer (Ultraphane II, LKB, Milton Keynes, UK).

Table 3.2

Arragement of cuvettes for ammonia assay

Type of cuvette	NH3 control	NH3 assay	Water	Gastric juice
BLANK	-	1ml	0.1ml	-
Control	0.1ml	1ml	-	-
Test	-	1ml	-	0.1ml

3.4.6 Calculation of ammonia concentration

A = absorbance of light

ΔA = INITIAL Absorbance - FINAL Absorbance

[NH3] = (ΔA test - ΔA blank) x 30.3

[NH3] = concentration of ammonia in mg/ml

Control ammonia concentration = (ΔA Control- ΔA Blank) x30.3

30.3 is constant factor which was obtained by :

1mmol of NH3 weighs 17mg

1mmol of NADPH has absorptivity of 6.22 millimolar

1mmol NH3 reduces absorption by 6.22 millimolar

6.22 millimolar absorption = 1mmol NH3

1 millimolar absorption =   1/6.22 mmol NH3

If this was contained in 0.1ml gastric juice, then millimolar absorption in 0.1ml of gastric secretion would be:

$$\frac{1}{0.1} \quad X \quad \frac{1 \text{ mmol NH3}}{6.22}$$

But this was obtained after diluting 0.1 ml of gastric juice to 1.11 ml [1ml ammonia assay solution + 0.1 ml gastric juice + 10µl (0.01ml) of glutamine dehydrogenase].

$$\frac{1}{0.1} \quad x \quad \frac{1 \text{mmol of NH3}}{6.22} \quad x \; 1.11$$

1mmol of NH$_3$ weighs 17mg, and therefore the equation can be written as;

$$= \frac{1.11 \times 17}{0.1 \times 6.22}$$

which is equal to 30.3

The concentration of NH3 is calculated as follows:

if the INITIAL absorption of light by gastric juice sample = 1.540

the INITIAL absorption of light by BLANK = 1.440

the FINAL absorption of light by gastric juice sample = 1.290

the FINAL absorption of light by BLANK = 1.400

Difference of absorption for light by gastric juice sample would be:

ΔA= 1.540 - 1.290 = 0.250

The difference of absorption of light by BLANK would be:

ΔA =1.440 - 1.400

= 0.040

Therefore the concentration of ammonia in the gastric juice samples would be:

(ΔA of gastric juice - ΔA of BLANK) x 30.3

(0.250 - 0.040) x 30.3

= 6.36 is concentration of ammonia in mg per milliltre (ml)

1mg/ml of ammonia is equal to 58.8mmol/L.

Therefore the concentration of ammonia in the gastric juice sample would be:

6.36 x 58.8 = 374mmol/L

3.4.7 Principle of urea assay

The principle of this test is the production of a chromogen (colour) by the reaction of colourless urea with colourless diacetyl monoxime. The urea concentration is directly proportional to the intensity of the colour produced, which is measured spectrophotometrically between 515-540 nm.

Diacetyl Monoxime + Urea ———→ Pink chromogen + Hydroxylamine
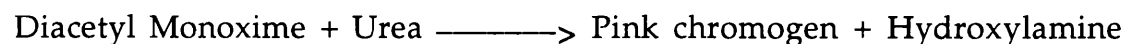
3.4.8 Protocol for urea assay

1. A standard curve was prepared by serial dilution of the urea control solution (150mg/dl).

2. Cuvettes were labelled for Blank, Standard and Specimens, the latter being case samples of gastric juice.

3. To each cuvette 3ml of BUN acid reagent and 2ml BUN colour reagent were added. Each cuvette was thoroughly mixed.

4. To the Standard 20 μl of the Urea Nitrogen standard solution 30mg/dl was added. To the specimen cuvettes 20 μl of gastric juice were added. All cuvettes were mixed thoroughly. No addition was made to the Blank (which had 3ml of BUN acid and 2ml of BUN colour reagent).

5. All cuvettes were simultaneously placed in a boiling water bath for exactly 10 minutes.

6. All cuvettes were quickly removed and placed in cold tap water for 3-5 minutes.

7. Absorbance of light at 535nm was measured with a spectrophotometer and the Standard and Specimen (gastric juice) samples were compared with the BLANK.

3.4.9 Preparation of Standard curve for urea

i). Five cuvettes were labelled from 1-5 for standards. The volumes of Urea Nitrogen standard solution (150mg/dl) and water are shown in Table 3.3 (p.131), to keep the total volume after adding water at 5ml.

ii). Another five cuvettes were labelled from 1-5, and a 6th tube for BLANK. To each of these tubes 3 ml of BUN acid reagent and 2ml of BUN colour reagent were added; these tubes were mixed thoroughly.

iii). 0.02ml of each of the diluted standards was pippetted and added to the tube marked appropriately as shown in Table 3.4 (p.131).

iv). Simultaneously all cuvettes were placed in boiling water for exactly 10 minutes.

v). All cuvettes were than removed quickly and placed in cold tap water for 3-5 minutes.

vi). The absorbance of cuvettes 1-5 was compared with the Blank.

3.4.10 Determination of *H pylori* status.

Once the concentration of ammonia and urea has been determined in the gastric juice, the ratio of $(ammonia)^3$/urea was calculated.

$CO (NH2)_2 + H2O <===========> CO2 + NH3 + NH3$
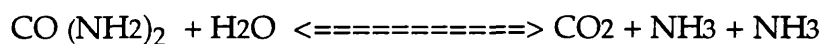
This reaction occurs in presence of urease enzyme.

By the Law of Mass Action,

$$\frac{[CO2] + [NH3]^2}{[CO (NH2)_2]} = K, \text{ (the equilibrium constant)}$$

When equilibrium is reached, the rate of the forward reaction i.e. the formation of CO2 and NH3 is equal to the rate of the backward reaction, i.e. formation of urea $(CO (NH2)_2)$, and the ratio remains constant. If there is urease enzyme present this equilibrium is reached more rapidly than in its absence, resulting in decrease in the urea concentration and an increase in the production of CO2 and NH3. As CO2 was not measured, I have used $[NH3]^3$ /$[CO (NH2)_2]$ instead. My results in combination with the results of others (Neithercut et al 1991, 1993, Kim et al 1989) suggested 1.25 as a cut off point; those who had ammonia[3]: urea ratios more than 1.25, were considered positive for *H pylori* infection.

Table 3.3

Preparation of standard curve for urea (stage 1)

Diluted standard Number	Volume of Urea nitrogen standard solution (150mg/dl) in mls	Volume of water in mls
1	0.5	4.5
2	1.0	4.0
3	1.5	3.5
4	2.0	3.0
5	2.5	2.5

Table 3.4

Preparation of standard curve for urea (stage 2)

Tube	Addition [0.02ml]	Urea N [mg/dl]
Blank	Water	0
1	Diluted std. No.1	15
2	Diluted std.No 2	30
3	Diluted std. No 3	45
4	Diluted std. No 4	60
5	Diluted std. No 5	75

METHOD 5

3.5 Statistical analysis

All the statistical tests used in the preparation of this thesis were performed using the Statview 4.02 (Non-FPU) software programme (Abacus Concepts, Inc., Berkeley, CA, 1992).

3.5.1. Chi-squared test (χ^2) for contingency tables

Contingency table analysis explores whether the proportional incidence of a particular quality in one set of observations is statistically significantly different from the incidence of that quality in a different set of observations. The presence of the quality defines one row of the table and its absence a second row. Each set of observations defines a column. In the simplest case (and the only situation in which this test is used in this thesis) the qualities considered are only the presence or absence of *H pylori*, and there are only two populations. This gives rise to a 2 x 2 table (two-by-two table), i.e. a table with two rows (presence of infection with *H pylori*, absence of such of infection) and the columns (e.g. DU, controls).

The chi-squared (χ^2) works by calculating expected frequencies. The null hypothesis is that there is no association between the incidences, the alternative being an association of some kind. For each cell the frequency we would expect if the null hypothesis were true is calculated. The chi-squared test is not valid when the minimum expected values is less than five. This test is used to determine quantitatively whether a set of frequencies follows a particular distribution; i.e. do observed frequencies

differ significantly from those expected on the basis of some specified theory or hypothesis.

Example:

	Group A	Group B	Total
No. of *Hp* positive	163	119	282
No. of *Hp* negative	260	289	549
Total	423	408	831

According to the null hypothesis the proportion of *Hp* positive in group A is the same as proportion of *Hp* positive in group B. In that case, the best estimate of the proportion *Hp* positive is that derived from both groups A and B together as:

$$282/831 = 0.3394$$

Therefore the expected number of *Hp* positive in group A would be:

$$423 \times 0.3394 = 143.55$$

and number of *Hp* positive in group B would be:

$$408 \times 0.3394 = 138.48$$

It is therefore possible now to construct a table of expected values, similar to that given above for the observed values.

	Group A	Group B	Total
No of *Hp* positive	143.5	138.5	282
No of *Hp* negative	279.5	269.5	549.0
Total	423	408.0	831.0

χ^2 is then calculated by the formula:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

"O" = observed value and
"E" = the expected value

$$\frac{(163 - 143.5)^2}{143.5} + \frac{(119 - 138.5)^2}{138.5} + \frac{(260 - 279.5)^2}{279.} + \frac{(289 - 269.5)^2}{269.5}$$

= 8.17 on 1 degree freedom (df)

The p value is obtained by looking up the χ^2 value under the appropriate degree of freedom in the χ^2 distribution table. The degree of freedom (df) is derived by:

$$df = (No\ of\ Rows\ -1)\ X\ (\ No\ of\ Column\ -1)$$

therefore   df= (2-1) X (2-1) = 1

Chi-squared test is only valid for comparing frequencies; it will not work on percentages. I have used chi-squared test with Yates's continuity correction.

$$\chi^2 = / \sum \frac{(O - E - 1/2)^2}{E}$$

"O" = observed value and
"E" = the expected value

This correction was shown by Yates to be important if there was only 1 degree of freedom, i.e. the table was of the 2 x 2 form.

3.5.2. Fisher's exact test

If any expected value in a two-by-two table is less than 5, the p value given by the χ^2 is not strictly valid. In this case, an exact test is available called Fisher's exact test. Unlike χ^2 this test involves the calculation of the p value directly, without the use of a particular test statistic. Fisher's exact test is calculated by enumerating all possible rearrangements to the sets of observations and comparing the number of possible rearrangements to the observed counts under the assumption of no association between two variables.

Example.

Fisher's exact test works like this; suppose we carry out a clinical trial and randomly allocate 4 patients to treatment A, and 4 to treatment B. The outcome is as follows:

	Survived	Died	Total
Treatment A	3	1	4
Treatment B	2	2	4
Total	5	3	8

We want to know whether the difference in mortality between two treatments is due to chance or is evidence of a difference between the treatments. If we keep the row and column totals constant, there are only 4 possible tables:

(i)

	S	D	T
A	4	0	4
B	1	3	4
T	5	3	8

(ii)

	S	D	T
A	3	1	4
B	2	2	4
T	5	3	8

(iii)

	S	D	T
A	2	2	4
B	3	1	4
T	5	3	8

(iv)

	S	D	T
A	1	3	4
B	4	0	4
T	5	3	8

S = Survived   D = Died   T = Total

Now suppose we labelled these patients from a to h, the survivors we shall call a to e, and the deaths f, g, h, tables (i & iv) can be arranged in 5 different forms, tables (ii & iii) can be arranged in 30 different ways. Therefore there are 70 ways of arranging these 8 patients in two groups, each with 4 patients, 5+30+30+5=70. Now, the probability of any one arrangement arising by chance is 1/70, since they are equally likely. If there are 3 deaths, table (i) arises from 5 out of 70 arrangements so the probability

5/70 = 0.071, with table (ii) arrangement has probability of 30/70 = 0.429.

Similarly table (iii) has probability 30/70 = 0.429 and table (iv) has

probability 5/70 = 0.071 (Bland Martin 1987).

Table (ii) has the same arrangement as what was observed and it has a

probability of 0.429, which could easily have arisen by chance and is

consistent with the null hypothesis.

Now we calculate the probability in a more extreme table like i which has a

probability 0.071, where all deaths occur in one group so the probability of

the observed table or a more extreme one is 0.071+ 0.429 = 0.5

This is the method of Fisher's exact test: we calculate the probability of

each possible table , we then find the probability of the observed or more

extreme tables arising by chance and if this total probability is small (say

less than 0.05) the data are not consistent with the null hypothesis and we

can conclude that there is evidence that association does exists.

3.5.3 Type 1, 2 errors, power of test and sample size formula

If the null hypothesis is <u>true</u> and a <u>non-significant</u> result is obtained

everything is fine and a correct decision is made. If, however, the null

hypothesis is <u>true</u> and <u>a significant</u> result is obtained, the decision to reject

the hypothesis is incorrect and error has been made. This type of error is

called type 1 error or an alpha error (α). The probability of making a **type 1**

error, is by definition the probability of rejecting the null hypothesis when

it is in fact true. The p value for any result can be alternatively interpreted

as the chance of making a type 1 error.

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If the null hypothesis is _false,_ a statistically significant result leads to a correct decision. If, however, in this situation a non-significant result is obtained, a decision error has been made, and this is called **a type 2** or beta (β) error. For non-significant results, it is necessary to calculate the probability of making this error (Daly et al 1991).

Power of the test (1-β): the power of a test increases as the difference between the hypothesised value of the mean and the real value increases, and a high power means a low chance of type 2 error.

Sample size formula:

The size of sample required for comparison of proportions in two independent groups is calculated by the formula given below.

$$n > \frac{K(p_1 q_1 + p_2 q_2)}{\Delta^2}$$

In this equation n is the number of individuals required in each group, p_1 and p_2 are the presumed proportions in the two groups being compared, $q_1 = 1-p_1$, and $q_2 = 1-p_2$, Δ is the difference to be detected which, of course, is $p_1 - p_2$ (Daly et al 1991).

Example:

The prevalence of _H pylori_ was detemined in 14 controls and 13 DU subjects. There were 4 controls and 7 DU who had _H pylori_ infection. On Fisher's exact test there is no difference in the prevalence of _H pylori_ in controls and in DU subjects, but one cannot rule out the possibility of type 2 errors in this case. The possibility of finding a type 2 error is calculated as given below:

	Controls	DU
$Hp+ve$	4	7
$Hp-ve$	6	6

therefore $p_1 = 4/10 = 0.4$ $p_2 = 7/13 = 0.54$

$q_1 = 1-p_1 = 1-0.4 = 0.6$ $q_2 = 1-p_2 = 1-0.54 = 0.46$

$\Delta = 0.54-0.40 = 0.14$ (subtracting smaller value of proportion from the larger value). If these values are filled in the above equation:

$$n = K \frac{(0.4 \times 0.6) + (0.54 \times 0.46)]}{(0.14)^2}$$

The value of K is obtained from the appropriate table, it is 8.6, at the significance level of 0.05 if the probability of a type 2 error is 0.1 or 10% (Daly et al 1991).

$$n = \frac{8.6 (0.24 + 0.25)}{0.02} = 210$$

therefore one needs 210 subjects in each group before one reaches the level of significance. With the prevalence values given above for controls and DU, if one claims that there is no difference in the prevalence of *H pylori* in controls and DU, one may be making a type 2 error.

