

A S T U D Y O F G A S T R I C
Helicobacter pylori
I N F E C T I O N

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Thesis submitted for the degree of
Doctor of Philosophy
University of Edinburgh
1993

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ABSTRACT

Helicobacter pylori (*H.pylori*) was studied in duodenal ulcer (DU) and gastric cancer patients.

The relationship between dietary linoleic acid, antral *H.pylori* infection and DU was studied. A detailed dietary history was obtained from DU patients and matched non-ulcer dyspepsia controls. The fatty acid content of abdominal adipose tissue biopsies was measured in both groups. Levels of dietary and adipose tissue fatty acids were related in controls and non-smokers. The adipose fatty acids did not reflect dietary intake in DU patients or controls who smoked. DU is associated with reduced adipose tissue linoleic acid content and this is accentuated by smoking.

In an *in vitro* test, linoleic acid was not shown to be metabolised to more saturated fatty acids by *H.pylori*.

Eradication of *H.pylori* from the stomach prolongs remission in DU disease but gastric re-infection is common and leads to relapse. Tooth plaque was cultured from DU patients and from matched controls. *H.pylori* was cultured from the antrum in 89% and from tooth plaque in 19% of duodenal ulcer patients. The identity of *H.pylori* was confirmed by biochemical testing and DNA analysis. Some strains were examined by electron microscopy. Ribotyping

showed that the organisms present in tooth plaque and the antrum were identical but each individual carries a separate strain. Gastric re-infection by *H.pylori* could originate from dental plaque.

A general population of hospital inpatients with and without teeth were studied by bacteriological culturing of tooth plaque, by urease testing of tooth plaque and by measuring plasma antibody to *H.pylori*. No significant difference in antibody levels was found in either group.

In an *in vitro* experiments, bile acids inhibited or prevented growth of *H.pylori* supporting the view that biliary post-reflux gastritis and type B *H.pylori* gastritis are of different aetiology.

Gastrectomy specimens from 83 patients who underwent gastrectomy for primary gastric cancer and from 34 controls who underwent gastric surgery for peptic ulcer were reviewed histologically for *H.pylori*. No associations were found between *H.pylori* and gastric intestinal metaplasia, tumour extent or stage.

A cheap alternative "in-house" urea solution was developed and is described. This is satisfactorily sensitive and specific for the diagnosis of *H.pylori* and could be used for epidemiological studies in field work. These results are discussed and related to the work of others.

Statement

I declare that the work contained in this thesis was performed by myself. Any work performed by others has been acknowledged in the text.

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ETHICAL PERMISSION

Ethical permission for these studies was obtained from the Lothian Health Board Ethical Committee for Medicine and Oncology. Informed consent was obtained from each volunteer and patient.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the followings for their support and guidance during this study.

I am particularly grateful to Dr MA Eastwood, Dr AC Scott, and Dr KR Palmer, Western General Hospital, Edinburgh.

I am grateful to Mr G Brydon, G I Laboratories, Western General Hospital, Edinburgh, for his help and support. I am also grateful to Dr RJ Owen, Central Public Health Laboratories, National Collection of Type Cultures, London, for nucleic acid analysis of *Helicobacter pylori*, Mr C Rajgopal, Western General Hospital, Edinburgh, for adipose tissue biopsy, Dr AM Lessells, Western General Hospital, Edinburgh, for helping in histopathological study, Miss Alice Michie, Senior Dietician, Western General Hospital, Edinburgh, for dietary analysis, Mr F Donnelly, Western General Hospital, Edinburgh, for electron microscopic examination of *Helicobacter pylori*, Dr RA Elton, Medical School, Edinburgh, for statistical analysis of data, Mrs Olga Greenan, Western General Hospital, Edinburgh, for helping during culturing of *Helicobacter pylori*, Sister Crosbie and Sister Crichton, Western General Hospital, Edinburgh, for helping during collection of samples, Miss A Stewart and Mrs A Jenkinson, GI Annexe, Western General Hospital, Edinburgh, for assistance with editing and word processing.

I am grateful to the Ministry of Health and Family Welfare, People's Republic of Bangladesh, for leave of absence.

None of these would have been possible without the generous support of World Health Organisation.

ABBREVIATIONS

- BAO = Basal acid output
- BRENDA = Bacterial restriction-endonuclease
digest-analysis
- cAMP = Cyclic adenosine monophosphate
- CLO = *Campylobacter* like organism
- cm = Centimetre
- DNA = Deoxyribonucleic acid
- DU = Duodenal ulcer
- duTP = Deoxyuridine triphosphate
- EDTA = Ethylenediaminetetra-acetic acid
- Fab = Antigen-binding fragment
- FAME = Fatty acid methyl esters
- FFA = Free fatty acid
- GLC = Gas-liquid chromatography
- gm = Gram
- H₂ = Histamine 2 receptors
- HETE = Hydroxyeicosa tetraenoic
- HLA = Human leucocytic antigen
- HP = *H. pylori* (*Helicobacter pylori*)
- Kg = Kilogram
- KV = Kilovolt
- LA = Linoleic acid
- μl = Microlitre
- MAO = Maximal acid output
- m = Metre

ml = Millilitre
mm = Millimetre
 μm = Micrometre
mg = Milligram
mmol = Millimol
min = Minutes
M = Molar
MUSFA = Monounsaturated fatty acid
NADH = Reduced nicotinamide adenine dinucleotide
NCTC = National collection of type cultures
nm = Nanometre
NSAID = Non-steroidal anti-inflammatory drug
OFN = Oxygen free nitrogen
PAO = Peak acid output
PG = Prostaglandin
PUSFA = Polyunsaturated fatty acid
QF = Quick fit
RFLP = Restriction fragment-length polymorphism
rpm = Revolutions per minute
rRNA = Ribosomal ribonucleic acid
TX = Thromboxane

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CHAPTER 1

INTRODUCTION

Peptic ulceration results from a break in the integrity of the upper gastrointestinal tract mucosa. The ulcer crater extends through the muscularis mucosae (Ivy et al., 1950) and is surrounded by acute and chronic inflammatory cells.

Peptic ulceration is a very common disease with serious complications and great economic significance, and has therefore been extensively studied. Despite this, its exact pathogenesis is not yet known. Until 10 years ago study of the cause and treatment of peptic ulceration concentrated on the view that it resulted from either a lowering of the defences in the mucosa or an increase in digestive attack by acid and pepsin (Graham, 1989).

One of the most interesting findings during the past decade has been that of the close association between the bacterium *Helicobacter pylori* (*H.pylori*), type B gastritis, and peptic ulceration. There are now strong grounds for believing that peptic ulceration is, at least in part, an infectious disease.

The work described in this thesis explores aspects of peptic ulcer pathogenic factors and addresses the following issues:

The relationship between dietary fatty acid intake and duodenal ulcer; the capacity of *H.pylori* to metabolise linoleic acid; a possible oral reservoir for infection of the stomach by *H.pylori*; the prevalence of *H.pylori* in a population of hospital inpatients; the influence bile acids upon the growth of *H.pylori*; an exploration of the possible

link between the presence of *H.pylori* and gastric cancer; and the development of a simple, rapid urease test to identify *H.pylori*.

1.1 THE EPIDEMIOLOGY AND CAUSES OF PEPTIC ULCERATION - A REVIEW OF THE LITERATURE

1.1.1 EPIDEMIOLOGY

1.1.1.1 Prevalence

Epidemiological studies are made to elucidate patterns of disease incidence and prevalence that may highlight causal or protective factors. The first report of prospective studies of the prevalence and epidemiology of peptic ulceration appeared in 1950 (Ivy et al., 1950). These showed considerable differences between and within countries. Thus, the prevalence of active duodenal ulcers (DU) found in Finland, by an endoscopic survey of 358 normal subjects, was 1.4% with a life-time prevalence of 5.9% (Ihamaki et al., 1979). The life-time prevalence in American males is 10% and 4% for American females (Langman, 1979; Grossman, 1980). The yearly incidence of DU in Denmark between 1963 and 1968 was 0.15% with a linear trend of age-specific increase reaching 0.3% by the age of 75 to 79 years in males and 0.3% in females (Bonnievie, 1975). The annual incidence of DU in Yorkshire, England, was 0.21% in males and 0.06% in females (Pulvertaft, 1959) and in the United States was 0.08% in males and 0.04% in

females (Kurata et al., 1985). DU is four times commoner than gastric ulcer in the United States (Kurata et al., 1984).

1.1.1.2 Regional variation of peptic ulceration

There appears to be a real geographical variation in the prevalence of DU. Its presentation, course and complications also differ from country to country and region to region (Pulvertaft, 1959; Langman, 1979; Tovey, 1979; Moshal et al., 1981; Hugh et al., 1984). The overall prevalence of DU is higher in Scotland and (to a lesser extent) in the north of England than in the south of England (Langman, 1979). DU is commoner along the west coast of Africa than along the east coast, in northern India than in the south, and amongst Chinese rather than Japanese in Sumatra (Lam, 1989). In Japan, gastric ulcer is five to ten times commoner than DU (Sonnenberg, 1985).

1.1.1.3 Race and heredity

Data from different sources diverge over the importance of race in predisposing to DU. DU prevalence, hospitalisation and mortality are higher for whites than for non-whites (Kurata and Haile, 1982). Age-specific mortality rates indicate a somewhat higher mortality rate from ulcer disease in non-whites than in whites up to the age of 65, with the reverse being true at a more advanced age (Kurata and Haile, 1982). Even with the limitations of different data, there is little reason to suspect that race

is a major predisposing factor for DU (Kurata and Haile, 1982).

1.1.1.4 Sex

DU is estimated to be 1.5 to 3 times commoner in males than females (Pulvertaft, 1959; Bonnevie, 1975; Grossman, 1980), but the prevalence of gastric ulcers is approximately the same for men as for women (Ostensen et al., 1985). The male to female ratio for DU has been reported as 18:1 in India, 9:1 in Africa and in Bangladesh, 4:1 in Hong Kong, 2:1 in the United Kingdom, and 1:1 in the United States (Lam, 1989). The male to female ratios for hospitalisation for DU and mortality rates were respectively 1.8:1 and 2.4:1 in 1970. By 1983 both ratios had decreased to 1.3:1 (Kurata et al., 1985).

1.1.1.5 Changes in incidence and severity

The incidence and severity of peptic ulcer disease have been declining for the past few decades (Hollander and Tarnawski, 1986) and have also declined throughout the West during this century (Mendeloff, 1974; Bonnevie, 1985). For instance, there has been a gradual decline in the number of hospital admissions for any reason associated with peptic ulceration over the past few decades (Elashoff and Grossman, 1980; Brown et al., 1976). This may reflect decreasing incidence, change in the severity of the disease or it may be due to revised criteria for admission; nowadays peptic ulcer patients are mainly treated as outpatients. There has been a decline in the incidence of

perforated peptic ulceration (Mackay, 1966; Illingworth et al., 1944; Jamieson, 1955). This is a good index of severity because almost all the patients are hospitalised. Most importantly, age-sex-specific death rates due to peptic ulcer disease have also been declining during the past few decades (Susser, 1982; Kurata et al., 1983; Sonnenberg et al., 1985).

A prospective study of first time diagnosed cases of DU in males in New York city showed a significant fall over the years 1952 to 1963 (Pulvertaft, 1959; Pulvertaft, 1968). In Iceland, Jonasson et al. (1983) noted a less marked decline.

The reasons for these falls in incidence, in severity and in mortality have been widely studied, and many hypotheses have been advanced to account for them (Friedman et al., 1974; Langman, 1979; Kurata and Haile, 1984). One aspect that has not been carefully considered is the increased consumption of, and interaction between, dietary essential fatty acids and the natural defence mechanisms of the gastroduodenal mucosa (Hollander and Tarnawski, 1986). Other reasons for the decline include powerful new ulcer healing drugs such as H₂ receptor blockers, a decline in smoking, a change in environment including industrialisation, and changes in work patterns, and climate (Kurata and Haile, 1984; Friedman et al., 1974).

Effective ulcer healing drugs have been available since the late 1970's and their use has been associated with a marked fall in the number of operations performed for

uncomplicated peptic ulcer. These drugs do not affect the development of an ulcer but they do induce temporary remissions. However, the falling incidence of peptic ulcer disease pre-dated the introduction of these new agents.

1.1.2 CAUSES OF PEPTIC ULCERATION

In gastric ulcer patients, acid and pepsin secretion are often normal or lower than normal. By contrast, DU is more closely related to greater acid and peptic activity (Baron, 1970). Peptic ulceration can be seen as the consequence of imbalance of defence and attacking forces.

1.1.2.1 Mucosal Defence

Many defences (fig. 1.1) resisting attack by acid and peptic activity have been described and are reviewed below, drawing from the reports published by Allen et al., 1988; Hudson et al., 1990; Mertz et al., 1991; and Zandomeneghi et al., 1991. Defence mechanisms include: a) a barrier preventing contact between the mucosa and luminal contents, formed by an adherent mucus (gel) layer of normal viscosity, and bicarbonate secretion by the mucosa; b) normal cellular integrity and resistant lipoprotein / phospholipid membrane of epithelial cells; c) vascular integrity maintaining a proper blood supply to the mucosa; d) adequate cell replication in the mucosal crypts, normal mucosal regenerative capacity and cell turnover; e) epidermal growth factor; f) normal levels of locally elaborated cytoprotective substances especially

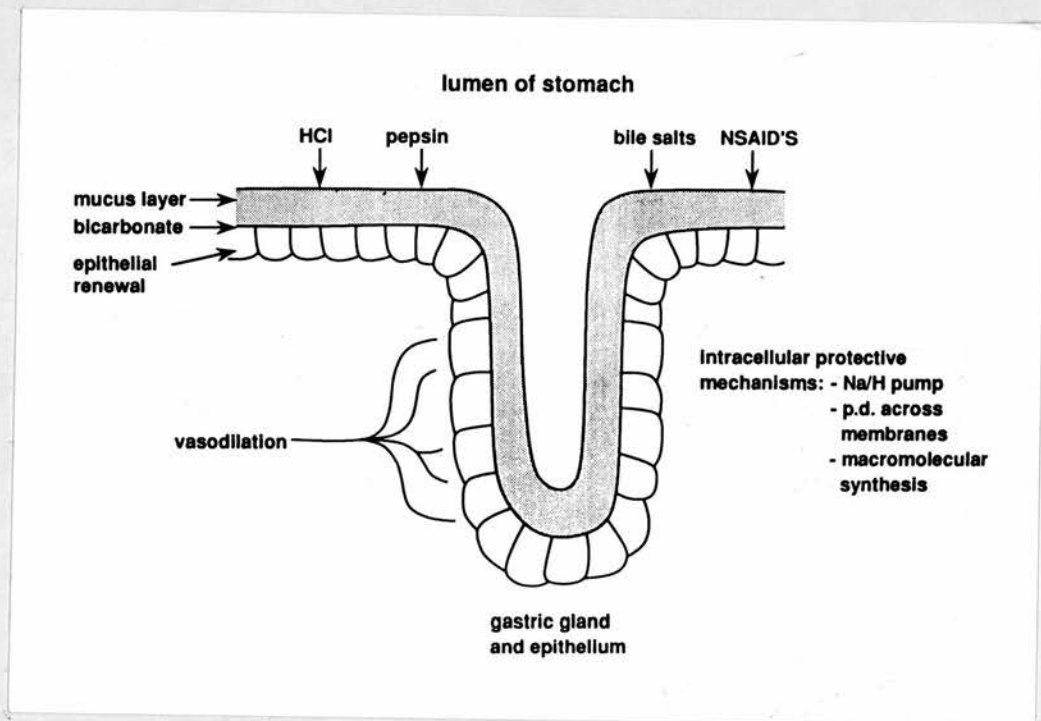


Fig. 1.1 Pathophysiology of duodenal ulcer
-aggressive and defensive factors-

prostaglandins (PG). It is believed that the mucosal defences may be breached and peptic ulceration follow if any of these are deficient.

1.1.2.1a The mucus barrier

A 180 μm thick adherent layer of water-insoluble mucus gel forms a continuous cover over the gastroduodenal mucosal surface and is the first line in the mucosal defence against acid and pepsin. This gel provides a stable layer that supports surface neutralisation of acid by mucosal bicarbonate secreted from the mucosa and is a diffusion barrier to pepsin, so preventing proteolysis of the underlying epithelial cells. Mucosal viscosity is also maintained by the secretion of new mucus by epithelial cells. The mucus gel does not provide a significant diffusion barrier to hydrogen ions (Allen et al., 1988; Freston, 1989).

1.1.2.1b Cell layer integrity and lipoprotein membrane

The luminal surfaces of the mucosal cells are connected by a trilaminar lipoprotein membrane which forms a major defence barrier. The basement membrane on which the cells rest is also a barrier. The integrity of the layer of cells is essential to mucosal defence (Bardhan, 1981).

1.1.2.1c Gastric mucosal blood supply

Gastric mucosal blood flow and ischaemia are important in the pathogenesis of acute mucosal injury. An adequate blood flow maintains the secretory and metabolic activity of the cells and facilitates removal of hydrogen ions that have diffused back into the mucosa (Whittle, 1977; Guth, 1982). Taha et al. (1991) reported that duodenal, but not

gastric mucosal, blood flow is reduced in nonsteroidal anti-inflammatory drugs users who smoke and patients with DU or *H.pylori*.