3.5.4. Mann-Whitney 'U' test

This is a non-parametric test, i.e one that is independent of the magnitudes of the observations, dependent only on their rank. It is equivalent to the two-sample "t" test (parametric test). The advantage of this test over the "t" test is that it relies only on the distribution of the data,

the observations being ranked, whereas for the t test we assume the data are from a population of normal distribution with uniform variances in the two groups studied. The normal distribution curve (Gaussian) is bell shaped, it is symmetrical about the mean, 95% of all observation will lie within the range -2 standard devations to +2 standard devations from the mean. There are two disadvantages of Mann-Whitney test: firstly, for those data which are normally distributed this test is less powerful than t test i.e. the t test ,when valid, can detect smaller differences for the given sample size; and secondly, this test gives no idea of the size of the difference. This test is similar in principle to the Wilcoxon rank sum test.

Non-parametric tests employ the sequential ranking of observation from all groups or variables of interest, or compare two groups observation by observation, in order to test the null hypothesis. The Mann-Whitney U test explores the hypothesis that the distributions of ranks of the two groups are the same.

The test is carried out as follows: first combine the two groups in a single array arranged in order of magnitude, but in such a way that two groups can still be identified, now rank the observations in the first group, and those in the second group, with the smallest observation given rank 1; tied values within same population receive successive rank numbers, but tied values across the two populations receive the mean of the two rank numbers at the position concerned. The rank numbers in one group are summed (see example below).

Example:

Systolic blood pressure was measured in 9 patients with diabetes mellitus and 8 controls patients of similar age; the diabetic patients have systolic blood pressures of 114,120,120,128,130,135,138,140 and 141. The controls have blood pressures of 110,112,112,118,120,122,125 and 130.

Blood pressure	Rank	Ranks for controls adjusted	Ranks for diabetics adjusted
110	1	1	
112	2	2	
112	3	3	
114	4		4
118	5	5	
120	6	7	
120	7		7
120	8		7
122	9	9	
125	10	10	
128	11		11
130	12	12.5	
130	13		12.5
135	14		14
138	15		15
140	16		16
141	17		17

Blood pressure readings in bold are from the control group whereas the remaining are from the diabetic group. In the Mann-Whitney test the rank numbers in each group are added. For the control group the total of the ranks equals 49.5, whereas for the diabetic group it is 103.5. If the two populations are similar in blood pressure then it would be expected that the sums of the ranks in each group would be close to each other. If the distribution is different it would be expected that the group with the lower median would have a lower sum of the ranks. There were 8 observations in the control group (n_1) and 9 observations in the diabetic group (n_2).

The Mann-Whitney test statistic is calculated from the sum of the ranks in one of the groups (chosen arbitrarily), as shown in the equation given below:

$$U = (n_1 + n_2) + n_1(n_1 + 1)/2 - R_1$$

U = the Mann-Whitney test statistic

n_1 = the number of observation in group 1 (control)

n_2 = the number of observation in group 2 (diabetic group)

R_1 = is the sum of the ranks in the group 1

If we put the values of above example in this equation:

$U = (8 \times 9) + 8(8+1)/2 - 49.5$

$U = 72 + 36 - 49.5 = 58.5$

The choice of which group of dependent variable values is defined is arbitrary, and the value of the Mann-Whitney test statistic will be different, depending on the choice. To solve this problem, one calculates the Mann-Whitney U by choosing either group as group 1 and then determining what the Mann-Whitney U value would have been if the choice had been different (alternative value is called as U'). To determine what the Mann-Whitney U value would have been if one had selected the group to be called 1 differently:

$U' = (n_1 \times n_2) - U$

U' = the value of the Mann-Whitney test statistic if the other group had been chosen as group.

$U' = (8 \times 9) - 58.5 = 13.5$

For the two-tailed alternative hypothesis, the appropriate test statistic to use in Mann-Whitney test is the larger of U and U', since 58.5 is larger then 13.5; this is used for finding the significance (0.05) from the appropriate table for the critical values of Mann-Whitney U test (Hirsch & Riegelman 1991).

3.5.5. Spearman's rank correlation

This is a non-parametric measure of the degree of association between numerical variables for a given set of individual subjects. This indicates the proportion of the variation in the dependent variable (y) that is shared with independent variable (x). The values of both dependent and independent are ranked, that is to say rank numbers are assigned to values of the dependent variable, and a separate set of ranks are assigned to the values of the independent variable. The difference between the ranks of the two variables (d_i) is calculated for each subject at each point of the data-pairs. The Spearman's rank correlation is then calculated by the following formula:

$$r_s = 1 - \frac{6 \times \Sigma d_i^2}{n^3 - n}$$

r_s = sample estimate of Spearman's correlation coefficient
n= number of matched pairs of dependent and independent variables
d_i = is difference between the ranks of the values of the independent and dependent variable for individual data pair.

Example: Eight patients had treatment for hypercholesteraemia for one month. Serum cholesterol was measured before and after the treatment.

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Patient	Before	Rank$_1$	After	Rank$_2$	d_i	d_i^2
1	281	6	269	5	1	1
2	259	2	245	2	0	0
3	272	4	271	6	-2	4
4	264	3	248	3	0	0
5	253	1	229	1	0	0
6	297	7	276	7	0	0
7	312	8	289	8	0	0
8	275	5	260	4	1	1

Before= Cholesterol level before the treatment

After= Cholesterol level after the treatment

d_i= difference of ranks

d_i^2= Square of the difference of ranks

Σd_i^2= sum of the square of the ranks

Rank$_1$= the ranks for the variables before the treatment

Rank$_2$= the ranks for the variables after the treatment

Now if we put these value in above equation to find the Spearman's correlation:

$$r_s = 1 - \frac{6 \times 6}{8^3 - 8}$$

$$r_s = 0.9286$$

Spearman's correlation coefficient (r_s) ranges in value from -1, indicating a perfect negative relationship, through 0, indicating no correlation between the variables, to +1, indicating a perfect direct relationship (Hirsch & Riegelman 1991)

3.5.6. Wilcoxon signed rank test

The Wilcoxon signed rank test is appropriate in the same cases that a paired t test would be used, but is a non-parametric version of the t test.

Thus it is applicable in cases where the t-test in not appropriate, i.e. where the distribution of the data is unknown or is known not to be normal. This test is based on the differences between each pair of observations in the dataset and tests the hypothesis that sum of the rank numbers of the ranked differences is equal to zero under the assumption that the distribution of the ranks is symmetrical (Statview manual reference).

This test first calculates the difference between the values for each pair, then it ranks these differences from smallest to the largest ignoring the sign of the difference (- or +) during ranking; once the ranking has been calculated the sign of the difference is given back to each rank, add up all positive (+), call this T+, its significance is then looked up in the appropriate table.

3.5.7. Regression Analysis

This is used to verify the linearity of the relationship between two variables; one of these variables is dependent (y) and the other one independent (x). These variables are quantitative (example, 100, 145, 180 etc.) rather than qualitative (example, positive and negatives, male and female, smokers and non-smokers etc.). The strength of the association is described as the correlation coefficient and denoted by "r". If the relationship between the two variable is linear i.e. a graph plotting the points of the paired observations on axes representing x and y is a straight line, r is called the coefficient of linear correlation or Pearson's product moment correlation.

The value of r varies between +1 and -1, the sign of r depends on whether there is a direct or negative relationship between the two variables. If the relationship between the two variables is perfect or exact, that is if all the points on the scatterogram lie on the regression line, r will be equal to +1 or -1 (Daly 1991). If there is no relationship at all between the two variables, r will be 0. The greater the numerical value of r, the stronger the relationship between two variables. The value of r^2 describes the proportion of the value of y that can be predicted from the corresponding value of x.

3.5.8. Descriptive Analysis

MEAN: The mean is the sum of the observations divided by the number of observations. Each observation plays a part in the calculation of the mean, there could be some difficulties which can arise if there are some outliers in the data which could distort the mean; these outliers can be discarded or corrected if they arise from obvious errors in the data.

MEDIAN: The median is the middle value in a set of observations that has been ordered from lowest to highest value. When there are an even number of observations, the median is the average of the two numbers on the either side of the middle.

STANDARD DEVIATION: The standard deviation measures the average divergence of all observations in a set of data from the mean.
The divergence of any one observation from the mean= x-m, where m is the mean and x is the observation.
The sum of all divergences from the mean = Σ (x- m)

Example 3,4,5,6,7 are the observations, therefore the mean of these observations is 5

Observation	x-m
3	-2
4	-1
5	0
6	1
7	2

In practice S (x- m) will be zero, in order to get around to this problem, the standard deviation is calculated by using the square of the individual divergences from the mean i.e. Σ (x- m)^2.

Observation	x-m	(x- m)^2
3	-2	4
4	-1	1
5	0	0
6	1	1
7	2	4
25	0	10

So the average squared divergence, Σ (x- m)^2/n, where n is the number of observations which in this case was 5, will be 10/5=2, this is called the variance; and the square root of this variance is called the standard deviation.

$\sqrt{2}$= 1.414, this is standard deviation of the mean which was 5 in this example.

Data from a normal (Gaussian or bell-shaped) distribution follows the empirical rule of statistics: 68% of the data are contained in the range of the mean plus or minus the standard deviation; 95% in the range of mean plus or minus twice the standard deviation; 99.7% in the range of the mean plus or minus three times the standard deviation. Thus a quick rule

of thumb for normally distributed data is that the vast majority of

observations (95%) fall within two standard deviations of the mean.

CHAPTER FOUR

RESULTS

RESULTS 1

4.1 Epidermal growth factor data (EGF)

The detailed information about subjects regarding age, sex, and smoking habits are given in table 4.1 (p.168).

The data comprise salivary EGF concentrations and gastric juice EGF concentrations measured in three groups, controls (those subjects without any symptoms), DU (those with duodenal ulcer) and NUD (those subjects with symptoms of dyspepsia but negative findings on oesophago-gastro-duodenoscopy), and during two situations, basal and during maximal stimulation with histamine. During the infusion of histamine, all the patients complained of a dry mouth and had difficulty in providing a sample of saliva.

The mean EGF concentrations in the three groups in basal and histamine periods are given in table 4.2 (p169); also given in the table (in parentheses) are the standard deviations of the mean concentrations. It is notable that the standard deviations are of much the same magnitude as the corresponding means. This implies that the distributions are not normal and so non-parametric statistics have been used to test significance. Superscript symbols are used to indicate a significant difference.

The results are described under ten headings:

1. Basal period. There was no significant difference in EGF concentration between control and NUD groups in either saliva or gastric juice. Salivary EGF in DU exceeded the EGF concentration in NUD ($p < 0.05$) but was not

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different from controls (p=0.313). The gastric EGF concentration in the DU group was not different from the gastric EGF concentrations in the control and NUD groups (see Table 4.2, p169).

2. Histamine period. After histamine stimulation commenced there was an increase in EGF concentration in all three groups, both in saliva and in gastric juice.

In saliva the effect was approximately to double the concentration, but the levels reached in the three diagnostic groups have the same pattern as that observed in the basal period. There were no differences between control and NUD (p=0.074) or between DU and control (p=0.069), but there was a highly significant difference between the DU and the NUD groups (p=0.0002), the concentration in DU being higher than in the control group.

In gastric juice the levels attained in the control and NUD groups were almost identical, but the concentration in the DU group was significantly higher than the concentrations in the other two (DU vs control p=0.0004, DU vs NUD p=0.0011) and indeed was double their value (see table 4.2, p169).

3. Basal-Histamine difference:
In saliva the rises in EGF concentration after histamine were just not significantly different between DU and control (p=0.065) and controls and NUD (p=0.171), but were significantly different between DU and NUD (p=0.004). The EGF concentration in the NUD increased by 64%, in the controls by 98% and in the DU by 131% .

In the gastric juice the increases were almost identical, about six-fold, in the control and NUD groups, but the increase in the DU group was double (53.7ng/ml) that in control and NUD groups (23.3, 24.8ng/ml respectively) (DU vs control p=0.0004, DU vs NUD p=0.001, see Table 4.2, p 169).

4. Salivary - gastric EGF differences:

During the basal period, there was a tendency for the gastric EGF concentration to exceed the salivary EGF concentration in the controls and NUD but not in the DU, see table 4.3 (p 170). The means of the individual differences between gastric EGF and salivary EGF concentrations (gastric EGF minus salivary EGF) during the basal period in controls were +1.0, in DU -0.8 and in NUD +2.8ng/ml. These gradients were not different in control vs DU (p=0.597) or in NUD vs control (p=0.136), but were significantly different between NUD and DU (p=0.043).

After histamine stimulation the gastric concentrations of EGF always exceeded those in saliva. The means of the differences between gastric EGF and salivary EGF concentrations in controls were +18.6, in DU +43.0 and in NUD +20.3ng/ml. These gradients were great in the DU group and less in controls and in NUD groups (DU vs controls p=0.0032, DU vs NUD p=0.023) but did not differ between controls and NUD (control vs NUD p=0.432, see table 4.3,p 170).

5. Correlation: There were no correlations between contemporaneous salivary EGF and gastric EGF concentrations during the basal or the histamine period in any diagnostic group (see table 4.4, p 171).

6. Gender: None of the average values of EGF concentrations, whether salivary or gastric, or whether in the basal period or during histamine

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stimulation showed any difference between genders (p>0.05 table 4.5, p 172).

7. <u>Age</u>: There was no correlation between age and EGF concentration either in saliva or in gastric juice, during the basal or histamine stimulation periods in any group except for the histamine stimulated salivary EGF in the DU group (p=0.024 table 4.6, p.173). This correlation was positive.

8. <u>Smoking</u>: There was no difference between smokers and non-smokers; the average values of EGF concentrations in saliva and in gastric juice during basal or histamine stimulation periods were not significantly different within the same diagnostic group or when compared to other groups (p>0.05 table 4.7, p.174).

In Smokers the relative values of EGF in saliva were 3.4 , 3.2, and 4.3 ng/ml in controls, NUD and in DU respectively during the basal period. After histamine stimulation the EGF concentrations in saliva increased to 5.8, 6.3, and 13.9 in controls, NUD and in DU respectively. The EGF concentrations in gastric juice were 3.6, 5.2 and 4.9 ng/ml during the basal period in controls, NUD and DU respectively. After histamine stimulation commenced the EGF concentrations in gastric juice increased to 27.4, 28.5, and 56.2 ng/ml in controls, NUD and in DU groups respectively.

In non-smokers the EGF concentrations in saliva during the basal period were 3.8 , 2.5 and 4.7 ng/ml in controls, NUD and DU respectively. The EGF concentrations in saliva after histamine stimulation started were 8.1, 3.6, and 8.9 ng/ml in controls, NUD and in DU subjects respectively. The

EGF concentrations in gastric juice during the basal period were 5.4, 5.6, and 3.1 ng/ml in controls, NUD and in DU patients respectively. In the histamine stimulation period the EGF concentrations in gastric juice increased to 20.6, 23.1 and 52.3 ng/ml in controls, NUD and in DU patients respectively.

9. Duodeno-gastric reflux: There was no correlation between the gastric EGF concentration and duodenogastric reflux during basal period or after histamine stimulation in any group. Duodenogastric reflux during the basal period was higher than during histamine stimulation (p=< 0.0001 table 4.8, p.175).

10. DU and Non-DU group : In order to see any difference between the DU group and all subjects without DU, the controls and the NUD were combined into a Non-DU group.

Basal salivary EGF concentrations in the Non-DU and DU group were 5.1, and 4.6ng/ml respectively, and there was no statistically significant difference between them (p=0.094). The EGF concentrations in gastric juice during the basal period in Non-DU and DU groups were 3.2 and 3.8ng/ml respectively , and there was no difference between them (p=0.689). There was no difference between salivary and gastric EGF during the basal period in Non-DU and DU groups (see table 4.9, p.176).