1.1.2.1d Gastric mucosal cell replication

Gastric epithelial cells normally survive for only two to six days and therefore need to be constantly replenished by new cells which arise from the neck of the gastric glands. Any interference with cell replication and maturation weakens mucosal defence (Bardhan, 1981).

1.1.2.1e Epidermal growth factor (EGF)

Epidermal growth factor is a polypeptide found in a number of body fluids, including saliva and gastrointestinal secretions. It inhibits gastric acid secretion, accelerates cell maturation, stimulates cellular proliferation and increases PG synthesis (Itoh et al., 1988; Konturek et al., 1989; Freston, 1989; Zandomenighi et al., 1991).

1.1.2.1f The protective role of prostaglandins

The existence of PGs was demonstrated by Kurzok (1930) when he noted that strips of uterus relax or contract when exposed to semen. Bergstrom and Sjoval (1960) isolated PGs in a pure form and later defined their chemical structure. PGs are synthesised by the gastroduodenal mucosa from the biological precursors the polyunsaturated fatty acids, linoleic acid and arachidonic acid (Pesker, 1977). Linoleic acid and arachidonic acid are released from membrane bound phospholipids (fig. 1:2) by the action of phospholipase A₂ in response to a variety of physical, chemical and neuro-

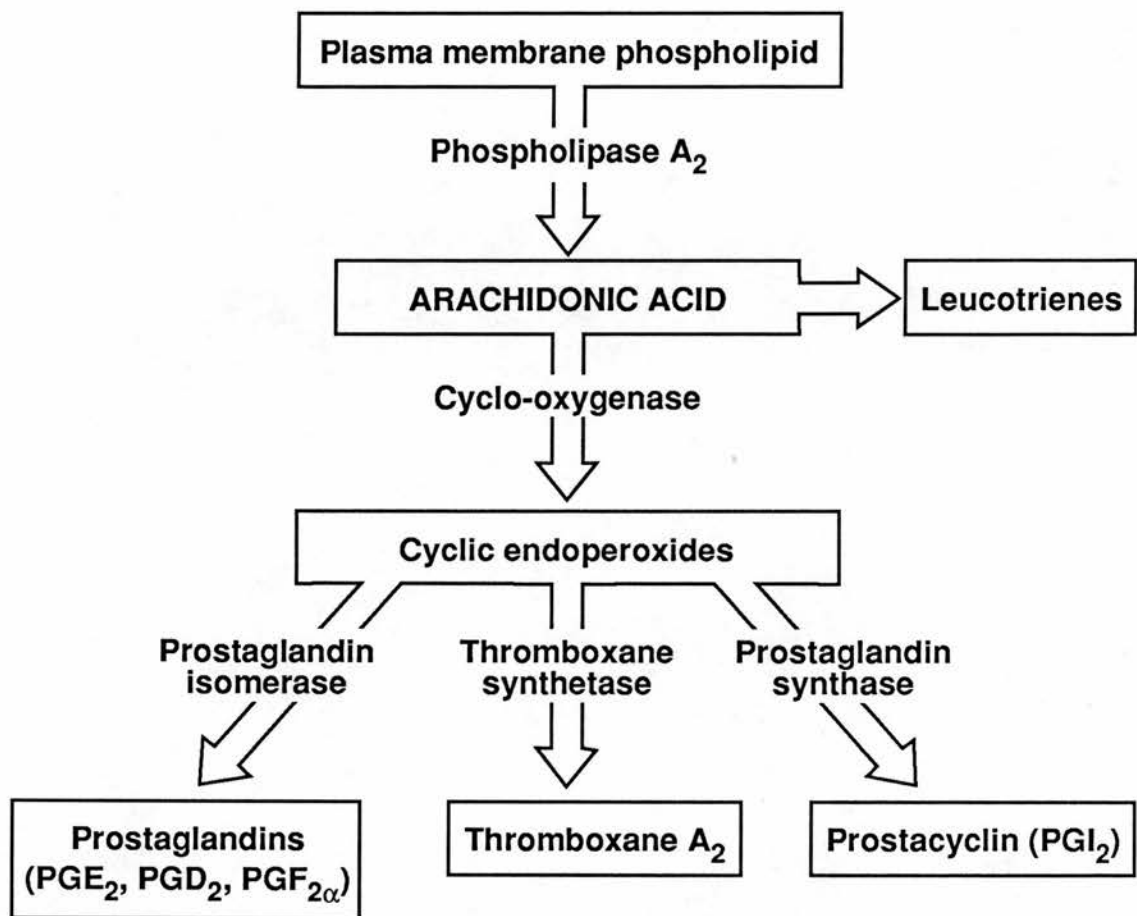


Fig. 1.2 Arachidonic acid metabolism. Arachidonic acid is converted by cyclo-oxygenase to cyclic endoperoxides which are then converted by tissue enzymes to prostaglandins. Vascular endothelium synthesizes a prostaglandin with a different structure, prostacyclin (PGI₂). Platelet enzymes produce thromboxane A₂. Leucotrienes are important regulators of inflammatory and hypersensitivity reactions.

hormonal factors. Arachidonic acid is rapidly metabolised to oxygenated products by the cyclo-oxygenase and lipoxygenase pathways. The intermediate cyclo-oxygenase products are converted to PGs, the lipoxygenase products to leukotrienes (fig. 1.2).

There are four groups of such substances, cyclic compounds and the prostanoids which include the PGs, prostacyclins and thromboxanes.

The prostanoids are cyclic hydroxy fatty acids with two side chains one of which carries a carboxyl group, the other a hydroxy group at C 15. They are very potent biologically and are important local hormones generated *in situ*, rapidly metabolised and are briefly active in the immediate vicinity of their synthesis.

PGs (fig. 1.3) and prostacyclins have a cyclic ring of 5 carbon atoms, whilst thromboxanes have a ring of 6 carbon atoms. They have potent effects on smooth muscle, platelet stickiness and blood vessel contractility. Prostacyclins (vessel wall constituents) inhibit platelet stickiness, relax arterial wall tone, and decrease systemic blood pressure. Thromboxanes (platelet constituents) by contrast cause platelets to clump, increase arterial wall tone and increase systemic blood pressure.

The chemical nomenclature is based on the fully saturated 20-carbon acid with C₈ to C₁₂ closed to form a five membered ring prostanoid acid. There are different classes - the suffices A, B, C, D, E, F, G, H denote different constituents of the ring and the numerical subscripts - 1, 2 3 denote the number of double bonds at

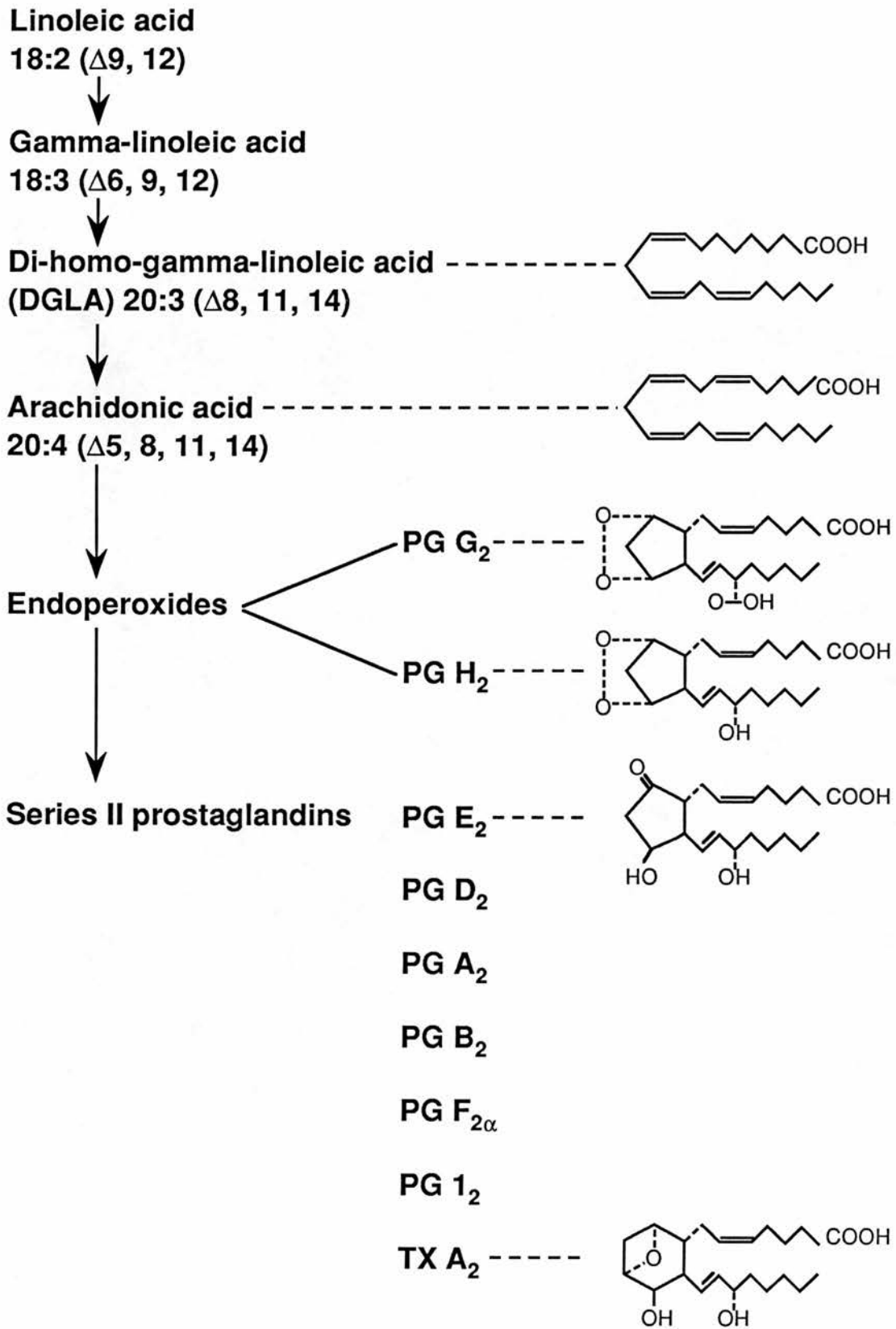


Fig. 1.3 Structure of prostaglandins

the side chain. The difference between the E and F series lies in the keto and hydroxyl groups at position 9 respectively.

As shown in fig. 1.3, the precursor of the prostanoids (eicosanoids) is arachidonic acid, which can fold and allow the appropriate groups to come into juxtaposition for the ring to close. This occurs in the microsomal fraction and is catalysed by prostaglandin endoperoxide synthetase, a multifunctional protein. This enzyme inserts 2 molecules of oxygen to yield a 15- hydroperoxyl-p, endoperoxide with a substitute cyclopentane ring PGG. This is a cyclo-oxygenase activity of the enzyme. A peroxidase activity reduces PGG to its 15-hydroxy analogue. Free radical intermediates inactivate the enzyme as do certain nonsteroidal anti-inflammatory drugs, e.g., aspirin or indomethacin. These drugs compete with the fatty acid substrate for the cyclo-oxygenase active site. Aspirin also acetylates a serine hydroxyl at or near the active site which permanently inactivates the enzyme. The acetylenic fatty acids such as eicosa-5,8,11,14 tetraenoic also have the same effect.

The range of eicosanoids is considerable and their effect varies from tissue to tissue. The various eicosanoids are found throughout the whole of the biological system. They inhibit platelet aggregation, increase platelet cAMP content, affect membrane receptors, act as peripheral vasoconstrictors, pulmonary vasoconstrictors and bronchoconstrictors. PGE₂ reduces ADH production and alters water reabsorption and controls renin release and thus influences kidney function.

Thromboxane (TX) A_2 is labile, has a short half life and is converted into TXB_2 . TXA_2 influences platelet aggregation. Leukotrienes are epoxy fatty acids active in respiratory, vascular and intestinal smooth muscle. Three lipoxygenases (5-,12-,15-) have been found in mammalian tissue. 5 lipoxygenase is important in leukotriene production in neutrophils, eosinophils, monocytes, mast cells, keratinocytes and lungs, spleen, brain and heart. Products of 12 lipoxygenase activity inhibit collagen induced platelet aggregation. The biological relevance of 15 hydroxyeicosatetraenoic (HETE) is unknown. Leukotrienes have potent biological functions. The peptido-leukotrienes cause constriction of bronchi, especially small airways. They also have microvascular effects on arteriolar constriction, venule dilatation and plasma exudation. They are chemotactic agents for neutrophils, eosinophils and also are involved in the contraction of small gastrointestinal smooth muscle.

After linoleic acid is absorbed from the gastrointestinal tract it is converted into longer chain fatty acid in the liver e.g., di-homo-gamma-linoleic acid (fig. 1.4), and then stored as a tri-acyl-glyceride. In man, arachidonic acid is by far the commonest and gives rise to the series II PGs. It is either derived from linoleic acid or ingested itself. It is esterified and stored as tri-acyl-glycerides throughout the body as a component of phospholipids in cell membranes. The first step for eicosanoid formation is by hydrolysis of these

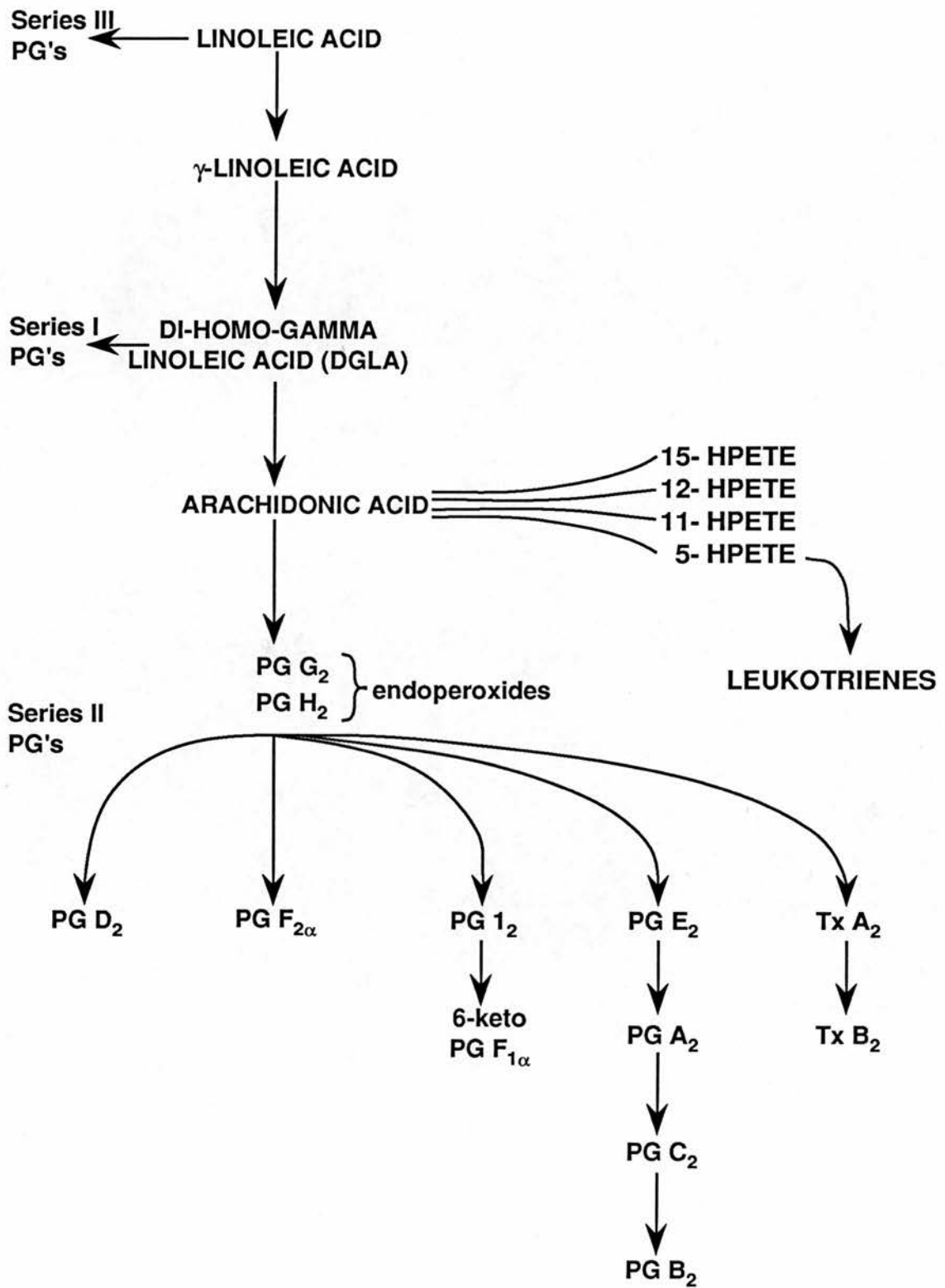


Fig. 1.4 Prostaglandin pathway

esters or is an activation of the release of arachidonic acid from the 2 position o-phosphoacylglycerides. The important phospholipids are those which are a source of arachidonic acid phosphatidylcholine and phosphoinositides. Only those fatty acids capable of being converted into the delta -5,8,11,14 tetraenoic fatty acids of chain length 19,20,22 are essential because these give rise to physiologically active eicosanoids. Eicosanoids are metabolised very rapidly and are excreted in urine or bile. There is a great divergence between the 10 gm of essential fatty acid which are thought to be necessary in the diet and the 1 mg of PG metabolites formed per 24 hours in man.