After histamine stimulation started EGF concentrations in saliva and gastric juice increased in both Non-DU and DU groups. DU produced more EGF in saliva than Non-DU group and this was statistically significant (p=0.0013), and DU produced more EGF in gastric juice than the Non-DU group (p<0.0001). There was more EGF in the gastric juice than

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in saliva in both Non-DU and DU groups and this was statistically significant (p<0.0001 see table 4.9 p.176).

RESULT 2

4.2 Prevalence of *H pylori* in Controls

One hundred healthy volunteers were included in this study; the prevalence of *H pylori* was determined by the ^{13}C-Urea breath test. Their results, broken down into the various age groups are given in table 4.10, (p.177). The overall prevalence of *H pylori* was 39%.

In most groups the proportion infected with *H pylori* increased with age although there was some tendency towards a fall in those aged over 65years. The prevalence of *H pylori* was commoner in men than in women (χ^2, p=0.0003 table 4.11. p.178). There was no difference between smokers and non-smokers, although there appeared to be a tendency for smoking to protect against *H pylori* (χ^2, p= 0.068 see table 4.12, p.179).

RESULT 3

4.3 EGF and *H pylori* status

The distribution of those *H pylori* +ve and those -ve in the three groups is given in table 4.13, (p.180). The relative values of EGF concentration in saliva during the basal period were respectively 3.6 and 3.66 ng/ml in *H pylori* +ve and *H pylori* -ve subjects. The basal gastric EGF concentrations in the gastric juice were respectively 4.52 and 4.75ng/ml in *H pylori* +ve

and *H pylori* -ve subjects. After histamine stimulation the EGF concentrations in the saliva and gastric juice increased in both *H pylori* +ve and *H pylori* -ve subjects. The EGF concentrations in saliva after histamine stimulation became respectively 8.2 and 6.8ng/ml in *H pylori* +ve and *H pylori* -ve subjects. The EGF concentrations in the gastric juice after histamine stimulation became respectively 31.0 and 36.0ng/ml in *H pylori* +ve and *H pylori* -ve subjects (table 4.14, p 181). The EGF concentrations in the gastric juice in *H pylori* +ve and -ve controls were 25.9 and 21.3ng/ml respectively. The EGF concentrations in the gastric juice in *H pylori* +ve and -ve NUD subjects were 22.5 and 26.6ng/ml respectively.

EGF concentrations in saliva and in gastric juice were examined for significant differences between *H pylori* +ve and *H pylori* -ve subjects. Whether one examined the samples obtained during the basal period or with histamine stimulation, no significant difference emerged (table 4.14, p.181). There was no difference in EGF concentration between controls and NUD group whether they were *H pylori* +ve or -ve.

Control and NUD groups were combined to form a single Non-DU group as a contrast group to the DU patients. Those DU patients who were *H pylori +ve* had a higher EGF concentration in their gastric juice during the histamine period than *H pylori +ve* Non-DU subjects ($p < 0.001$); similarly *H pylori -ve* DU had a higher EGF concentration in their gastric juice during the histamine period than *H pylori-ve* Non-DU ($p < 0.01$ see table 4.15, p. 182) . The prevalence of *H pylori* in this group was 45% in controls, 45% in NUD and 35% in DU.

CHAPTER FIVE

ANALYSIS OF RESULTS

Analysis of results 1

5.1 Quantification of swallowed saliva

Previous studies from this Department have shown that the electrolyte concentrations in gastric aspirate can be interpreted as a mixture of a primary gastric juice with a volume V_G and duodenogastric reflux with a volume V_R. However, this method of calculation ignores the volume and electrolytes of any swallowed saliva. Whitfield (personal communication) had further speculated that the calculation of V_G and V_R could be modified to take into account and indeed quantify swallowed saliva. These calculations have not been published, because it was impossible to test their validity due to the lack of a suitable marker for the swallowed saliva. Whitfield theoretically calculated the amount of swallowed saliva in aspirated gastric juice, by taking into account the published concentrations of electrolytes (HCO_3^-, K^+, Na^+, Cl^-) in saliva. Using this method, the aspirated juice V_{cor} was partitioned into three distinct volumes, the primary acid component V_{acid}, the saliva V_{sal} and the modified estimate of duodenogastric reflux V_{reflux}.

EGF is present in both saliva and gastric juice, and therefore at first sight does not appear to be suitable as a marker for the saliva. However, the literature available to me at the start of this study (Konturek et al 1989) claimed that the concentration of EGF in saliva was greater than that in gastric juice. I have therefore explored the possibility that EGF would act as a suitable marker for the swallowed saliva in aspirated gastric juice.

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The criterion for validating Whitfield's hypothesis that the volume of swallowed saliva can be estimated from electrolyte concentration, is that such a calculation should yield an estimate of the concentration of EGF in the swallowed saliva that is consistent with the EGF concentration measured in a simultaneously expectorated samples of saliva. I have measured salivary contamination of gastric juice during basal conditions as it forms a greater part of gastric aspirate during basal circumstances.

Method of Calculation

The total EGF output in aspirated gastric juice ($Q_{EGFVcor}$) is the sum of the EGF output in primary gastric juice (Q_{EGFVG} or V_{acid}), the EGF output of swallowed saliva ($Q_{EGFVsal}$) and the EGF output of duodenogastric reflux ($Q_{EGFVreflux}$). All outputs, both volumes of gastric juice, saliva or reflux; and of EGF are expressed per 10 minutes.

$$Q_{EGFVcor} = Q_{EGFVG} \text{ or } V_{acid} + Q_{EGFVsal} + Q_{EGFVreflux}$$

$Q_{EGFVcor}$ = the total EGF output, obtained by multiplying the V_{cor} and the measured EGF concentration in aspirated gastric juice.

V_{cor} = Volume of gastric juice corrected for pyloric loss.

V_G = Volume of primary gastric secretion calculated from the FORTRAN computer programme, i.e. V_{cor} further corrected for duodenogastric reflux (V_R).

V_{acid} = Volume of primary gastric acid, calculated from the FORTRAN computer programme, after correction both for duodenogastric reflux (V_{reflux}, not the same as V_R) and for swallowed saliva, V_{sal}.

V_{sal} = Volume of swallowed saliva calculated with the FORTRAN computer programme.

The EGF concentration in the primary gastric secretion is obtained from the slope of the regression line relating EGF output per 10 minutes to the calculated primary acid component volume in 10 minutes (see Fig. 1 & 2, pp.190,191). A different value was obtained for this EGF concentration according to whether the estimate of the primary acid component was V_G or V_{acid}. Multiplying the appropriate concentration by the volume of the primary acid component gives the output of EGF in the primary acid component (Q_{EGFV_G} or V_{acid}). Subtracting this output from the total EGF output ($Q_{EGFV_{cor}}$) gives the output of EGF in the extragastric component i.e. swallowed saliva and duodenogastric reflux.

In the first instance I have considered V_G as the correct estimate of the primary acid component. Since with this method we have no separation of the duodenogastric reflux component from salivary component, the extragastric component is simply $V_{cor} - V_G$ or V_R. The extragastric output of EGF is then obtained from the formula $Q_{EGFV_{cor}} - Q_{EGFV_G}$. Dividing this calculated extragastric output of EGF by the volume in which it has been secreted, i.e. V_R (from the FORTRAN programme) gives the EGF concentration in the extragastric component.

These calculations are summarised as:.

Formula 1

$$[EGF]_{extragastric} = \frac{Q_{EGFV_{cor}} - Q_{EGFV_G}}{V_{cor} - V_G}$$

This value was negative, and I therefore assumed that the analysis in terms of V_G is not satisfactory.

I then used formula 2, to calculate the EGF concentration in the extragastric component according to Whitfield's hypothesis. In formula 2, I have used the EGF concentration in V_{acid} and the calculation is represented by:

Formula 2

$$[EGF]_{extragastric} = \frac{Q_{EGF}V_{cor} - Q_{EGF}V_{acid}}{V_{sal}}$$

Remembering that $[EGF]_{reflux}$ was apparently negligible, this value of $[EGF]_{extragastric}$ component represents the concentration of EGF in swallowed saliva. It was compared for fit with the [EGF] in saliva expectorated at the same time. Further details of these calculations are given below.

Mean EGF concentrations during the basal period in combined (control, DU, and NUD) group were 3.42 ng/ml in saliva, 3.40 ng/ml in gastric juice (see table 10). The concentration of EGF in the V_G obtained from the appropriate regression slope was 3.43 ng/ml, and the concentration of EGF in the V_{acid} obtained from the other regression slope was 5.47 ng/ml (see Figures 1&2, pp. 190,191).

The concentration of EGF in the extra-gastric component was now calculated by using formula 2 for V_{acid}. It should be borne in mind that the V_G formula gave a meaningless negative value (-1.2 ng/ml) of EGF in the extragastric component. With the V_{acid} formula, the EGF

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concentration obtained in the extragastric component was 3.73 ng/ml, which showed a close correspondence with the measured EGF concentration in expectorated saliva, 3.40 ng/ml.

Analysis of results-2

5.2 EGF and volume rate of gastric secretion (VG & Vacid)

My results so far have shown that subjects in the DU group produce more EGF in the gastric juice, in terms of both concentration and output after histamine stimulation, than the subjects in the control and NUD group. These DU subjects differ from the other two groups in two ways: firstly they produce a greater volume of gastric juice after histamine stimulation, secondly these subjects have a duodenal ulcer. I wanted to explore whether the DU subjects had a higher EGF concentration because they had a DU or because they were secreting a greater volume of gastric juice.

I used the plateau values of V_G and V_{acid} of individual subjects during the histamine stimulation period to obtain the median value of the plateau values. I divided these sixty subjects into two groups according to whether their plateau average was greater or less than the median. Median values of V_G and V_{acid} during histamine stimulation period were 32.4 ml/10min, and 29.2 ml/10min respectively. There were 30 subjects above the median value and 30 below. The distribution of these subjects according to their V_G or V_{acid} in the different diagnostic groups is given in table 5.1 (p.183).

i. Those with VG more than 32.4ml/10min

There were 13 DU, 10 NUD and 7 controls subjects in this group.

The mean (S.D) EGF concentrations in DU, NUD and controls were 56.5 (43.8), 16.9 (15.1), 23.3 (9.1) ng/ml respectively. There was a statistically significant difference between DU and controls (p=0.0126) and between the DU and NUD group (p=0.0036) but there was no difference between the control group and NUD (p=0.204).

ii Those with VG less than 32.4ml/10min

There were 7 DU, 10 NUD and 13 controls in this group. The mean (S.D) EGF concentrations in DU, NUD and controls were 48.4 (21.7), 32.6 (10.6) and 23.9 (14.4) ng/ml. There was a statistically significant difference between the DU and control (p=0.007), but no difference between the DU and NUD (p=0.079) or between the NUD and controls (p=0.136).

iii. Those with Vacid more than 29.2ml/10min

There were 12 DU, 11 NUD and 7 controls in this group. The mean (S.D) EGF concentrations in DU, NUD and controls were 57.9 (45.4), 19.1 (16.1) and 22.3 (9.1) ng/ml respectively. There was a statistically significant difference between DU and controls (p=0.018), and between DU and NUD (p=0.008), but there was no difference between controls and NUD (p=0.389).

iv. Those with Vacid lower than 29.2ml/10min

There were 8 DU, 9 NUD and 13 controls in this group. The mean (S.D) EGF concentrations in DU, NUD and controls were 47.3 (20.3), 31.7 (10.8) and 23.9 (14.4) ng/ml respectively. There was no difference between NUD and controls (p=0.171) and between DU and NUD (p=0.067) but there were statistically significant differences between DU and controls (p=0.007).

There was no relation between the volume of gastric secretion and the concentration or the output of EGF in the gastric juice. There was a strong relationship between the presence of a DU and a high EGF concentration, irrespective of the 10-minute volume of gastric juice.

Analysis of results 3

5.4 Prevalence of *H pylori* in controls and dyspeptics

The studies I carried out with regard to the prevalence of gastric infection with *H pylori* consisted of 100 control (asymptomatic) subjects examined by a non-invasive technique. The reason for undertaking this part of the investigation was to act as a control for a previous investigation on the prevalence of *H pylori* performed in this Department. The previous study was carried out by Chandrakumaran (1991), on 252 dyspeptic subjects referred for diagnostic endoscopy between 1987 and 1990. The main thrust of their study was to determine whether *H pylori* was more frequently present in patients undergoing endoscopy for the complaint of dyspepsia who turned out to have DU, than in similar patients who were found not to have a DU.

These were the results of Chadrakumaran's study. Oesophagitis was diagnosed in 30 (12%); 23 patients had miscellaneous (MISC) macroscopic findings, including five with gastric ulcer; a duodenal ulcer (DU) was diagnosed in 76 (30%) and no abnormality, that is no ulcer disease (NUD) was found in 123 (49%). The prevalence of *H pylori* in these dyspeptics

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was determined by the CP-urease test, culture and histology of endoscopic biopsies. The CP urease test was positive in 145 (57%) and negative in 107 (43%) see table 5.2 (p 184).

Every patient who was urease test positive was also positive by either culture or histology, they therefore used as their "gold standard" for the diagnosis of infection with *H pylori*, that either one (or both) of the results of culture and histology was positive. Using this criterion, none of the CP-urease positive patients was classified as *H pylori* negative but 14 (13%) of the 107 CP-urease negative patients were positive for *H pylori* by the later tests. The prevalence of *H pylori* in each of the four diagnostic groups was 70% (DU), 67 % (NUD), 61% (Misc) and 30% (reflux oesophagitis), i.e., overall 63% (see table 5.2 and 5.3, pp. 184,185).

The weakness of the previous study was that there were no normal controls. The reason for this was that at that time there was no reliable non-invasive method of diagnosis available to us and it was ethically unacceptable to use an invasive method such as an endoscopy and biopsy in normal healthy controls, to detect the prevalence of *H pylori* infection in them. When the new non-invasive methods became available for the diagnosis of *H pylori*, I chose the ^{13}C-urea breath test to investigate the prevalence of *H pylori* in controls. This test is non-invasive and has a high sensitivity and specificity, 92%, 100% respectively using histology and/or culture as 'gold standard' (Logan et al 1991).

I studied one hundred healthy control volunteers who were well matched for age with the dyspeptic subjects of the previous study. I did this study between November 1992 and January 1993. The prevalence of *H pylori* in controls is given in the table 4.10 (p 177). The results of the urea breath test

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were broken down into the age groups 16-25yrs, 26-35yrs, 36-45yrs, 46-55yrs, 56-65yrs and 66 and above; that is, the same as those of Chandrakumaran's dyspeptic subjects. The overall prevalence of H $pylori$ in controls was 39%.

I have analysed the results of this control group and the results of Chandrakumaran's study on the dyspeptic subjects, taking into account the relations to gender, smoking habits and age.

In most groups the proportion infected with H $pylori$ increased with age although there was some tendency towards a fall in those aged over 65years. In the controls, H $pylori$ was commoner in males than in females (χ^2, p=0.0003, table 4.11, p. 178). There was no statistical difference between smokers and non-smokers, although there appeared to be a tendency for smoking to protect against H $pylori$ (χ^2, p= 0.068 , table 4.12, p.179). There was no effect of gender or of smoking in any of the patient groups (60 of 100 male patients H $pylori$ positive, 99 of 152 female patients, 65%, H $pylori$ positive, χ^2, p= 0.448; 22 of 33 smokers positive, 19 of 29 non-smokers positive in a subgroup of 62 subjects whose smoking habits were recorded, χ^2, p> 0.99).

The effect of age, clearly demonstrable in the normal subjects, could be seen to be carried through to some extent in the dyspeptic groups although the effect was reduced by a larger prevalence in the patients of the youngest three age groups; by contrast, there seemed to be no effect of smoking or of gender in the patient groups.