The released fatty acids are adsorbed to plasma albumin to be taken to other tissue for metabolic utilisation by beta-oxidation pathways. The determinant of beta oxidation or acyl glycerol synthesis is dictated by available acyl-CoA molecules by the acyl transferase and the carnitine palmityl transferase of the mitochondrial membrane. This latter is increased during starvation. The n-6 unsaturated fatty acids, linoleic and arachidonic acid, give rise to the 3-series and 4-series leukotrienes respectively, whereas the alpha-linoleic acid n-3 of fish oil origin gives rise to the 5-series leukotrienes. Linoleic acid and arachidonic acid give rise to the 1 and 2 series PGs respectively whereas alpha-linoleic acid gives rise to the 3 series. The relative contribution of n-3 and n-6 fatty acids in the diet will, therefore, dictate the spectrum of types and biological potencies of the prostanoids and leukotrienes.

Other than this dietary control, the production of eicosanoid formation is dependent upon phospholipase A₂ activity. This activity is activated and inhibited by a number of messengers in a similar manner to the inositol cyclase system.

In man, the most prominent PG in the gastrointestinal tract is PGE (Bennett et al., 1968). When linoleic acid or arachidonic acid is administered intragastrically, the concentration of PG in the stomach rises several thousandfold within a matter of minutes (Hollander et al., 1982), promoting mucosal protection and accelerating the repair of mucosal ulceration (Robert et al., 1979; Hollander et al., 1982; Tarnawski et al., 1985). Evidence is accumulating that endogenous elaboration of PG, mainly of the PGE group, by the gastroduodenal mucosa is of great importance in the intrinsic defence against ulceration (Hollander et al., 1982). These PGs, administered exogenously (Robert et al., 1979) or synthesised endogenously (Hollander et al., 1982), can prevent mucosal ulceration induced experimentally by aspirin, alcohol, bile acids, and even boiling water (Hollander et al., 1982).

Lack of linoleic acid in the diet and consequently in the gastric mucosa results in a decrease of PGE to the extent that gastric mucosal protection is no longer sufficient to maintain the integrity of the mucosal barrier; consequently, DU follows (Grant et al., 1988 and 1990). These workers have also shown that the linoleic acid content of adipose tissue in DU patients was

significantly lower than that of control subjects.

It has been suggested that *H.pylori* could metabolise gastric polyunsaturated and monounsaturated fatty acid to saturated fatty acids and consequently, by reducing PG production cause a lowering of the gastric mucosal barrier and increase the likelihood of peptic ulceration. (Experiments designed to test this theory are described in chapter 4 of this thesis).

PGs are, therefore, important for cytoprotection (fig. 1.5), or more properly and appropriately, "gastric mucosal protection". This involves many mechanisms (Robert, 1981; Miller, 1983; Hollander and Tarnawski, 1986; Malagelada et al., 1986; Rask-Madsen and Lauritsen, 1987; Hawkey, 1989; Tovey et al., 1989; Northfield and Goggin, 1991) including: the maintenance of an intact mucosal barrier by increasing mucosal blood flow (Jacobson, 1986; Wilson, 1991); production of adherent mucus gel; increase in mucus viscosity; increase in the hydrophobicity of the mucus layer; increased mucus output (Johansson and Kollberg, 1978; Bolton et al., 1978; Wilson, 1991); increased bicarbonate output (Feldman, 1983; Isenberg et al., 1986, Wilson, 1991); increased cell turnover; increased macromolecular synthesis; providing cellular protection by increasing cAMP in the sodium pump. PGE in the stomach also inhibits acid secretion (Befrits and Johansson, 1985). This occurs whether the PG is given intravenously or orally (Horton et al., 1968; Classen et al., 1971). Pharmacological analogues of PGs, for instance arbaprostil and misoprostol, are also potent acid inhibitors when given

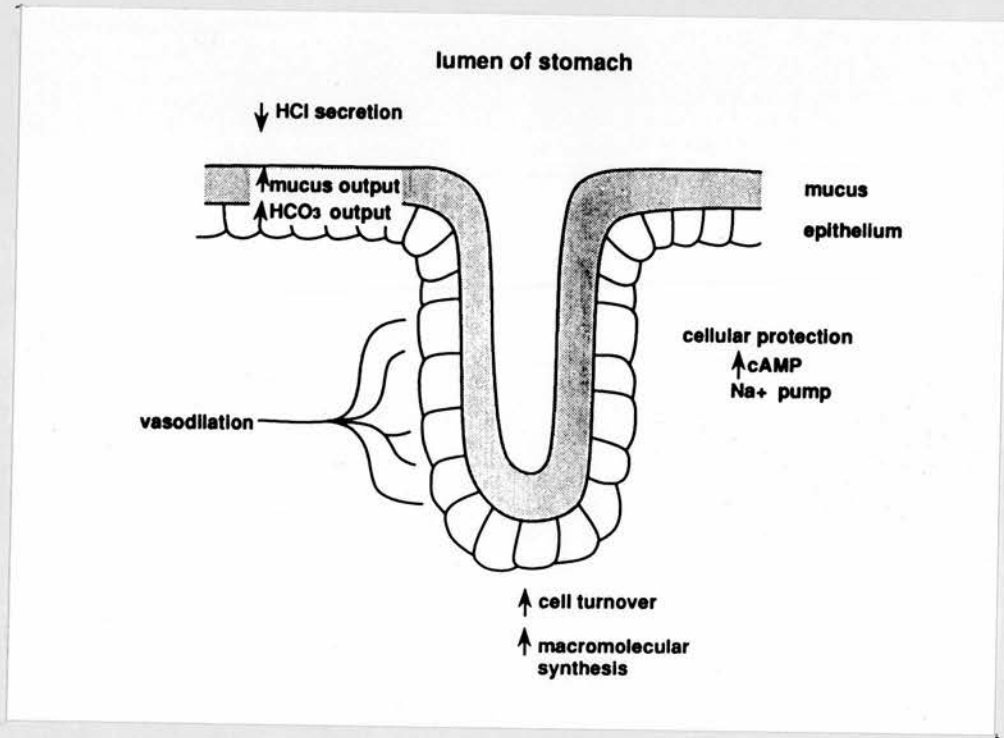


Fig. 1.5 Modes of action of prostaglandin E on the gastric mucosa

intravenously or orally (Konturek et al., 1976).

It is possible that an increase in the intake of polyunsaturated (linoleic acid) and mono-unsaturated fatty acid in the diet could result in greater production of PGs by the gastric mucosa with a consequent increase in the gastric mucosal defences and a corresponding lowering of the risk of peptic ulceration. Long term dietary fatty acid intake is said to be reflected in the fatty acid levels in the adipose tissue of an individual (Wood et al., 1987). In chapter 3 of this thesis the relationship between the essential fatty acid levels in the diet and in the adipose tissue of DU patients and control subjects is examined.

1.1.2.2 Attack upon the mucosa

The attack (see fig. 1.1) upon the mucosa is a combination of acid and pepsin secretion. The following factors (Soll and Berglindh, 1987; Graham, 1989) might be important: a) increased parietal cell mass and increased chief cell mass; b) increased basal acid secretory drive; c) increased postprandial acid secretory drive; d) rapid gastric emptying; e) abnormalities of motility and duodenogastric reflux; and f) type B (*H.pylori*) gastritis.

1.1.2.2a Increased parietal cell mass and chief cell mass

The maximum capacity of the stomach to secrete acid (maximal acid output or MAO) is a function of the total parietal cell mass (Grossman and Elashoff, 1980; Lam, 1984) which is 1.5 to 2 times greater in DU patients than in control subjects. The values for normal subjects and DU

patients overlap, and only between 20 and 50 per cent of DU patients hypersecrete acid (Cox, 1952). Approximately two-thirds of DU patients also have an increased blood level of pepsinogen 1 (Samloff et al., 1975).

1.1.2.2b Increased basal acid secretory drive

Basal acid output (BAO) varies with total acid secretory capacity, and therefore, might be increased in subjects with an increased MAO. By determining the ratio of BAO to MAO or peak acid output (PAO) and making adjustments for the variable gastric parietal cell number, an elevated BAO / PAO ratio suggests an increased basal secretory drive. Most investigators consider a BAO / PAO ratio above 0.3 to indicate an increased basal secretory drive (Feldman et al., 1980; Kirkpatrick and Hirschowitz, 1980; Lam, 1984; Blair et al., 1987).

The physiology of acid secretion is complex, and the parietal cell is regulated by input from endocrine, neural, and paracrine pathways; gastrin, acetylcholine, and histamine are the primary respective chemotransmitters delivered by these pathways. Increased activity of any of these pathways might increase basal secretory drive in DU patients (Soll and Berglindh, 1987).

1.1.2.2c Increased postprandial acid secretory drive

This might be due to postprandial hypergastrinaemia, decreased acid inhibition of postprandial gastrin response or increased parietal cell sensitivity to gastrin. Increased postprandial acid secretory drive was found in about 10% of the DU patients in one study, but the

pathophysiological significance of this abnormality has not yet been established (Cooper et al., 1985).