The prevalence of H pylori in patients with duodenal ulcer was greater than in controls in two age groups; 36-45 (χ^2, p =0.013) and 56-65 (Fisher's, p=0.05) but not in the other age groups (table 5.4, p. 186).

The prevalence of H pylori in patients with duodenal ulcer was next compared with the prevalence in the NUD group. There was no significant difference in any age group, the smallest p-value reached being 0.132 (table 5.5, p. 187).

The prevalence of H pylori was examined in patients with reflux oesophagitis. In view of the small numbers in the oesophagitis category and their absence from many of the age groups, the data were combined. In the combined age groups (36-75), the prevalence of H pylori was rather less in the oesophagitis group (8 out of 28) than in the controls (32 out of 67) but the difference was not significant difference (χ^2, p=0.084). Comparing with DU in the combined age-groups of reflux oesophagitis, 36-55 and 56 and above, the prevalence in the DU group (44/59) was greater than that in the oesophagitis group (9/29) and this difference was highly significant (χ^2, p <0.0001).

Comparing the DU with the miscellaneous group in the combined 36-55 age groups, there was no difference in the prevalence of H pylori (Fisher, p=0.708). Combining the NUD and Miscellaneous groups demonstrated that their prevalence of H pylori was no different from that of the DU group, 48 positive out of 65 in combined group (NUD plus miscellaneous) compared with 30 positive out of 43 DU in age group 36-55, χ^2 p=0.81, 28 positive out of 42 in combined group compared to 11 out of 12 in DU in age-group 56-65, Fisher's p=0.145 (see table 5.6, p. 188).

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The prevalence of *H pylori* in patients with NUD was greater than in controls in one age group (36-45, Fisher's, p =0.002) but not in any other age group (table 5.7, p. 189). The prevalence of *H pylori* in controls was compared with miscellaneous group. 18 were positive out of 38 controls as compared to 6 out of 10 in miscellaneous group in the age group 36-55 (Fisher's, p=0.505), 12 out of 21 controls as compared to 7 out of 11 in miscellaneous group, (Fisher's, p>0.999).

Table 4.1

Age, Sex , Smoking habits and Diagnosis of subjects studied

Group	Total	Male	Female	Age	Smoker	Non smoker
Control	20	13	7	35	8	12
NUD	20	11	9	53.3	6	14
DU	20	9	11	43	7	13

DU= Duodenal ulcer
NUD= Non ulcer dyspepsia

Table 4.2

EGF concentration (ng/ml) means (standard deviations)

Secretion	Saliva			Gastric		
Group	**Basal**	**Hist.**	**Diff**	**Basal**	**Hist.**	**Diff**
Controls	3.62 (2.21)	7.19 (4.64)	3.57	4.69 (6.32)	23.33 (12.57)	18.74
NUD	2.68 (1.90)	4.42 (2.98)	1.74	5.51 (7.18)	24.75 (15.05)	19.24
DU	4.59* (3.08)	10.64* (6.27)	6.05	3.77 (2.94)	53.66@ (37.08)	49.89@

* = DU significantly greater than NUD but not greater than controls
@= DU significantly greater than NUD and controls
Hist.= After histamine stimulation
Diff= Differences between basal and histamine EGF levels

Table 4.3

Difference between gastric and salivary EGF concentrations means and (standard deviation)

Period	BASAL			HISTAMINE		
Secretion	Gastric	Saliva	Diff	Gastric	Saliva	Diff
Controls	4.69 (6.32)	3.62 (2.21)	1.07	23.33 (12.57)	4.69 (6.32)	18.64
NUD	5.51 (7.18)	2.68 (1.98)	$2.83^{@}$	24.75 (15.05)	4.42 (2.98)	20.33
DU	3.77 (2.94)	4.59 (3.08)	-0.82	53.66 (37.08)	10.64 (6.27)	43.02^{*}

@= NUD higher than DU (p<0.05)
*= DU higher than controls and NUD (p<0.05)
Diff= Difference between gastric and salivary EGF concentration

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Table 4.4

Spearman's correlation between salivary & gastric EGF concentration

Group	Basal	Histamine
Control	0.069 NS	0.49 NS
NUD	0.01 S	0.018 S
DU	0.168 NS	0.876 NS

NUD= Non-ulcer dyspepsia   NS= Non significant
DU= Duodenal ulcer   S= Significant

Table 4.5

EGF concentration (ng/ml): means (standard deviations) in Male (M) & Female (F) and p value for the difference

Group	Basal Gastric	Hist. Gastric	Basal Saliva	Hist. Saliva
Controls	M 3.78 (3.64) F 6.34 (9.75) p=0.781	M 26.25 (13.5) F 17.9 (9.1) p=0.165	M 3.35 (1.97) F 4.13 (2.69) p=0.476	M 6.1 (3.28) F 9.2 (6.3) p=0.322
DU	M 3.63 (3.61) F 3.93 (2.02) p=0.305	M 42.63 (20.6) F 67.15 (48.6) p=0.470	M 5.65 (3.31) F 3.3 (2.34) p=0.138	M 12.2 (7.13) F 8.73 (4.73) p=0.270
NUD	M 6.44 (7.24) F 4.75 (7.35) p=0.624	M 26.2 (13.74) F 23.6 (16.6) p=0. 790	M 3.31 (2.55) F 2.2 (1.00) p=0.470	M 5.17 (3.2) F 2.82 (2.8) p=0.305

Table 4.6

Spearman's correlation between age and EGF concentration in Saliva and
Gastric juice (p values)

Secretion	Saliva		Gastric	
Group	**Basal**	**Hist.**	**Basal**	**Hist.**
Control	0.674 NS	0.081 NS	0.378 NS	0.439 NS
NUD	0.138 NS	0.898 NS	0.251 NS	0.686 NS
DU	0.177 NS	0.023 S	0.841 NS	0.288 NS

Table 4.7

EGF concentration (ng/ml): means (standard deviations) in Smokers (SM) & Non-smokers (NSM) and p value for the difference

Group	Basal Gastric	Hist. Gastric	Basal Saliva	Hist. Saliva
Controls	SM 3.6 (4.6) NSM 5.4 (7.3) p=0.105	SM 27.4 (16.8) NSM 20.6 (8.5) p=0.396	SM 3.4 (2.2) NSM 3.8 (2.2) p=0.787	SM 5.8 (3.7) NSM 8.1 (5.1) p=0.316
DU	SM 4.9 (4.2) NSM 3.1 (1.6) p=0.322	SM 56.2 (46.6) NSM 52.3 (32.9) p=0.905	SM 4.3 (3.4) NSM 4.7 (3.1) p=0.606	SM 13.9 (7.1) NSM 8.9 (5.2) p=0.075
NUD	SM 5.2 (3.7) NSM 5.6 (8.4) p=0.322	SM 28.5 (9.5) NSM 23.1 (16.9) p=0.620	SM 3.2 (1.8) NSM 2.5 (1.9) p=0.409	SM 6.3 (3.4) NSM 3.6 (2.7) p=0.069

Table 4.8

Duodeno-gastric reflux (Wilcoxon rank sign test)

Basal V reflux	3.35 ml/10min	p value
Hist. V reflux	0.399 ml/10min	<0.0001

Table 4.9

EGF concentration (ng/ml) means (standard deviations) in DU and Non-DU groups and p values for differences

Secretion	Saliva		Gastric	
Group	Basal	Hist.	Basal	Hist.
Non-DU	3.15 (2.09)	5.82 (4.11)	5.1 (6.69)	24.04 (13.71)
DU	4.6 (3.1) p=0.094	10.64 (6.3) p=0.001	3.8 (2.93) p=0.689	53.66 (37.08) p<0.0001

Table 4.10

Prevalence of *H pylori* in healthy Controls, subdivided by age

Age(yrs)	Total	Positive	% Positive
16-25	19	03	15.8
26-35	14	04	28.6
36-45	19	05	26.3
46-55	19	13	68.4
56-65	21	12	57.1
66 & above	08	02	25.0

Table 4.11

Effect of gender on prevalence of *H pylori* in healthy controls

Hp status	Male	Female	χ^2 p value
Positive	28	11	0.0003
Negative	21	40	

Table 4.12

Effect of cigarette smoking on the prevalence of *H pylori* in healthy
controls

Hp status	Smokers	Non smokers	χ^2 p value
Positive	10	29	0.068
Negative	28	33	

Table 4.13

Prevalence of *H pylori* in those who had gastric secretion test

Group (No)	Hp +ve	Hp -ve
Controls (20)	9	11
NUD (20)	9	11
DU (20)	7	13
Total (60)	25	35

Table 4.14

EGF concentration in *H pylori* +ve/ -ve subjects
mean ng/ml (std)

Secretion	*Hp* +ve	*Hp* -ve	p value
Basal saliva	3.6 (2.6)	3.66 (2.5)	0.964 NS
Basal gastric juice	4.52 (6.1)	4.75 (5.5)	0.626 NS
Hist. saliva	8.23 (6.4)	6.84 (4.6)	0.294 NS
Hist. gastric juice	30.97 (31.1)	36.02 (25.2)	0.222 NS

NS= not significant
Hist= Histamine stimulated

Table 4.15

EGF mean (std), in *H pylori* +ve/-ve DU, Non-DU

subjects

Secretion	*Hp +ve*		*Hp-ve*	
Group	**Basal gastric EGF**	**Hist. gastric EGF**	**Basal gastric EGF**	**Hist. gastric EGF.**
Non-DU	4.41 (6.72)	20.69 (14.64)	5.66 (6.7)	26.04 (12.56)
DU	4.8* (4.52)	57.4 (46.2) p<0.001	3.2* (1.5)	51.66 (33.1) p<0.01

*= not significant

Table 5.1

VG and Vacid (ml/10min), mean (std) EGF levels ng/ml, (number of subjects)

Primary gastric juice	VG		Vacid	
Group	High VG (> 32.2 ml)	Low VG (< 32.2ml)	High Vacid (> 29.2ml)	Low Vacid (< 29.2ml)
Controls	22.26 (9.13) (7)	23.91 (14.4) (13)	22.26 (9.13) (7)	23.91 (14.4) (13)
NUD	16.9 (15.1) (10)	32.6 (10.64) (10)	19.1 (16.1) (11)	31.67 (10.85) (9)
DU	56.52 (43.8)** (13)	48.4 (21.7)* (7)	57.91 (45.4)** (12)	47.3 (20.1)* (8)

* =   DU higher than control but not different from NUD ($p<0.05$)
** = DU higher than controls and NUD ($p<0.05$)

Table 5.2

Incidence of *H pylori* in the macroscopic diagnosis groups,
assessed by the CP-urease test (urease +ve) and by the culture & histology
test (*H pylori* +ve)

Group	CP-Urease positive	*H pylori* positive
NUD	75 (61%))	83 (67%)
DU	49 (64%)	53 (70%)
Oesophagitis	8 (28%)	9 (30%)
Miscellaneous	13 (57%)	14 (61%)
Total	145 (58%)	159 (63%)

Table 5.3

Prevalence of *Hp*- Fractions (%) in Subject groups, subdivided by age

Age (yrs)	Controls	DU	NUD	Misc:	Oesoph:
16-25	3/19 (16)	2/4	2/5	-	-
26-35	4/14 (29)	7/13 (54)	6/11 (55)	-	-
36-45	5/19 (26)	13/18 (72)	14/18 (78)	2/3	2/8
46-55	13/19 (68)	17/25 (68)	28/37 (77)	4/7	3/13 (23)
56-65	12/21 (57)	11/12 (92)	21/31 (68)	7/11 (64)	2/4
66-75	2/8	3/4	12/19 (63)	-	1/3
76-85	-	-	2/4	1/1	1/1

Percentages are only given when justified by the number in the group.(DU=Duodenal ulcer, NUD=Non ulcer dyspepsia, Misc:=Miscellaneous, Oesoph:=oesophagitis)

Table 5.4

Comparison of prevalence of *H pylori* in Controls and DU subjects

Age (yrs)	*H pylori*	Control	DU	p-value
16-25	Positive Negative	3 16	2 2	0.194 (Fisher)
26-35	Positive Negative	4 10	7 6	0.252 (Fisher)
36-45	Positive Negative	5 14	13 5	0.005 (χ^2)
46-55	Positive Negative	13 6	17 8	0.976 (χ^2)
56-65	Positive Negative	12 9	11 1	0.05 (Fisher)
66 & above	Positive Negative	2 6	3 1	0.222 (Fisher)

DU= Duodenal ulcer

χ^2 = Chi square

Fisher =Fisher's exact test

Table 5.5

Comparison of prevalence of *H pylori* in DU and NUD groups

Age (yrs)	*H pylori*	NUD	DU	p-value
16-25	Positive Negative	2 3	2 2	>.0999 (Fisher)
26-35	Positive Negative	6 5	7 6	0.972 (χ^2)
36-45	Positive Negative	14 4	13 5	0.721 (Fisher)
46-55	Positive Negative	28 11	17 8	0.745 (χ^2)
56-65	Positive Negative	21 11	11 1	0.132 (Fisher)
66 & above	Positive Negative	12 7	3 1	>0.999 (Fisher)

NUD= Non ulcer dyspepsia
DU= Duodenal ulcer
Fisher = Fisher's exact test
χ^2 = Chi square

Table 5.6

Comparison of prevalence of *H pylori* in DU and
the combined group (NUD + Miscellaneous)

Age (yrs)	*H pylori*	DU	NUD & Misc:	p-value
36-55	Positive Negative	30 13	48 17	0.807 (χ^2)
56-65	Positive Negative	11 1	28 14	0.145 (Fisher)

NUD=Non ulcer dyspepsia
Misc:= Miscellaneous
Fisher = Fisher's exact test
χ^2 = Chi square

Table 5.7

Comparison of prevalence of *H pylori* in Controls and NUD groups

Age(yrs)	*H pylori*	Control	NUD	p-value
16-25	Positive Negative	3 16	2 3	0.271 (Fisher)
26-35	Positive Negative	4 10	6 5	0.241 (Fisher)
36-45	Positive Negative	5 14	14 4	0.002 (Fisher)
46-55	Positive Negative	13 6	28 11	0.715 (χ^2)
56-65	Positive Negative	12 9	21 11	0.533 (χ^2)
66 & above	Positive Negative	2 6	12 7	0.103 (Fisher)

NUD=Non ulcer dyspepsia
χ^2 = Chi square
Fisher = Fisher's exact test

Regression Plot VG vs EGF output (QEGF Vcor)

Regression Plot (Basal)
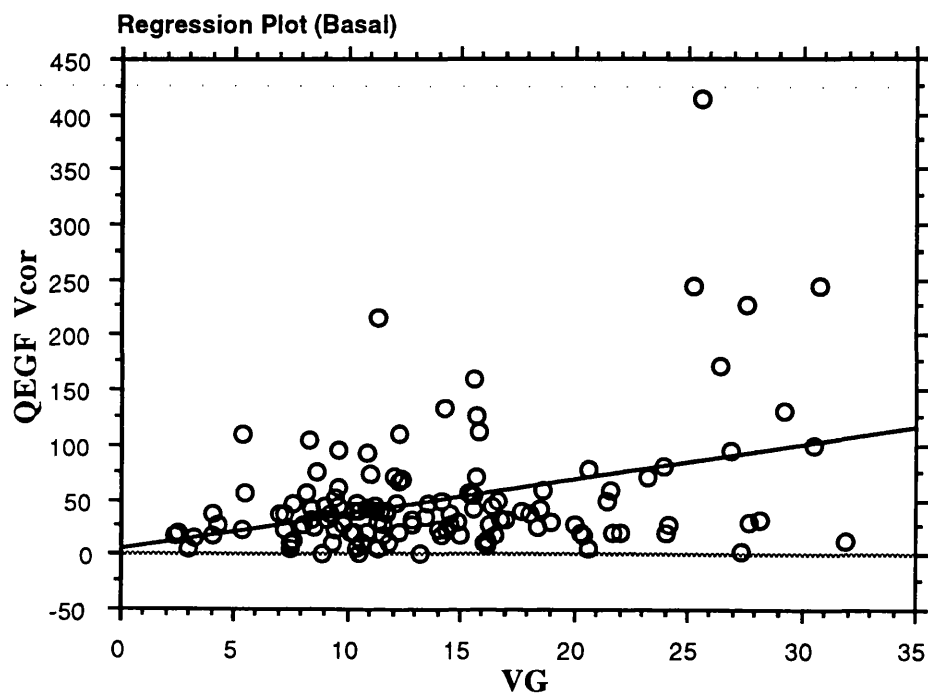
Fig.1   Y = 6.691 + 3.151 * X; R^2 = .134

Regression Plot V_acid vs EGF output (QEGF Vcor)

Regression Plot (Basal)
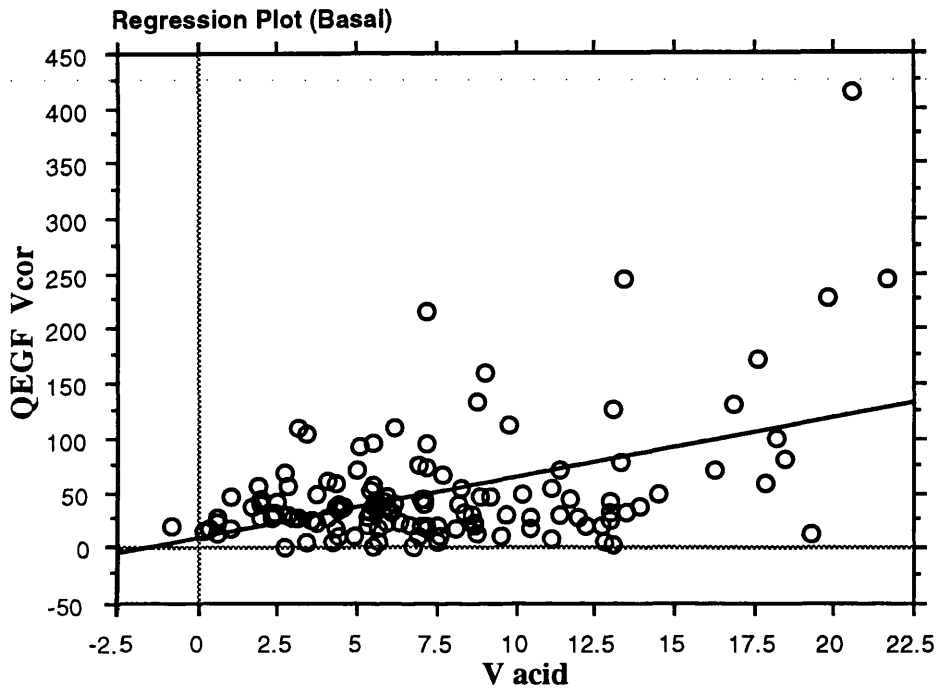
Fig.2   Y = 9.929 + 5.471 * X; R^2 = .214

Comparasion of UBT and ammonia[3]: urea ratio
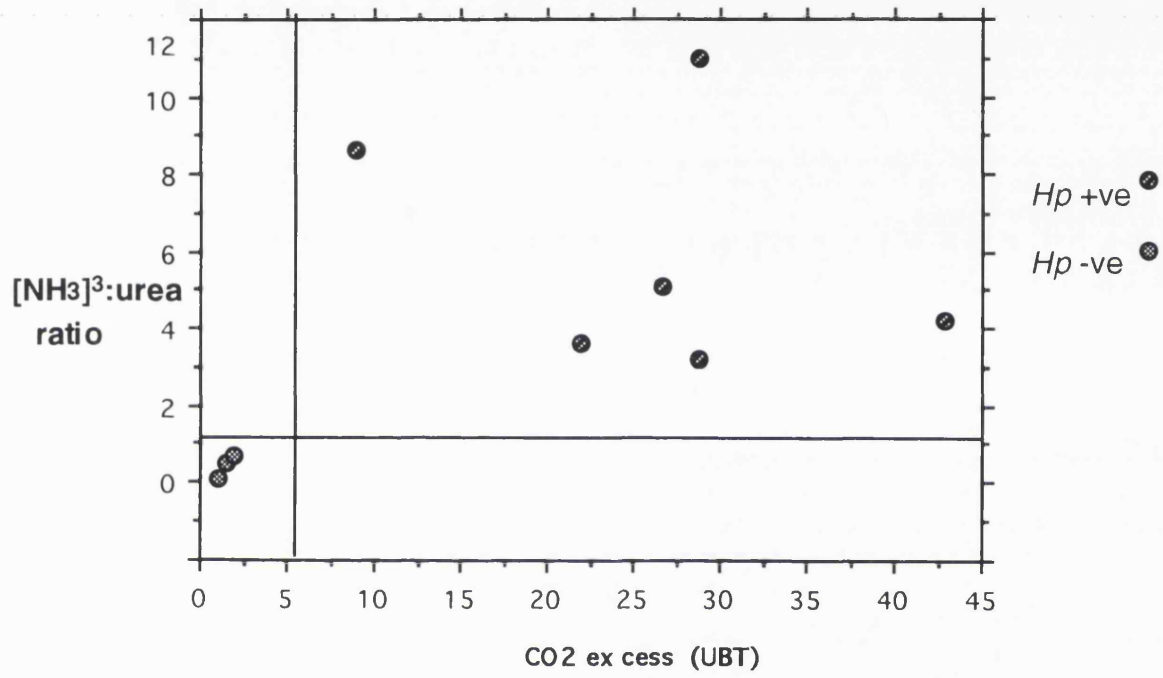
Fig. 3

CHAPTER SIX

DISCUSSION

5.1 DISCUSSION OF METHODS

5.1.1. Gastric secretion test

Sixty subjects attended for the gastric secretion test and a careful detailed recording of each patient's age, smoking history, height and weight was kept. For smoking history, the maximum period of smoking and stated average rate of smoking per day was used. Subjects may understate their cigarette consumption or deny being smokers for the fear of disapproval; thus it had to taken on trust that the subjects had told the truth.

The method of stimulating maximal gastric secretion was at variance with common practice, the most popular stimulatory method being that of the single bolus of pentagastrin. The method used in this Department follows that of Lawrie, Smith and Frost (1964), in using an intravenous infusion of histamine, with the use of an antihistamine to reduce side effects. This gives a more reliable plateau of secretion compared to the transient peak obtained with a single injection of histamine (Kay 1953). It is claimed that a continuous infusion of pentagstrin is a more accurate method of stimulating the gastric secretion (Aubery 1970), but some workers have shown that the effect of pentagastrin fades (Emås & Svensson 1972).

The plateau of secretion obtained with maximal histamine stimulation was, on average, of 40 minutes duration. A plateau was felt to be preferable to a peak response as it enabled the detection of, and correction for, sequestration and poor aspiration of gastric juice. Using a dose of 13μmol kg^{-1} h^{-1} histamine acid phosphate, very few unpleasant side-effects have been observed other than flushing and occasional headaches.

In previous work from this Department done on 30 duplicate maximal histamine tests, the difference between two tests on the same individual was approximately 10%, which can be considered to be a measure of the accuracy of any one test. The three measurements of gastric secretion, MAO (volume of mean acid output), V_{obs} (volume observed), and V_G (volume of gastric juice corrected for pyloric loss and duodenogastric reflux), had variations which, expressed as a percentage of the total variation, were 4%, 5%, and 1.5% respectively. This result confirms that V_G, which should be theoretically more accurate, since it is the result of modifications for pyloric loss and duodenogastric reflux, is indeed less variable.

The pyloric loss and duodengastric reflux may not vary much. In an individual there is a significant rise in V_G from basal to maximal secretion (300%-400%) but pyloric loss remains constant. It seems that the rate of pyloric loss in an individual is relatively constant and is not determined by the secretory state of the individual at the time (Roxburgh 1989). Groups of controls and DU patients with a similar median V_G have similar median rates of pyloric loss and thus it appears that the presence of DU has no specific effect upon pyloric loss. Duodenogastric reflux is normal phenomenon: it is found in control subjects regardless of the method used (Muller-Lissner et al 1983). It has also been shown in previous work from this Department that duodenogastric reflux is greatest under basal conditions and decreases significantly at maximal stimulation, both in controls and DU subjects (Roxburgh, 1989).

5.1.2 Correction of swallowed saliva in gastric juice

During gastric secretion studies one source of error is swallowed saliva, and the measurement of swallowed saliva has proved difficult. Many workers have attempted to prevent swallowing of saliva, and their reports are referred to on pp.47-48. All these methods increased salivary secretion and no one was certain that salivary contamination had been prevented. Boulos et al (1980) used thiocyanate as a marker of swallowed saliva in the aspirated gastric juice, but found it unsatisfactory. If one accepts that it is impossible to prevent swallowing of the saliva, then a different marker must be found.

Previous studies from this Department have shown that the electrolyte concentrations in gastric aspirate can be interpreted as a mixture of a primary gastric juice with a volume V_G and duodenogastric reflux with a volume V_R. However, this method ignores the volume and electrolyte concentration of any swallowed saliva. Whitfield speculated that the calculation of V_G and V_R can be modified to take into account and indeed quantify swallowed saliva. He theoretically calculated the amount of swallowed saliva in aspirated gastric juice, by taking into account the published concentration of electrolytes (HCO_3^-, K^+, Na^+, Cl^-) found in saliva. These calculation were written on a FORTRAN computer programme; with this method aspirated gastric juice corrected for pyloric loss (V_{cor}), was subdivided into three distinct parts, the primary acid component (V_{acid}), the swallowed saliva (V_{sal}) and duodenogastric reflux (V_{reflux}). The reason that Whitfield's hypothesis has not been published was the lack of a suitable marker of saliva to check the calculations.

I have used epidermal growth factor (EGF) as a marker of swallowed saliva to test Whitfield's mathematical model of the composition of aspirated gastric juice. EGF is present in both saliva and gastric juice, and therefore at first sight does not appear to be suitable as a marker of the saliva. However, the literature available to me at the beginning of this study claimed that the concentration of EGF in saliva was greater than in gastric juice. I therefore investigated the possibility that EGF could act as a marker to quantify the swallowed saliva in aspirated gastric juice.

5.1.3. Measurement of EGF in gastric juice and saliva

Samples of gastric juice and saliva were kept at -20°C for 5 months due to unavailability at that time of a radioimmunoassay kit. I am not aware of any literature which suggests that changes occur in the concentration of EGF due to storage of the samples. I have analysed these samples in the Research laboratory in Stoke-on-Trent, and it was suggested by Professor Elder (who is in charge of that laboratory) that no change would be likely to occur in the EGF concentration in the samples provided they were frozen at -20°C (personal communication).

I chose radioimmunoassay to quantify the EGF in gastric juice and in saliva because this method is highly sensitive: thus it is possible to measure extremely low concentrations of EGF in these samples. Other methods used for measuring EGF in gastric juice and in saliva is ELISA (enzyme-linked immunosorbent assay); with this method one can measure EGF concentration in the sample as low as 10^{-9}g, whereas with radioimmunoassay one can measure EGF as low as 10^{-12}g. The antiserum

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used in radioimmunoassay has 100% specificity to human EGF, and does not cross-react with any other peptide.

The technique of radioimmunoassay found its first and to date its widest application in the study of protein and polypeptide hormones of which insulin was the first to be assayed in this way by Yalow and Berson (1960). Radioimmunoassay is a competitive immunoassay in which the antigen labelled with an isotope competes with the unlabelled antigen to bind to antibodies. The radioimmunoassay technique is used for both quantitative and qualitative analysis. Initially it was limited to the measurement of a few naturally antigenic polypeptide hormones, but it has now been extended to studies of a large number of substance such as proteins, peptides, steroids, drugs and vitamins. Any compound can be measured by radioimmunoassay provided that the substance is immunogenic (induces an immune response), is available in pure form and can be labelled.

Calculation of radioimmunoassay results

There are various methods to calculate radioimmunoassay results. The method I used for the calculation of results of epidermal growth factor in saliva and in gastric juice was the Spline function interpolation technique.

Spline interpolation technique

The interpolation method for calculation of the concentration of an unknown by radioimmunoassay relies on the experimental information from the results of a series of standards. Even with several standard points, one makes only very general assumptions about the form of the standard curve. Methods for deriving the standard curves range from linear interpolation, where the curve is constructed from straight lines

joining individual points, to spline function interpolation, where the standard curve is constructed from curved segments that join together to form a smooth, complete curve (Rawlins and Yrjönen 1978). The spline function tries to find the ideal equation for each part of the curve; these different segments are then coupled to each other in a smooth, continuous way as described on pp.122-123.

The advantage in using the spline function to describe the standard curve in radioimmunoassay arises from the fact that the formula describing the curve over one concentration range can be completely different from, and therefore independent of the function used for another range. This means that it is simple to handle the different effects that may become important in different concentration regions, e.g., extra curvature at high concentrations due to non-specific binding or extra curvature at low concentrations due to multiple binding sites (Rawlins and Yrjönen 1978).

5.1.4. ^{13}C-urea breath test

I have used the ^{13}C-urea breath test to detect the prevalence of *H pylori* in controls, as this method is non-invasive, without any side effects and ethically acceptable. ^{13}C-urea is a stable isotope and can be used without any restriction, including in children and women of child-bearing age. The test can be repeated as often as necessary and, as no biological certification is needed, samples for ^{13}C-urea breath test can be sent by post. None of the subjects included in this study was under the age of 18 or pregnant, but the test would still have been safe to use in these groups.

Using histology as the "gold standard", the ^{13}C-urea breath test has a sensitivity of 98% and specificity of 92% (Logan et al 1991). Unlike

histology and culture, the ^{13}C-urea breath test is non-invasive,

intrinsically less liable to observer variation and allows frequent

assessment. Due to patchy distribution of *H pylori* in the stomach, both

histology and culture are liable to observer variation. The urease test (CP-

urease test) on biopsies is the quickest and simplest test, but again like

histology and culture this test is also invasive and like all biopsy-based

methods this method is also liable to sampling error. The ^{13}C-urea breath

test is better than other diagnostic tests such as serology (ELISA), as

serology relies on the presence of antibodies in the serum which are found

in the serum for a long time, maybe up to a year after the eradication of *H*

pylori infection, whereas the ^{13}C-urea breath test is only positive in

infection at that time. With the ^{13}C-urea breath test, it is possible to define

a sharp cut-off between positive and negative results, and it can detect very

low levels of *H pylori* colonization within stomach. The disadvantage of

the ^{13}C-urea breath test is that it is expensive, and the mass spectrometer

used to detect ^{13}C in expired gas is not widely available.

The principle of the urea breath test is that, in the presence of the enzyme

urease, orally administered urea is hydrolysed into carbon dioxide and

ammonia. If the urea carbon is labelled with ^{13}C isotope, the latter can be

detected in the breath as labelled carbon dioxide.

5.1.5. Measurement of ammonia and urea in gastric secretion
(ammonia test)

The principle of this test is similar to that of the ^{13}C-urea breath test; the

presence of *H pylori* will utilize urea present in the gastric juice, resulting

in an increase in the concentration of ammonia and lowering of the urea

concentration in the gastric juice. This method of detection of *H pylori*

has been used by others (Kim et 1990, Neithercut et al 1991 & 1993).