Impaired acid inhibition of gastrin release has been proposed to explain the enhanced gastrin release found in some DU patients, but this has not yet been confirmed (Walsh and Grossman, 1975; Malagelada et al., 1977; Cooper et al., 1985).

Gastrin, which is elaborated by antral mucosal endocrine cells (G cells), is a trophic hormone but its main function is to stimulate acid production. It also stimulates growth of parietal cells when its concentration in the circulation is excessive (Walsh et al., 1975). If the pH of the antral contents is lowered to 2.5, gastrin release is almost totally inhibited in normal subjects but only moderately reduced in DU patients (Walsh et al., 1975). Basal or postprandial hypersecretion of acid results from an enhanced sensitivity of parietal cells to secretagogues. At least some DU patients are more sensitive to both exogenous gastrin infusion and to endogenously released gastrin than are control subjects (Lam et al., 1980; Lam and Koo, 1985). Increased sensitivity to gastrin in DU patients probably follows enhanced vagal tone, an altered affinity of the parietal cell receptors for gastrin and an impairment of an inhibitory pathway, such as that mediated by somatostatin. This hypothesis is disputed by others (Hirschowitz, 1984).

Gastric secretion is stimulated primarily by the act of eating. Two mechanisms are involved: first, via the vagus by direct cholinergic action on parietal cells; and,

second, by indirect action through the release of several peptides such as gastrin, somatostatin and bombesin (Tache, 1988). The sight and smell of food lead to modest, vagally mediated secretion by the stomach, but chewing and swallowing food provide a particularly powerful vagal stimulus. Gastrin interacts with the vagus at the receptor site to stimulate powerful secretion of gastric juices. There are receptors (histamine, muscarinic, cholinergic and gastrin receptors) on the parietal cell for vagal and humoral stimuli. These stimuli act largely through histamine (probably produced by mast cells) to provide the final stimulus to the parietal cell (Colin-Jones, 1986; Colin-Jones, 1987).

1.1.2.2d Rapid gastric emptying

A meal evokes acid secretion, but in normal subjects the food stays long enough in the stomach to buffer the acid produced. In contrast, in DU patients rapid gastric emptying removes the food, leaving the acid unbuffered, so that an abnormally large acid load is delivered into the duodenum (Howlett et al., 1976; Graham, 1989).

1.1.2.2e Gastroduodenal motility disorder and duodenogastric reflux

Motility defects are mainly found in relation to gastric ulcer. An incompetent pyloric sphincter might allow reflux of duodenal contents into the stomach causing gastritis and gastric ulcer. Delayed gastric emptying, perhaps due to antral hypomotility, may lead to stasis and delayed clearance of refluxed duodenal contents. Duodenal

contents might injure the gastric mucosa by damaging the gastric mucosal barrier leading to increased hydrogen ion back diffusion. If emptying is delayed, food is retained in the antrum; this could lead to increased gastrin release and greater acid secretion (Burge, 1966; Dragstedt and Woodward, 1970). Acid delivered into the duodenum is neutralised by pancreatic bicarbonate. In patients with DU, because the frequency of duodenal contractions is reduced, acidity in the proximal duodenum is not adequately reduced enhancing the risk of duodenal ulceration (Borgstrom and Arborelins, 1978).

1.1.2.2f Type B (*H.pylori*) Gastritis

This is described in detail later on in this chapter under the heading of *H.pylori* and peptic ulceration.

1.1.2.3 Other risk factors for peptic ulceration

Additional risk factors include: a) cigarette smoking; b) diet and drinks; c) alcohol; d) drugs (nonsteroidal anti-inflammatory drugs and aspirin); e) corticosteroids; f) occupation; g) socioeconomic status; h) familial aggregation, blood group and tissue antigen; i) psychology and personality type; j) mental stress.

1.1.2.3a Cigarette smoking

Cigarette smoking appears to be a risk factor for the development, maintenance, and recurrence of peptic ulcer disease (Freston, 1989). Smoking has an inconsistent effect on gastric acid secretion, but it does have other effects on upper gastrointestinal function which could contribute to the pathogenesis of peptic ulcer disease. These include

(Solomon and Jacobson, 1972; Eastwood, 1988; Hudson et al., 1990): the inhibition of mucosal PG synthesis by interfering with the cyclo-oxygenase pathway; decreased pancreatic and duodenal bicarbonate secretion; increased gastric emptying; increased duodenogastric reflux; decreased gastric mucus secretion; decreased gastric blood flow; decreased secretion of salivary epidermal growth factor (Hixson et al., 1987); increased gastrin. Smoking also interferes with the action of histamine-2 antagonists (Eastwood, 1988; Hudson et al., 1990; McCready et al., 1985).

There is a positive correlation between the quantity of smoking and peptic ulceration (especially for DU) (Friedman et al., 1974; Harrison et al., 1979; McCarthy, 1984). Cigarette smoking is associated with decreased ulcer healing (Sontag et al., 1984), high DU recurrence rates and increased DU complications. Whether smoking is associated with ulcer development remain unclear but smoking increases the risk of DU in subjects who are infected with *H.pylori* (Martin et al., 1989).

Recently, a defect in ventilatory function has been found in patients with chronic gastric ulcer and to a lesser extent in patients with DU (Kellow et al., 1986; Stemmermann et al., 1989). The defect in gastric ulcer patients was more severe in smokers than in non-smokers. Whether smoking led to the ventilatory defect, which in turn led to ulceration, or whether there was simply an association with smoking, ventilatory defects and gastric

ulcer is not known (Dippy et al., 1973). Death rates from ulcer disease are greater in patients who smoke, although it is not clear whether this apparent increase in mortality reflects more severe ulcer disease or the cardiac and pulmonary consequences of smoking (Harrison et al., 1979).

Aspects of the relationship between cigarette smoking and linoleic acid content in the adipose tissue of duodenal ulcer patients and controls have been examined in this thesis in chapter 3.

1.1.2.3b Diet and drinks

Although certain foods, beverages and spices may cause dyspepsia, there are no data to show convincingly that specific substances in the diet cause, perpetuate or reactivate DU. Some studies suggest that increasing dietary fibre reduces DU incidence and recurrence rates whilst other studies show the opposite (Malhotra, 1978; Langman, 1979, Tovey, 1979, Rydning and Berstad, 1986; Holcombe, 1992). Marotta and Floch (1991) reported that "vitamin U" found in cabbage and in green vegetables helps peptic ulcer healing. Spices have long been implicated as a cause of gastric mucosal injury. Myers et al. (1987) showed that there is no significant effect of capsaicin, red or black pepper on gastric mucosa. Tovey et al. (1989) reported that black tea without milk and sugar resulted in an increased acid output which was more pronounced with DU patients. Coffee (Cohen, 1980), tea (Dubey et al., 1984), and other, non-caffeine containing beverages (McArthur et al., 1982) greatly stimulate acid secretion but have not yet been proven to be a cause of peptic ulceration.

A statistically significant relationship has been found between salt consumption, urinary sodium chloride excretion and death rates from gastric ulcer disease in several countries (Sonnenberg, 1986). Recently, evidence has begun to accumulate to suggest that increasing the amount of linoleic acid in the diet protects against the development of peptic ulceration (Grant et al., 1988 and 1990).

1.1.2.3c Alcohol

Alcohol in high concentrations causes upper gastrointestinal mucosal erosions, gastritis and bleeding. These acute effects do not appear to progress to chronic peptic ulcer, and alcohol was not found to be a risk factor for DU (Friedman et al., 1974; Paffenbarger et al., 1974). The extent to which alcohol by itself stimulates acid secretion in man is controversial. A standard test meal used to consist of 50-100 ml of 10% ethanol in water. There is, however, no doubt that wine, beer and other alcoholic beverages are acid secretagogues (Lenz et al., 1983; Peterson et al., 1986), probably due to components other than alcohol.

1.1.2.3d Drugs (nonsteroidal anti-inflammatory drugs - NSAIDs and aspirin)

Some studies (Gillies and Skyring, 1969; Levy, 1974, Cameron, 1975; Bugat et al., 1976; Piper et al., 1981) suggest an association between treatment with aspirin or other NSAIDs, chronic ulceration and peptic ulcer bleeding. Hawkey, (1990), in a review article concluded that aspirin and other NSAIDs cause chronic gastric

ulceration. NSAIDs consumption has been firmly associated with the development of gastric ulcers, but the association with DU remains controversial (Levy, 1974; Piper et al., 1981; Freston, 1989). In the elderly, i.e., over 60 years of age, NSAID consumption is a risk factor for bleeding from both DU and gastric ulcers (Somerville et al., 1986). Damage from aspirin and NSAIDs in the upper gastrointestinal tract is probably dose-dependent. Tarnawski et al. (1990) showed that aspirin and NSAIDs damage both superficial and deep gastric microvessels with a 71% reduction of healthy mucosal cells and also cause increased vascular permeability, stasis, mucosal oedema and release of inflammatory mediators.