However, none of these studies has compared this method of detection of

H pylori with the ^{13}C-urea breath test. I have therefore done the ^{13}C-urea

breath test on 9 controls who also had ammonia and urea measured in the

gastric juice. I chose to compare this method of finding out the *H pylori*

status with the ^{13}C-urea breath test because the latter has a sensitivity and

specificity of 98% and specificity of 92% (Logan et al 1991)

There was 100% concordance (Figure 3, p.192) between this method and

^{13}C-urea breath test: all those who were positive by the ^{13}C-urea breath test

were also positive by this method and all those who were negative on ^{13}C-

urea breath test were also negative by this method. The gastric juice

samples were analysed by Dr J Holton; he was not aware of the results of

the ^{13}C-urea breath test in the 9 controls, and I repeated the tests myself.

There was complete concordance between Dr J Holton's results and my

results for both urea and ammonia. These two set of assays were done two

months apart, which suggests that freezing these gastric juice samples does

not change the urea or ammonia concentration in the gastric juice. The

gastric juice samples were frozen at -20°C for 5 months before the first

assay, and I am not aware of any evidence in the literature that freezing for

that long would alter the concentrations of urea and ammonia in the

gastric juice. Niethercut et al (1991) kept gastric juice samples frozen for 21

days and found no significant change in ammonia concentration in them.

5.2. DISCUSSION OF RESULTS

5.2.1. EGF in gastric juice and saliva

In this study EGF concentrations have been measured simultaneously in both saliva and gastric juice in patients with duodenal ulcer and non-ulcer dyspepsia in comparison with normal controls. Calabrò et al (1990) have measured EGF concentration in gastric juice only under basal conditions in patients with DU, GU and in controls. I have measured the salivary and gastric EGF concentrations not only under basal conditions but also after histamine stimulation. A similar study has been carried out by Konturek et al (1989), who measured EGF concentrations in saliva and gastric juice only in controls, both in basal and in stimulated secretion although they stimulated the gastric secretion with pentagastrin which, unlike histamine, has a 'fade' effect (Aubery 1970, Emås & Svensson 1972). In another study Hansen et al (1989) measured EGF in saliva and in gastric juice in patients with DU, GU and in controls under basal condition. Ohmura et al (1987), Hirasawa et al (1991) and Maccini et al (1990) have measured only salivary EGF in controls and in patients with peptic ulcer.

The results of my study have shown that during the basal period the salivary EGF concentrations were not significantly different between DU and control groups, and between controls and NUD groups, but the salivary EGF concentration in the DU group was significantly higher than that in NUD group. The relative values of salivary EGF concentrations in controls, DU and NUD were 3.6, 4.6, and 2.7ng/ml respectively. Hirasawa et al (1991) have also reported an increase in basal salivary EGF output in patients with DU and GU, almost double as compared to the controls; like myself they also used radioimmunassay to measure EGF. They included

129 controls and 232 patients with peptic ulcer. Kingsnorth et al (1989) and

Hansen et al (1989) found no difference in salivary EGF concentrations

between patients with DU and controls. However, Maccini et al (1990)

found lower EGF concentrations in saliva in patients with DU as

compared to controls. There are two problems with this study. Firstly,

their controls groups consisted of dyspeptics who had normal endoscopic

findings, which was equivalent to the NUD group of my study. My

control group were healthy volunteers who had no gastrointestinal

symptoms and they were not on any medication; those who had previous

history of peptic ulcer or were taking any medication were excluded.

Secondly, in their study (Maccini et al 1990) the subjects were asked to

collect saliva for 15 minutes in a cup prior to endoscopy, whereas I

collected a point-sample of saliva every half hour without giving them

any warning; they were not given any stimulus, oral or visual, which

might have increased the flow of saliva. It is possible that continuous

spitting, the method of collection used by Maccini et al (1990) increases the

salivary flow and this may be the reason for the difference between their

results and mine. Ohmura et al (1987) also found lower salivary EGF

concentrations in patients with DU as compared to controls; the authors of

this study have not made it clear how they collected the saliva samples,

whether they used any stimulus or a dental sucker to collect the saliva

samples. The authors of this study have classified DU as active, healing

and scarring on endocopic examination; I am not aware that any such

naked eye classification of DU is reliable. They found no difference in

salivary EGF concentration in controls subjects and patients in the scarring

group of DU.

During basal conditions the gastric juice EGF concentrations were similar

in all three groups, and I found no statistically significant difference

between them. The values of EGF concentration in gastric juice in controls, DU, NUD were 4.7, 3.8 and 5.5ng/ml respectively. Similarly Hansen et al (1989) also found no difference in gastric juice EGF concentration in patients with DU and in controls. However, Calabrò et al (1990) found lower EGF concentrations in the gastric juice in patients with DU than in the controls. This study was slightly different from mine, in that their subjects were asked to discontinue H2 receptor antagonist 12 hours before the samples of gastric juice were collected, whereas I asked my patients to stop H2 antagonists or proton-pump inhibitors 48 hours before the gastric secretion study. Secondly, they collected gastric juice samples at the time of endoscopy through the suction channel, whereas I collected the gastric juice samples by nasogastric tube, and discarded gastric juice collected in the first 20 minutes of the test. It is well known that during endoscopy or the insertion of a nasogastric tube there is an increase in salivary flow due to pharyngeal irritation and this may be the explanation for the difference in EGF concentrations in gastric juice between their study and mine.

In this study I have found no statistically significant difference between basal salivary EGF concentration and basal gastric juice EGF concentration in any diagnostic group. One may argue that the EGF concentration in the saliva and in the gastric juice is similar because the swallowed saliva is not being much diluted, and in that case basal gastric juice is merely swallowed saliva. However, previous work form this Department (Faber et al 1977) has shown that under basal conditions the stomach does produce some acid and therefore swallowed saliva should be diluted. The fact that there is no evidence of dilution suggests that the stomach itself produces EGF and thereby maintains the EGF concentration in gastric juice at about the same levels as in saliva. However, Konturek et al (1989) have found that

the concentration of EGF in gastric juice was several times less than that in saliva. This study was different from mine in two ways. Firstly, Konturek et al (1989) aspirated saliva for 15 minutes, which might result in an increase in flow of saliva. Secondly they have used a different method (dextran-coated charcoal) of separation of free and bound antibodies in the separation stage of radioimmunoassay, whereas I have used the double antibody method of separation, and this could explain the difference in results.

After histamine stimulation I found an increase in the salivary EGF concentration in each of the three groups; it became about double that observed during the basal period. I noticed that during the histamine period subjects complained of a dry mouth and had difficulty in providing salivary samples. There is, therefore, a possibility that the rise in the EGF concentration in the saliva in all three groups could have been the effect of sialo-concentration rather than an increased output of salivary EGF. In favour of this interpretation is the fact that the salivary EGF concentration increased in similar proportion in all these groups. Similarly Hansen et al (1989) found that salivary EGF concentration increases during thirst.

The gastric juice EGF concentrations and outputs increased after histamine in all three groups in my study. The increase was much greater in the DU than in the other two groups. This difference between DU and the other subjects could not be explained by the fact that the DU subjects also secreted more acid than the other two groups, because EGF concentration in stimulated secretion was independent of the rate of secretion. However, I know from previous studies that after histamine stimulation the secretion of hydrochloric acid and pepsin increases and these increases are on average greater in DU than in the other groups (Baron 1969). It is therefore

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possible that histamine stimulates EGF production in some manner that is linked to its stimulation of gastric acid or pepsin. In this respect it is interesting that there is evidence that EGF is secreted by the chief cells (Hansson et al 1990). Such a linkage would also destroy the very unlikely, but at present still just tenable hypothesis that the increase in gastric EGF production is a response to promethazine rather than histamine. Perhaps less unlikely is the possibility that the mechanism of the EGF release in response to histamine is mucosal damage produced by histamine itself.

One may argue that the possible sources for the increase in gastric juice EGF concentration after histamine in my study group include not only the stomach itself but also reflux from the duodenum, as Brunner's glands and pancreatic juice are known to contain EGF (Konturek et al 1989) and so does the pharyngo-oesophageal secretion (Sarosiek et al 1993). Duodenogastric reflux is most unlikely as a source because I found no correlation between gastric EGF concentration and calculated reflux (VR) in the basal circumstances during which there is considerable reflux present in the aspirate, and because there was virtually no reflux detectable after histamine stimulation (mean 0.7 ml/10 min). The oesophagus remains a possible source. Sarosiek et al (1993) have measured EGF secreted by the oesophagus. The authors of this study perfused a 7cm segment of lower oesophagus which was sealed off by two balloons to prevent salivary and gastric juice contamination, with saline and acid (HCl). The results of this study showed that the oesophagus secretes EGF after perfusion with saline, the mean concentration of EGF in recovered perfusate varied between 1.8 to 2.1 ng/ml, while EGF output varied between 9.3 and 11.1ng/minute. have also reported reduction in EGF produced by oesophagus when perfused with HCl. The EGF concentrations in gastric juice were 2.6, 3.6 and 4.6ng/ml during basal conditions in NUD, controls and in patients

with DU respectively in my study group. I have not used any balloons to prevent contamination of gastric juice by oesophageal mucus or swallowed saliva. I am not aware that the presence of a nasogastric tube in the oesophagus can stimulate the production of EGF by the oesophagus, though I cannot rule out the possibility. However, the massive twentyninefold increase in EGF output after histamine cannot conceivably have been accounted for by contamination from extra-gastric sources such as swallowed saliva or oesophageal mucus. Perhaps less unlikely is the possibility that the mechanism of the EGF release in response to histamine is mucosal damage produced by histamine itself, or by other agents such as *H pylori* (Lynch et al 1994) or aspirin (Konturek et al 1994), although none of these patients had been taking aspirin. The effects of *H pylori* infection on gastric juice EGF concentration in my study group have been discussed separately (p 211).

The results of my study agree with those of others (Ohmura et al 1987, Maccini et al 1990, Hanssen et al 1989) that basal EGF concentrations in saliva and gastric juice are not affected by gender, age or chronic smoking. However, Konturek et al (1989) found a significant reduction in salivary EGF during acute smoking, and Jones et al (1992) found a reduction in salivary EGF concentration in chronic smokers, and suggested that the decrease in salivary EGF predisposes to peptic ulcer in smokers. I also found no correlation between gastric juice EGF concentration and height.

I found no correlation between the salivary EGF concentrations and the gastric EGF concentrations in any of the groups either in the basal or histamine period, and this implies that gastric EGF is not just swallowed salivary EGF.

This study also showed that the difference in salivary EGF concentrations between basal and histamine periods was most marked in patients with DU. The EGF concentration increased by 64% in the NUD group, 98% in the control group and 131% in the DU group. A similar pattern was seen in the gastric juice EGF concentration; in the NUD and control groups there was an increase of sixfold whereas in the DU group it was twelvefold. This increase in EGF concentration in saliva and gastric juice cannot be explained by "washout" theory, as each basal and histamine period was of 90 minutes duration and there was a sustained increase in the EGF concentration in gastric juice and saliva. If these increases in gastric juice EGF concentration were due to "washout" phenomenon, then I would have observed a sudden rise in EGF concentration followed by a sudden fall. Therefore it is unlikely that the increase in the EGF concentrations in both saliva and gastric juice were due to "washout".

In order to see any difference between the DU group and all subjects without DU, the NUD and the control were combined into a Non-DU group. There was no difference in basal salivary and gastric EGF concentrations between DU and Non-DU groups. After histamine stimulation the DU groups produced more EGF in the saliva and in the gastric juice, again suggesting that it is the presence of DU that increases the secretion of EGF in saliva and in gastric juice.

5.2.2. EGF concentration and volume rate of gastric secretion

From these results so far it has become clear that the DU subjects secrete more EGF in gastric juice, in terms of both concentration and output after histamine stimulation, than the NUD and control groups. The DU

subjects differ from the other two groups in two ways, firstly they have a duodenal ulcer, secondly DU subjects are known to produce on average greater volumes of gastric secretion after histamine stimulation. I have examined my results in this context and explored whether DU subjects had a higher EGF concentration because they produce more gastric juice or because they have a DU. I have used the plateau values of V_G and V_{acid} (primary gastric secretion) of individual subjects during the histamine stimulation period to obtain median values of the plateau values. I divided these sixty subjects into two groups according to whether their plateau average was greater or less than median. It became clear that there was no correlation between the volume of gastric secretion and the concentration of EGF in the gastric juice. There was a positive strong relationship between the presence of DU and high EGF concentration irrespective of the volume rate of primary gastric secretion (V_G or V_{acid}).

This study has shown that the stomach secretes considerable amounts of EGF in response to histamine. Swallowed saliva cannot be the only source of gastric EGF, even in basal circumstances, because had that been the case then I would have observed lower concentrations of EGF in the gastric juice than in the saliva.

Other studies have suggested that EGF concentration is lower in saliva as well as in unstimulated gastric juice in those with duodenal ulcer as compared to controls (Ohmura et al 1987, Konturek et al 1991a, Maccini et al 1990, Li et 1993, Jones et al 1992). My data indicate quite the opposite in basal saliva and gastric juice, and the greater concentrations of EGF produced both in the saliva and in the gastric juice are further emphasised by the greater effect of histamine in the DU patients. The explanation of these discrepancies may lie largely in semantics and sampling errors: for

example, expectoration every 10 seconds for 10 minutes (Li et 1993, Rourk et al 1994), repeated expectoration for fifteen minutes (Maccini et al 1990), using a dental sucker to collect saliva over 10 minutes after lingual application of citric acid (Jones et al 1992) are not true basal circumstances. Another source of error to explain these discrepancies could be the fact that the high viscosity of saliva can lead to pipetting inaccuracies. I have measured two to three saliva samples during basal conditions and after histamine stimulation; these sample were analysed in duplicate, and the between-sample error in my study was 5%.

In these circumstances it is difficult to accept the view that EGF deficiency in saliva or gastric juice may be a cause of duodenal ulcer as suggested by various studies (Maccini et al 1990, Ohmura et al 1987, Calabrò et al 1990, Konturek et al 1991a). However, it seems more likely that the presence of an ulcer stimulates the production of greater amounts of EGF, both in saliva and gastric juice, and that this response represents the attempt at ulcer healing. Zandomeneghi et al (1991) have found hyperactive EGF-producing cells around the ulcer in patients with DU and they have suggested that this hyperfunction of EGF-producing cells might contribute to the in *vivo* repair of tissue damage. Wright et al (1990a, 1990b) and Hanby & Wright (1993) have reported that ulceration of the epithelium anywhere in the human gastrointestinal tract induces the development of a novel cell lineage from the gastrointestinal stem cells. This lineage initially appears as a bud from the base of the intestinal crypts adjacent to the ulcer. This lineage contains and secretes abundant EGF-like substance. They have suggested that after mucosal ulceration EGF-like substance is secreted to stimulate cell proliferation, regeneration and ulcer healing. This is a possible explanation of the high concentration of EGF in the gastric juice in patients with DU in my study; it is likely that the patients

with DU produce more EGF than controls in response to the presence of an ulcer.