There are at least two mechanisms whereby aspirin and other NSAIDs might cause mucosal damage. First, aspirin alters ion transport and the potential difference across the gastric mucosa (Davenport, 1964 and 1966). Secondly, NSAIDs inhibit PG synthesis (Whittle, 1981; Ligumsky et al., 1982). Thus, NSAIDs have been shown in animal models to reduce both epithelial bicarbonate secretion (Rees et al., 1983), mucus synthesis and secretion (Menguy and Masters, 1965; Rainsford, 1978), resulting in a reduced pH gradient across the mucus gel layer and exposure of epithelial cells to a more acidic environment (Ross et al., 1981; Flemstrom and Kivilaakso, 1983). NSAIDs may augment acute inflammation caused by *H. pylori*, and inflammation can exacerbate the biochemical injury to these tissues caused by NSAIDs (McCarthy, 1991).

1.1.2.3e Corticosteroids

The effect of corticosteroids is controversial, but one study suggests that there is an increased risk of peptic ulceration following the administration of corticosteroids for more than 30 days or in a total dose greater than 1 gm of prednisolone (Conn and Blitzer, 1976). This risk was not substantiated by another study (Messer et al., 1983).

1.1.2.3f Occupation

Despite the belief that DU is more likely to affect highly stressed professional and executive workers, the available evidence suggests that, if anything, DU is slightly commoner among unskilled labourers (Pulvertaft, 1959; Langman, 1979).

1.1.2.3g Socioeconomic status

Data from the National Health Survey in the United States (1979) suggest an inverse relationship between peptic ulcer and family income. In one study, DU was commoner among persons of low educational achievement, an effect partly accounted for by their smoking more cigarettes (Friedman et al., 1974).

1.1.2.3h Familial aggregation, ABO blood group and HLA antigen

Some workers have recognised a familial aggregation of DU, but the patterns of inheritance are complex (Rotter and Rimoin, 1977). In some reports 20 to 50 per cent of DU patients have a family history of DU, compared to 5 to 15 per cent of non-ulcer subjects (McConnell, 1980; Rotter,

1983). The familial aggregation of both DU and gastric ulcers is distinct; the first degree relatives of patients with DU had a threefold increase in the prevalence of DU but not of gastric ulcer. In contrast, relatives of patients with gastric ulcer had a threefold increase in the prevalence of gastric ulcer but not of DU (Doll and Kellock, 1951). Twin studies showing that concordance of peptic ulceration is commoner in monozygotic than in dizygotic twins indicates the existence of genetic factors (McConnell, 1980; Rotter, 1983). Individuals with blood group O have about a 30% greater risk of DU, compared with individuals with blood groups A, B, or AB. Individuals who are not secretors have a 50% greater risk of DU, while individuals who are both blood group O and H-substance non-secretors have 150% greater risk (McConnell, 1980; Rotter, 1983), *i.e.*, the risk with both genetic markers is more than additive. In addition, DU is commoner in Caucasians with tissue type HLA-B5 than in controls (Rotter, 1983). HLA-B12 phenotypes are found in greater frequency (Ellis and Woodrow, 1979) with DU.

1.1.2.3i Psychology and personality type

There is a specific "ulcer" personality characterised by an exaggerated dependency-independency conflict. Peptic ulcer patients can display exaggerated self-sufficiency, driving ambition, or aggressiveness. Certain traits including hypochondriasis, lowered ego strength, and excessive dependency (Feldman et al., 1986) are commoner in peptic ulcer patients than in controls. Dependency, anxiety and neurotic tendencies are found more commonly amongst

ulcer patients (Magni et al., 1986).

1.1.2.3j Mental stress

Both patients and physicians believe that peptic ulcer disease often get worse during or after stressful life events. Occupational, educational, or financial problems or family illness often precede the development or recurrence of DU (Meikle et al., 1976). Whilst there is some evidence to suggest that stress is causally related to ulcer disease and its complications (Stewart and Winser, 1942), this is difficult to prove because the definition and quantification of stress is itself difficult (Rabkin and Struening, 1976). The important variable is probably not the intensity of external stress, but rather the individual's interpretation and reaction to the stress (Feldman and Sabovich, 1980). Anxiety, resentment, guilt, and feelings of humiliation might increase acid secretion through cholinergic mechanisms. A patient's mental state might not only alter BAO, but also might alter the sensitivity of parietal cells to exogenous stimulation.

1.2 *Helicobacter pylori* AND PEPTIC ULCERATION

During the past decade, it has become clear that there is a close inter-relationship between infection with the bacterium *H. pylori*, type B antral gastritis and DU.

H. pylori is a slender, curved, spiral gram-negative, microaerophilic rod, approximately 2.5-2.9 μm in length and 0.5-0.85 μm diameter with a smooth coat and four to six

unipolar unsheathed flagella with terminal bulbs and a divergent 16S rRNA sequence. It has no axial filaments. The enzymes urease, catalase, oxidase and alkaline phosphatase are present in this organism (Itoh et al., 1987; Warren and Marshall, 1983; Tytgat, 1987; Andersen et al., 1987).

That spiral bacteria can be found in the stomach has been known for a hundred years. Bizzozero in 1893 first described spiral microorganisms in the gastric mucosa of dogs. Krienitz in 1906 found spiral bacteria in the gastric contents of patients with ulcerating gastric cancer. He also found "spirochaetes" in 14% of patients with gastric ulcer in the same year, but little attention was paid to this finding. Doenges (1939) described several types of spiral organisms in 43% of 242 stained human stomach autopsy specimens. Freedburg and Barron (1940) found "spirochetes" in 54% of patients with gastric ulceration. These findings led to speculation about a possible pathogenic role of these spiral bacteria in peptic ulcer disease (Freedburg and Barron, 1940) but did not provide any evidence to corroborate such a suggestion. Steer and Colin-Jones (1975) observed Gram-negative bacteria under the mucus layer in 80% of 47 patients with gastric ulcer.

The present upsurge of interest in the role of *H. pylori* in peptic ulceration can be dated to 1983 when Warren and Marshall (1983 and 1984) in Australia observed curved and S-shaped bacilli (then named *Campylobacter pylori* and subsequently renamed *Helicobacter pylori* by Goodwin et al., 1989) in endoscopic antral mucosal biopsies of patients with gastritis and peptic ulcer. Marshall et al.

(1985) showed that ingestion of 10^9 colony-forming units of *Campylobacter pylori* led to an active inflammatory reaction in previously normal gastric mucosa thus fulfilling Koch's third and fourth postulates, i.e., that "the inoculation of germs obtained from the disease and grown in pure culture should produce the same disease in a susceptible animal and should be found thereafter in the diseased areas so produced in the animal".

Volunteers who ingested *H.pylori* remained symptom free for 3 to 7 days. Thereafter half of them experienced epigastric discomfort, nausea and vomiting with or without halitosis. The remainder were asymptomatic. This suggested that if the normal immune processes did not clear the infection, then mild gastrointestinal disturbances might persist, and the patient could develop achlorhydria. They found that hypo- or achlorhydric phase lasted 3 to 12 months, during which time the histological pattern of active chronic gastritis developed; polymorphonuclear leucocytes in the mucosa gradually being replaced by lymphocytes and plasma cells. As immunity increased, the inflammation in the corpus region decreased and acid secretion returned to normal. The most severely affected mucosa was in the antrum and pyloric canal possibly because bacterial growth is less inhibited by acid secretion. Marshall et al. speculated that at this stage the development of peptic ulceration may be likely, because the inflamed mucosa is probably more vulnerable to acid. *H.pylori* also affects the islands of metaplastic gastric

epithelium found in the duodenum, and by weakening the normal defence mechanisms of the cell against acid attack mucosal integrity is disrupted and DU occurs (Marshall et al., 1985).

A prospective study in Amsterdam showed that *Campylobacter pylori* like organisms are the causative factors of gastritis, gastric and duodenal ulcers and non-ulcer dyspepsia syndrome (Rauws and Tytgat, 1989).

1.2.1 EPIDEMIOLOGY OF *H.pylori*

H.pylori has been commonly isolated from the stomachs of diverse populations throughout the world (Cover et al., 1989). Although most studies have focused on the presence of *H.pylori* in the stomach of symptomatic persons, the organism has commonly been isolated from asymptomatic subjects (Blaser, 1987). The organism has been identified by culture, silver staining or both in material from the stomach of patients with histological gastritis at a frequency ranging from 62% to 97% (Yeomans, 1988).