5.2.3. EGF, ammonia and urea concentrations in gastric juice

In this study I have measured EGF concentration along with measurements of urea and ammonia concentrations in the gastric juice in three different diagnostic groups. I have used the $(ammonia)^3$: urea ratio for the presence or absence of *H pylori* infection. It is well known that *H pylori* produces the enzyme urease which splits the urea present in the gastric juice, converting it into ammonia and carbon dioxide. This results in a reduction of the concentration of urea and an increase in the ammonia in the gastric juice. I have chosen 1.25 as the cut-off point in the $(ammonia)^3$:urea ratio as this value has been calculated as my discriminant (as described in appendix 1). By comparison with data in the literature (Neithercut et al 1991) and my own data, I conclude that a value greater than 1.25 indicates the presence of more ammonia than I would expect in the absence of a catalyst, and therefore that the sample contains the urease of *H pylori*.

There were two purposes of this investigation, firstly to find out whether the presence of *H pylori* has any influence on gastric juice EGF concentration and to see if this explains the way in which *H pylori* is involved in the pathogenesis of DU, and secondly to test the sensitivity of this method of identifying the *H pylori* status, and to compare it with the ^{13}C-urea breath test which is well established as a diagnostic test for *H pylori* infection. I am aware of no study that has tested the sensitivity and specificity of the ammonia: urea ratio against the ^{13}C-urea breath test,

which is the one that I wished to use because it is non-invasive and has a high sensitivity (92%) and high specificity (100%) (Logan et al 1991). The results of this comparison have already been presented (p. 201) and I am confident that the $ammonia^3$:urea ratio is reliable.

The urease activity results in a reduction of urea in the gastric juice and an increase in ammonia concentration. In terms of the results of the breath test there was a significantly greater ammonia concentration in *H pylori* +ve subjects compared to *H pylori* -ve subjects; these results were similar to what others have found (Neithercut et al 1991). There was a reduced concentration of urea in the gastric juice in *H pylori* +ve subjects compared to *H pylori* -ve subjects, but there was no statistical difference between them. However, I did find a clear difference in the $(ammonia)^3$: urea ratio between those *H pylori* +ve and those -ve by the breath test.

There was no difference in the gastric juice EGF concentration between the 25 subjects who were *H pylori* +ve and 35 subjects who were *H pylori* -ve. Out of 25 *H pylori* +ve subjects there were 7 who had a DU while the remaining 18 were without; of these 9 were controls and 9 NUD. Of the *H pylori* infected subjects, those with DU had a much higher EGF than those without ($p < 0.001$). Of the subjects who were *H pylori* -ve there were 13 who had DU and the remaining 22 were equally divided between controls and NUD subjects. Again, DU who were *H pylori* -ve produced much higher concentrations of EGF in the gastric juice than those without DU ($p < 0.01$).

This study has shown that the presence or absence of a DU was related to the EGF concentration but the presence or absence of *H pylori* was not. A similar study by Lynch et al (1994) which included controls and *H pylori*

positive DU and *H pylori* +ve Non-DU, found that those subjects who were infected with *H pylori* had lower gastric juice EGF concentration, and there was no statistical difference between those who were *H pylori* +ve but also had DU, and those who were *H pylori* +ve and had no DU. These results are different from mine. I found that the presence of DU was associated with increased EGF in the gastric juice irrespective of *H pylori* status. The difference between Lynch et al (1994) and my study could be explained in two ways. Firstly, the method used in measuring EGF in the gastric juice differed. They used ELISA to measure EGF concentration in the gastric juice whereas I have used radioimmunoassay, which is more sensitive than ELISA. Secondly, they measured EGF in gastric juice only during the basal period, whereas I have measured during basal conditions and also after histamine stimulation.

The results of my study show that lack of EGF is not likely to be a cause of DU. Indeed, patients with duodenal ulcer produced much more EGF in gastric juice. This suggest the increased EGF concentration in such patients may be a response to the presence of an ulcer. The increased EGF concentration in patients with a DU bore no relationship to the *H pylori* status of the individual. In other words, I also appear to have shown that if *H pylori* causes DU, it does not do so by reducing the EGF of gastric secretion.

5.2.4. Prevalence of *H pylori* in dyspeptic subjects and controls

In this investigation I wanted to find the prevalence of *H pylori* in controls. Previous work carried out in this Department by Chandrakumaran (1991) examined the prevalence of *H pylori* in a

dyspeptic group of 252 patients who included subjects with DU, NUD (those dyspeptics with normal endoscopy finding), Oesophagitis and a Miscellaneous group which included patients with gastric carcinoma and gastric ulcer. The methods used for the diagnosis of H pylori in this study were endosocopy and biopsy for the CP urease test (CP = Campylobacter pylori, the old name for H pylori), histology and culture. There were no controls in that study as there was no reliable non-invasive method then available to find the prevalence of H pylori in controls. We felt it was ethically unacceptable to do endoscopy and biopsy on healthy controls. The aim of my study was to find the prevalence of H pylori in healthy controls and compare it with prevalence in the dyspeptic group. I included 100 healthy volunteers in my study, with age and sex matching with Chandrakumaran's dyspeptic subjects. I have used the ^{13}C-urea breath test to find the prevalence of H pylori in these volunteers. This test is non-invasive and has a high sensitivity and specificity, 92%, 100% respectively using histology and/or culture as 'gold standard' (Logan et al 1991).

The results of my study showed that the prevalence of H pylori in asymptomatic controls increases with age as observed by other workers (Jones et al 1986, Dooly et al 1988, Dill et al 1990, Meyer et al 1991, Peterson et al 1991, Graham et al 1991b, Asaka et al 1992, Lin et al 1993, Smoak et al 1994, Vaira et al 1994). The overall prevalence of H pylori was 39%: this was similar to the findings of other studies from developed countries, 41% (Vaira et al 1994), 32% (Dooley et al 1989) 36% (Wilhemsen et al 1994). However, Graham et al (1991a) have reported a higher (52%) prevalence of H pylori infection in the Houston metropolitan area. The prevalence of H pylori in asymptomatic controls is much higher in developing countries. Majmudar et al (1990) have reported 100% prevalence of H pylori in an

Indian population, Megraud et al (1989) found that 80-90% of populations of Algeria, Vietnam and the Ivory Coast were infected with *H pylori*.

The prevalence of *H pylori* in my study was highest in the age group of 46-55 years, being 68.4%. Similar observations were made by Mossi et al (1993) and Meyer et al (1991), although Vaira et al (1994) found the rather lower figure of 59.1% in same age group. There was a tendency towards reduction of *H pylori* infection in the age group over the 65 (25%) in my study; similar findings were also observed by Mossi et al (1993). My study also suggested that the prevalence of *H pylori* was greater in men than in women, and this finding could not be explained on the bases of socio-economic status, smoking habits or race. However there are many studies in the literature which have shown that there is no difference in the prevalence of *H pylori* in men and women (Graham et 1991a, Megraud et al 1989, Wilhelmsen et al 1994, Forman 1993, Rocha et al 1992, Mossi et al 1993), although Fich et al (1993) have reported an increased prevalence of *H pylori* among women smokers. In this last case, therefore, the result appeared to be in the opposite direction to mine. For the present, I think I am prepared to accept that my finding was a sampling error.

I have found no statistically significant difference between smokers and non-smokers although there was trend a towards lower prevalence among smokers (*H pylori* +ve 10 out of 38 in smokers, non-smokers 29 out of 62, χ^2 0.068). Most studies have found no difference in the prevalence of *H pylori* among smokers and non-smokers (Graham et 1991a, Forman 1993, Vaira et al 1994).

Chandrakumaran found a prevalence of *H pylori* in the DU group lower than expected. Many other studies have put that prevalence at as high as

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90-100% (Graham et al 1989, Tytgat et al 1992, Tygat et al 1993, Rohrbach et al 1993, Sandikci et al 1993, Ateshkadi et al 1993) whereas the overall figure for Chandrakumaran's study was only 70%. One reason for finding such a low prevalence of *H pylori* in DU could be that he carried out this investigation during 1988-1990, and it is possible that his investigative techniques were not as sensitive then as now. However, his study showed that no CP-urease positive subject was found to be *H pylori* negative on histology and/or culture, that all CP-urease positive subjects were positive by histology/culture, and that of the CP-urease negative subjects, 13% were found to be *H pylori* positive on histology/culture. These findings are in line with other studies and suggest that there is internal consistency between the various methods of examination. Moreover, *H pylori* and active chronic gastritis are known to be strongly associated and the remarkably accurate 100% correlation (both positive and negative) between these two entities in his results strengthen confidence in the accuracy of his techniques for diagnosing *H pylori*.

The prevalence of *H pylori* in Chandrakumaran's group (for all ages studied) in DU, NUD, miscellaneous and oesophagitis were 70, 67, 61 and 30% respectively which were within the ranges found in other clinics, [Spiliadis et al 1991 (53% in DU), Hsu et al 1992 (76% in DU), Kachintorn et al 1993 (67.7% in DU), Uyub et al (50% in DU)]. In all of these studies, the criterion for diagnosis was histology and/or culture. and in all these studies three to five biopsies were taken at the time of endoscopy.

The ^{13}C-urea breath test has a sensitivity and specificity of 92% and 100% compared with histology and/or culture (Logan et al 1991), and therefore I have compared the results of *H pylori* prevalence in controls in my study with those of Chandrakumaran's study of *H pylori* prevalence in

dyspeptic subjects. Thus at first glance it appeared to me that *H pylori* was present more frequently in DU, NUD and miscellaneous group than in controls, but that patients with oesophagitis have a low proportion with the infection. However, in view of the marked effect of age on the prevalence of *H pylori* as observed in other studies (Jones et al 1986, Dooly et al 1988, Dill et al 1990, Meyer et al 1991, Peterson et al 1991, Graham et al 1991b, Asaka et al 1992, Lin et al 1993, Smoak et al 1994, Vaira et al 1994) and in my control group, I decided to compare the prevalence of *H pylori* in the individual age groups. When this was done, a greater proportion of *H pylori* positive patients in DU was present only in two age groups 36-45y (p=0.005) and 56-65y age group (p=0.05).

There was a greater prevalence of *H pylori* in the NUD group as compared to the control group only in one age group 36-45 (p=0.002). The prevalence of *H pylori* in dyspeptic subjects (NUD) was 67%, compared with other studies in which its prevalence varies between 43 and 87% (Rohrbach et al 1993, Lambert et al 1993).

The prevalence of *H pylori* was greater in the controls than in the oesophagitis group but the difference was not significant (χ^2, p=0.084). The prevalence of *H pylori* was much higher in the DU than in the oesphagitis group (p<0.0001). There was no difference between the prevalences of *H pylori* infection in the Miscellaneous and control groups.

There was no effect of gender or smoking on the prevalence of *H pylori* in Chandrakumaran's dyspeptic group although the apparent effect of these factors was clearly demonstrable in the control group of my study. However, Bateson (1993) found an increase in *H pylori* prevalence among DU smokers; he suggested that the excess of peptic ulcer disease in cigarette

smokers may be explained by their increased susceptibility to *H pylori* infection. On the other hand, Lindell et al (1991) have found a lower prevalence of *H pylori* among smokers (18%) than non-smokers (45%), and this difference was statistically highly significant. They suggested that the lower prevalence of *H pylori* in smokers might be a direct toxic effect of nicotine on colonization with *H pylori*. This study was also carried out on subjects with non-ulcer dyspepsia.

The explanation of this observation may be related to our other observation that when the data are adjusted for age, the patients with reflux oesophagitis had a lesser infection rate than patients with DU and almost even than normal subjects. The difference between the oesophagitis and other groups is that the patients with reflux often have acid in the oesophagus and the controls do not. Acid is known to destroy *H pylori* (Marshall et al 1990), and if the organism usually enters the stomach from the mouth then an acid barrier in the oesophagus might reduce the chance of the organism reaching the antrum.

The mechanism whereby dyspepsia (originating in any cause other than reflux) is linked with an increased likelihood of infection with *H pylori* could be direct, i.e. dyspepsia might predispose to the organism or the organism might produce dyspepsia: alternatively, the relationship might be indirect through some factor which links the complaint of dyspepsia with a breach of defences against infection. The passage of an endoscope might itself constitute such a link: many patients of Chandrakumaran had undergone endoscopy on one or more previous occasion. It is known that the organism is often present in saliva (Krajden et al 1989, Ferguson et al 1993) and in dental tartar (Majmudar et al 1990, Krajden et al 1989, Desai et al 1991, Shames et al 1989), and this oral infection may constitute a

reservoir of organisms that the endoscope might carry down to the stomach. Whatever the mechanism, it is one that has overridden any possible protective effects of the female gender and cigarette-smoking that were manifest in the control subjects.

In Chadrakumaran's DU group aged 56-65 the prevalence of H pylori infection was 91%, although one must set against that the small number of 12 patients in that subgroup. It is also the case that in certain parts of the world the H pylori prevalence is low despite a high prevalence of DU (Holcombe 1992, Katelaris et al 1993). That the prevalence of the H pylori organism is approximately equal in controls, DU and NUD within certain age groups is supported by a report from Greece which recorded a prevalence of H pylori of 53% in patients with DU, 47% in patients with duodenitis and 55% in control subjects (Spiliadis et al 1991), although the numbers in that study were small. In any case, it would not be surprising to find very different figures for the prevalence of H pylori in various groups from different centres. Patients with a long history of duodenal ulcer would be likely to be older, and to have had more endoscopies than the average; the effect of previous endoscopies might be enhanced if some were carried out in the period before this organism was recognised and therefore before special steps were taken to prevent instrumental infection. Patients with a short history might be having their first endoscopy, would be likely to be younger than average, and would have an even smaller infection rate if they were female and smokers. The important point about the low prevalence in duodenal ulcer patients in Chadrakumaran's study was that it was not significantly different from that in the other dyspeptic groups (apart from reflux oesophagitis). It is difficult to believe that there was some error in detecting the organism in our duodenal ulcer group that did not also apply to the other dyspeptic

groups. The laboratories involved in the detection of *H pylori* had no knowledge of the endoscopic diagnosis, so it is unlikely that the results were biased.

In this study, *H pylori* infection was equally common in DU and NUD patients, and in those with other endoscopically diagnosed lesions (except reflux oesophagitis); and in normal volunteers in the age group of 16 - 35y, 46-55 and in those over the age of 66y. My suggested explanation for these relationships is that, rather than *H pylori* causing duodenal ulcer, duodenal ulcer (and other causes of dyspepsia) invite infection with *H pylori* (via endoscopy). A previous study from this Department has already failed to show any evidence of an aetiological link between DU and *H pylori* via increased maximal gastric secretion (Chandrakumaran, Vaira & Hobsley 1994). In other words, that study also argues against *H pylori* being involved in the production of duodenal ulcer. Where does the evidence of that, and my present study, leave the well-documented reports from several centres that eradicating the organism results in healing of the DU with a longer relapse-free interval than the effect of H2 antagonists? A possible explanation is that the agents that eradicate *H pylori* have some ulterior effect (for example, stabilisation of the mucosa) that is responsible for the healing and for the protection from relapse.

My finding that most patients attending an Endoscopy Unit for the investigation of dyspepsia are apparently no more likely to have the infection than normal controls requires further discussion. Only in two diagnostic age group categories (36-45 and 56-65) was the prevalence of *H pylori* in DU higher than in the controls, and indeed only in one age group (36-45) was the prevalence of *H pylori* in the NUD group higher than in controls. However, I cannot rule out the possibility that the

prevalence of *H pylori* was really greater in DU in all age groups than controls, because the numbers in each age group were small and type 2 errors might have been present (next paragraph). Again, I found no difference in the prevalence of *H pylori* in DU and NUD groups, but I cannot rule out the possibility of type 2 error.

In order to assess the likehood of a type 2 error, I have calculated the size of sample required in those age groups where the difference in prevalence of *H pylori* between two diagnostic groups was not significant (table 6.1, p.222). These values for the size of samples were calculated at 5% significance (0.05) and 10% (0.1) chance of making type 2 errors. As seen in the table, in most of the age groups the number of subjects studied was smaller but in some age groups the required numbers of subjects are huge: for example more than 200,000 subjects are needed in the age group of 46-55 y in control and DU groups. This suggest that there is indeed no difference between control and DU subjects in that age group. Similarly the prevalence difference of *H pylori* in smoker and non-smokers in the control group was nearly significant, but would have been significance if the number of subjects studied in each group was 91 rather than the 38 smokers and 62 non-smokers who were studied.

Table 6.1

Sample size required in each diagnostic group to find the significance in

H pylori prevalence

Age group	Diagnostic groups	Number of subjects studied	Number of subjects needed in each group
16-25y	Cont vs DU	19 vs 4	28
26-35y	Cont vs DU	14 vs 13	219
46-55y	Cont vs DU	19 vs 25	211,500
66 and above	Cont vs DU	8 vs 4	13
16-25y	NUD vs DU	5 vs 4	421
26-35y	NUD vs DU	11 vs 13	171,000
36-45y	NUD vs DU	18 vs 18	1280
46-55y	NUD vs DU	37 vs 25	597
56-65y	NUD vs DU	31 vs 12	44
66 and above	NUD vs DU	19 vs 4	254
36-55 y	DU vs combined	43 vs 65	2000
56-65 y	DU vs combined	12 vs 42	41
16-25 y	Cont vs NUD	19 vs 5	55
26-35 y	Cont vs NUD	14 vs 11	58
46-55 y	Cont vs NUD	19 vs 37	650
56-65 y	Cont vs NUD	21 vs 32	600
66 and above	Cont vs NUD	8 vs 19	25
36-75 y	Cont vs Oesoph	67 vs 28	106

DU = duodenal ulcer   Cont = controls

NUD =non-ulcer dyspepsia   Oesoph = oesophagitis

Combined = combined group of NUD and miscellanous

5.2.4. Quantification of swallowed saliva in gastric juice

There have been various attempts to solve the problem posed by the presence of swallowed saliva in the gastric juice. Many workers have used different methods to try to prevent the amount of swallowed saliva: continuous suction (Ihre 1938), asking the patients to expectorate saliva (Kirsner et al 1956, Baron 1963) or inserting dental plugs (Makhlouf et al 1966). All these methods may well increase salivary output, and none of these workers was certain that the salivary contamination had been prevented.

The only way to quantify the amount of swallowed saliva in the gastric juice seems to be to use a marker which is present only in saliva. It was Teichmann (1953) who suggested that thiocyanate in the gastric juice comes from swallowed saliva. There had been no studies to verify his statement until Ruddell et al (1977) measured thiocyanate in fasting and secreting stomach and in saliva, and concluded that thiocyanate in gastric juice was of salivary origin. Boulos et al (1980) measured thiocyanate in saliva and in gastric juice during basal conditions and after stimulation with histamine: they found that despite the increase in gastric secretion after histamine there was no matched fall in the concentration of thiocyanate in the gastric juice. Moreover, in one third of the gastric juice specimens, the thiocyanate concentration was greater than in the simultaneous samples of saliva. They concluded that thiocyanate was not an appropriate marker.

Previous work from this Department has suggested that aspirated gastric juice, its volume corrected for pyloric loss (Vcor), consist of a primary

(hypothetical) "pure" gastric juice in a volume VG ml/min, plus duodenogastric reflux in a volume VR ml/min.

$$V_{cor} = V_G + V_R$$

This calculation ignores the amount of swallowed saliva. Whitfield (personal communication) calculated a mathematical model of aspirated gastric juice which partitions the volume-corrected aspirate into three different volumes, Vacid ml/min of primary gastric juice, plus duodenogastric reflux (Vreflux ml/min) plus swallowed saliva (Vsal ml/min),. This method depended upon taking into consideration the published evidence about electrolyte concentrations in pure gastric juice (Hirschowitz 1961, Hobsley & Gardham 1970, Hobsley & Silen 1970, Hobsley & Whitfield 1977, Hobsely & Whitfield 1978) and in duodenogastric reflux (Hobsley 1974, Hollander 1932, Faber et al 1974, Fiddian-Green et al 1979) together with the published electrolyte concentrations in saliva at varying rates of secretion (Jenkins 1978, Schneyer & Levin 1955, Prader et al 1955, Kostlin & Rauch 1955).

$$V_{cor} = V_{acid} + V_{reflux} + V_{sal}$$

The EGF output in the aspirated gastric juice is contributed to by primary gastric secretion (VG or Vacid), duodenogastric reflux and swallowed saliva. I have calculated the EGF concentration in primary gastric secretion (VG or in Vacid) by simple regression of EGF output/10minute against the volume rate of primary gastric secretion (VG and Vacid) separately in the same unit time. The mean values obtained were 3.43ng/ml for VG and 5.47ng/ml for Vacid respectively. By subtracting the amount of EGF contributed by primary gastric secretion (VG and Vacid separately) I calculated the EGF which presumably originated from any

extragastric source such as duodenogastric reflux or swallowed saliva. The result obtained by these calculations, using the V_G formula (which ignores swallowed saliva) was that the EGF concentration in the extragastric component of gastric juice i.e. duodenogastric reflux, was -1.2ng/ml which suggests that there was no EGF in duodenogastric reflux. By contrast using the V_{acid} formula, the concentration of EGF in the extragastric component was 3.73ng/ml.

My hypothesis is that all the extragastric EGF comes from the swallowed saliva. In that event, the calculated EGF of the swallowed saliva is of the same order as is found in expectorated saliva. If this hypothesis is accepted, it follows that Whitfield's mathematical model is an accurate representation and can be used to estimate the amount of swallowed saliva in the gastric juice.

One could consider the possibility that the extragastric EGF comes at least possibly from one or both of two other sources. EGF is known to be present in the Brunner's glands of the duodenum so duodenogastric reflux might be a source. However, there is no correlation between EGF output and VR (or Vreflux). There is also EGF in oesophageal lining cells (Sarosiek et al 1993, Jankowski et al 1992); the output of EGF from these cells increases considerably if the oesophageal lumen is infused with pepsin or saline solution. However, no such infusate was used here, and the same study (Sarosiek et al 1993) demonstrated that infusion of the oesophagus with acid reduces EGF production. In the circumstances of my study, in which the only factor that might have produced an effect on the oesophageal mucosa was the refluxed acid from the stomach in response to the irritation of the in-dwelling nasogastric tube, it seems unlikely that the oesophageal contribution was important. Finally in this study

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regression analysis between volume of V_G or V_{acid} and EGF output suggests that the EGF is coming from the stomach.

Whitfield's calculations are available as a FORTRAN computer programme (see appendix 2).

I chose to apply these calculations during the basal period, as during basal conditions the contamination of gastric juice by duodenogastric reflux and swallowed saliva is maximal, and it would not be possible to calculate accurately the amount of swallowed saliva during histamine stimulation because of the massive increase in gastric secretion. Moreover, during the histamine period the subjects always complained of dry mouth and I feel this means that any swallowed saliva was a negligible contribution to the gastric aspirate

CHAPTER SEVEN

CONCLUSIONS

Conclusions

(New findings are in italics, confirmation of the findings of others in ordinary type)

1. *This thesis contends that after histamine stimulation DU subjects produce more EGF in gastric juice and saliva than do controls and Non-ulcer dyspepsia (NUD) subjects. The higher EGF concentration and output in patients with DU in this study could be a response to the presence of the ulcer, aimed at promoting healing.*

2. *There is no correlation between salivary and gastric EGF concentrations, which suggests that swallowed saliva is not the only source of the EGF in gastric juice and that the stomach is capable of producing EGF even under basal condition.*

3. There was no correlation between gender, age and smoking with either salivary or gastric EGF concentration.

4. *It is known that DU subjects produce more gastric juice than controls; this study suggests that DU subjects also produce more EGF in gastric juice in response to histamine stimulation, but there was no correlation between volume rate of gastric secretion and the concentration of EGF because the concentration of EGF was approximately constant. DU subjects produce more EGF irrespective of the volume rate of gastric secretion, a finding which suggests that the presence of ulcer was more important than the volume rate of gastric juice secretion in determining the EGF concentration.*

5. *There is no correlation in any diagnostic group between the presence of* H pylori *infection and the EGF concentration in gastric juice, either during basal or after histamine stimulation. This suggests that if* H pylori *causes DU it does not do so by reducing the gastric juice EGF concentration. DU subjects had a higher EGF concentration than control subjects, irrespective of their* H pylori *status.*

6. Prevalence of *H pylori* infection increases with the age and there was a tendency towards a fall in those over aged over 65 years in both dyspeptic and controls subjects.

7. *There appeared to be a higher prevalence of* H pylori *in healthy men than women; there was no difference between* H pylori *infection in smokers and non-smokers but (only in the control group) there was a tendency for smoking to protect against* H pylori *infection.   These findings are open to some doubt as the numbers studied have been quite small and the results run counter to other evidence.*

8. *The prevalence of H pylori was higher in DU than in controls in only two age groups (36-45 and 56-65); however, the numbers of subjects studied in the various subgroups were small, and possibility of a type 2 error cannot be ruled out.*

9. There was no gender or smoking effect on the prevalence of *H pylori* in dyspeptic subjects.

10. The presence of *H pylori* infection causes a decrease in urea concentration and increase in ammonia concentration in gastric juice. *H*

pylori status can be determined by measuring ammonia: urea ratio (ammonia test) and *this method may be as reliable as the* ^{13}*C-urea breath test.*

11. *Until now the volume of swallowed saliva in gastric juice has been ignored due to lack of a suitable marker of saliva. Whitfield's hypothesis based on a mathematical approach calculates the amount of swallowed saliva in gastric juice. In this thesis by using EGF as a marker of saliva, Whitfield's hypothesis has been tested and found accurate.*

APPENDIX I

The calculation of ammonia/ urea ratio in gastric juice

The urea molecule when hydrolysed forms carbon dioxide (CO_2) and ammonia (NH_3), as shown below,

$$CO(NH_2)_2 + H_2O <=====> CO_2 + 2NH_3,$$
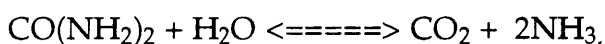
a reversible reaction. At equilibrium, i.e. when the rates of forward and reverse reactions are equal, the law of mass action states that the proportions of the products of the active masses (roughly, concentrations) on the two sides of the equation are constant, the equilibrium constant K.

$$K = [CO_2].[NH_3].[NH_3] / CO(NH_2)_2$$

In this equation, the value of $[CO_2]$ must be the same as the value of $[NH_3]$ since the two compounds are formed as one mole of CO_2 to each of the two moles of NH_3. Therefore the equation can be re-written as

$$K = [NH_3]^3 / [CO_2(NH_2)_2]$$

By comparison with data in the literature and our own data, we conclude that a value greater than 1.25 indicates the presence of more ammonia than we would expect in the absence of a catalyst, and therefore that the sample contains the urease of H pylori.

APPENDIX II

Calculation of Vacid, Vsal and V reflux in aspirated gastric secretion

$V_{cor} = V_{acid} + V_{alk}$

V_{cor} = volume of gastric juice corrected for pyloric loss
V_{acid} = volume of primary gastric juice
V_{alk} = volume of alkaline component in the gastric juice

Electrolyte output is calculated as;

$V_{cor} \times E_{cor} = V_{acid} \times E_{acid} + V_{alk} \times E_{alk}$

E_{cor} = electrolytes in V_{cor}
E_{acid} = electrolytes in V_{acid}
E_{alk} = electrolytes in V_{alk}

V_{acid} is replaced by;
$V_{acid} = V_{cor} - V_{alk}$

$V_{cor} \times E_{cor} = (V_{cor} - V_{alk}) \times E_{acid} + V_{alk} \times E_{alk}$
or

$V_{cor} \times E_{cor} = V_{cor} \times E_{acid} - V_{alk} \times E_{acid} + V_{alk} \times E_{alk}$

or

$V_{alk} \times E_{acid} - V_{alk} \times E_{alk} = V_{cor} \times E_{acid} - V_{cor} \times E_{cor}$

or
$V_{alk} (E_{acid} - E_{alk}) = V_{cor} (E_{acid} - E_{cor})$

or
$$V_{alk} = \frac{V_{cor} (E_{acid} - E_{cor})}{(E_{acid} - E_{alk})}$$

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If limits are set to the alkaline concentration, the upper and lower limit can be calculated for the V_{alk} for each electrolyte concentration. If the minimum of the upper values of V_{alk} is taken, and the maximum of the lower value of V_{alk}, and their mean is then taken, a reasonably viable value of V_{alk} can be calculated:

$$V_{alk} = V_{reflux} + V_{sal}$$

V_{reflux} = volume of duodenogastric reflux
V_{sal} = volume of saliva
then for any electrolyte,

$$V_{alk} \times E_{alk} = V_{reflux} \times E_{reflux} + V_{sal} \times E_{sal}$$

E_{alk}= electrolytes in alkaline component (V_{alk})
E_{reflux}= electrolytes in V_{reflux}
E_{sal}= electrolytes in V_{sal}

V_{reflux} can be replaced ($V_{alk} - V_{sal}$)
then
$$V_{alk} \times E_{alk} = (V_{alk} - V_{sal}) \times E_{reflux} + V_{sal} \times E_{sal}$$
or
$$V_{alk} \times E_{alk} = V_{alk} \times E_{reflux} - V_{sal} \times E_{reflux} + V_{sal} \times E_{sal}$$

If E_{sal} is replaced by a linear equation of the form $A. V_{sal} + B$, representing the linear relationship of $[Na^+]$ in saliva with the volume of saliva, then

$$V_{alk} \times [Na^+]_{alk} = V_{alk} \times [Na^+]_{reflux} - V_{sal} \times [Na^+]_{reflux} + V_{sal} \times (A.V_{sal} + B)$$
or
$$V_{sal} \times [Na^+]_{reflux} - (A.V_{sal}^2 + B.V_{sal}) = V_{alk} \times [Na^+]_{reflux} - V_{alk} \times [Na^+]_{alk}$$
or

$$V_{sal} \times [Na^+]_{reflux} - A.V_{sal}^2 - B.V_{sal} = V_{alk} \times [Na^+]_{reflux} - V_{alk} \times [Na^+]_{alk}$$
or
$$- A.V_{sal}^2 - B.V_{sal} + V_{sal} \times [Na^+]_{reflux} = V_{alk} \times [Na^+]_{reflux} - V_{alk} \times [Na^+]_{alk}$$
or

$$- A.V_{sal}^2 - V_{sal}(B + [Na^+]_{reflux}) = V_{alk}([Na^+]_{reflux} - [Na^+]_{alk})$$

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or

$$A \cdot V_{sal}^2 + V_{sal}(B - [Na^+]_{reflux}) + V_{alk}([Na^+]_{reflux} - [Na^+]_{alk}) = 0$$

this is in form of quadratic, and can be solved by usual formula.
In the equation, A is 1.87 (i.e. that in the straight line relationship between the $[Na^+]$ of whole saliva, and $V_{sal}/10minutes$), and (B - $[Na^+]_{reflux}$) is 150. i.e. B=-1, and that is the intercept

$$[Na^+]_{sal} = 1.87 \times V_{sal} - 1$$

This equation was calculated from a variety of sources, because at the time, there were no suitable studies. In particular, the study of whole saliva did not have a very great range of values for $[Na^+]$ or the volume.

By solving the quadratic, a volume for V_{sal} is calculated, and hence V_{reflux} can be calculated.

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