Serological and histological studies have been used in most population studies (Rautelin and Kosunen, 1991). An age-dependent increase in antibodies against *H.pylori* is well recognised (Jones et al., 1986; Morris et al., 1986b; Perez-Perez et al., 1988; Kosunen et al., 1989; Megraud et al., 1989; Sitas et al., 1991). In developed countries the presence of *H.pylori* infection seems to be low in children. It gradually increases with age, from 10% in young adults to reach a peak of 70% by the eighth decade (Rautelin and Kosunen, 1991). This is in accord with biopsy-verified

results of the prevalence of gastritis in Finland and Estonia, showing an overall gastritis prevalence 30% at the age of 30, and over 70% at the age of 60 (Siurala et al., 1968; Kekki et al., 1977). Approximately half of all adults are colonised by the age of 60 in developed countries (Taylor and Blaser, 1991). In France, less than 1% of children were found to be infected before the age of 6, while 5% of children aged 6 to 12 years and 15% of children aged 15 to 18 years were infected (Megraud et al., 1989). Similarly, in England, Jones et al. (1986) found that 10% of healthy blood donors under 20 years of age were seropositive for *H.pylori*, compared with 50% of persons over 50 years of age.

In developing countries, *H.pylori* infection begins earlier and also shows an age-dependent increase, reaching even higher rates of infection than in developed countries (Megraud et al., 1989; Perez-Perez et al., 1990). In Vietnam, 40% of 43 teenagers were found to be infected (Megraud et al., 1989). In the Ivory Coast, 55% of 116 children between the ages of 3 and 6 were infected (Megraud et al., 1989). In rural Thailand, 18% of children aged 5 to 9 years were infected, a rate that reached 55% by age 30 years and 75% by age 50 years (Perez-Perez et al., 1990). In contrast, in an urban Thai orphanage, 74% (27) of children aged 1 to 4 years were seropositive. 60% of persons from the People's Republic of China who were aged 20 to 39 years were infected (Lambert et al., 1990). In Thailand and Vietnam there appears to be a slight decrease

in seroprevalence in the elderly.

Prevalence rates plateau or decline after the age of 60 years in most populations. In a number of studies in both developed and developing countries the age-specific prevalence of infection was virtually identical in men and women (Megraud et al., 1989; Graham et al., 1991).

The acquisition of *H.pylori* continues with increasing age and appears to last for years, decades (Coghlan et al., 1987) or for life (Tytgat et al., 1990). Among adults in developed countries, the annual incidence of infection appears to be about 1 to 2% (Tytgat et al., 1991; Rautelin and Kosunen, 1991).

Several investigators have shown considerable differences in the prevalence of *H.pylori* infection in different ethnic groups (Graham et al., 1991). The Australian aboriginal population showed a low prevalence of *H.pylori* antibodies (Dwyer et al., 1988), whereas in the United States evidence of *H.pylori* infection was more often seen in persons originating from China and India than in those from Mexico or America (Graham et al., 1988). Bertram et al. (1991) also reported that the characteristics of gastritis associated with *H.pylori* infection are influenced by geographical factors.

H.pylori infection has an inverse relationship with socio-economic status in both developed and developing countries (Al-Moagel et al., 1990; Taylor and Blaser, 1991; Fiedorek et al., 1991; Sitas et al., 1991; Graham et al., 1991).

The natural reservoirs and mode of transmission of

H.pylori are still a matter of speculation. The gastrointestinal tract of human and other primates are the only known source or reservoirs of *H.pylori*. *Helicobacter mustelae* can be isolated from the gastric mucosa of ferrets (Fox et al., 1988; Tompkins et al., 1988); however, neither *Helicobacter mustelae* nor the organisms found in swine (Jones et al., 1987) or primates (Bronsdon and Schoenknecht, 1988; Baskerville and Newell, 1988) can explain the world-wide distribution of *H.pylori* infection.

It is tempting to incriminate water as a source of *H.pylori* (Klein et al., 1991; West et al., 1992) but no convincing evidence for this has yet been published. The motile spiral form of *H.pylori* has been shown to survive for at least a week in river water (Shahamat et al., 1989); while the coccoid form can survive in river water for at least a year or more (Tytgat et al., 1990).

Attempts to isolate *H.pylori* from food and soil have been unsuccessful (Cover and Blaser, 1992). It is likely that infection is transmitted from person to person. Evidence supporting this includes the higher prevalence of antibodies to *H.pylori* in institutionalised mentally retarded persons (Berkowicz and Lee, 1987), military barracks (Vaira et al., 1991), family contacts of *H.pylori* positive patients (Reiff et al., 1989; Drumm et al., 1990), household contacts of *H.pylori* positive subjects (Bologna et al., 1992), orphanage children (Reiff et al., 1989), and endoscopy staff (Mitchell et al., 1989; Reiff et al., 1989). Not all these trends are clear cut, however. Jones

et al. (1987), found no higher percentage of seropositivity in blood relatives of seropositive patients living in the same household. Collins et al. (1992) using a urea breath test reported that the prevalence of *H.pylori* infection is higher among younger age groups and spouses. This report also suggested person to person spread of this organism. Sexual transmission has also not yet been proved (Taylor and Blaser, 1991).

Recently, Thomas et al. (1992) have reported for the first time the isolation of *H.pylori* from stool in 10 of 24 Gambian subjects. They used very careful handling of materials in a suitable gas mixture before culture. Thus there is now a real possibility that faecal-oral transmission of *H.pylori* is the main means of transmission. If so, it would certainly explain much of the organism's pattern of distribution.

Mouth to mouth transmission still remains a possibility. Although *H.pylori* has not been isolated from saliva (Rosenthal et al., 1988; Krajden et al., 1989), Gobert et al. (1990), using the polymerase chain reaction, detected the *H.pylori* genome in saliva from six of 10 infected persons. An identical strain of *H.pylori* was found in the stomach and dental plaque of a single individual (Krajden et al., 1989). Maternal pre-mastication of food has been shown to be a risk factor for acquisition of *H.pylori* infection in young children (Albenque et al., 1990). Occasionally *H.pylori* has been transmitted from person to person through improperly cleaned endoscopes (Langenberg et al., 1990).

The oral cavity and especially dental plaque might be the natural source or reservoir of *H.pylori* for infection or re-infection of stomach and consequently relapse of DU.

Part of this thesis describes attempts to isolate *H.pylori* from the mouth and examines the relationship of oral *H.pylori* strains to those isolated from the antrum of the same patients.

1.2.2 PATHOGENIC MECHANISMS OF *H.pylori*

H.pylori resides very closely apposed to and indents the apical membrane of mucus cells (Lee et al., 1985; Steer, 1985; Buck et al., 1986; Chen et al., 1986; Goodwin et al., 1986, Bode et al., 1988) or the luminal surface of mucus secreting cells within gastric pits. It does not invade the tissues (Jones et al., 1984; Rollason et al., 1984; Tricottet et al., 1986; Blaser, 1987). A few lie within the mucus layer or more usually between the mucus layer and the underlying gastric epithelium (Hazell et al., 1986). *H.pylori* clusters above the tight junction between adjacent cells and can also be found between cells (Fung et al., 1979; Chen et al., 1986; Hazell et al., 1986; Goodwin et al., 1986; Bode et al., 1988). The cell wall of *H.pylori* contains a lectin (Evans et al., 1988) which selectively binds the organism to the mucus cell layer and the epithelial cell membrane. Lectins allow *H.pylori* to attach tightly to gastric epithelial cells (Slomiany et al., 1989). After attaching to the epithelial cells, *H.pylori* leads to polymerisation of actin below the

epithelial cell membrane and generation of a cell structure called "attachment pedestals" by which it is attached to the plasma membrane (Goodwin et al., 1986; Smoot et al., 1989; Rosenberg et al., 1991).

H.pylori is protected from the harmful effect of acid by its production of urease which metabolises urea to ammonia (Hazell et al., 1986; Marshall et al., 1990; Mobley et al., 1991). Ammonia may in turn damage the gastric mucosa. Other toxic factors produced by *H.pylori* (Cover et al., 1989) are: ureolysin, a mucin-degrading enzyme; proteases and endopeptidases which damage mucosal glycoproteins (Slomiany et al., 1987; Blaser, 1990); catalase, which inhibits the bactericidal effect of neutrophils (Babiour 1978; Tytgat et al., 1991; Marshall, 1991; Newell, 1991); phosphatase; cytotoxin which is a 13 kilodalton polypeptide producing cellular toxicity (Leunk et al., 1988; Figura et al., 1989; Leunk et al., 1990; Leunk, 1991; Blaser, 1992).

H.pylori flagella allow it to penetrate the mucus gel (Hazell et al., 1986). In addition, *H.pylori* exerts a mucolytic effect (Siderotham and Baron, 1990; Sarosiek et al., 1991) and decreases the hydrophobicity of the mucus layer (Mertz and Walsh, 1991). The cytotoxic effect of ammonia (Blaser, 1990; Cover et al., 1991; Mobley et al., 1991; Triebeling et al., 1991) follows damage to the surrounding cells at the tight junction between epithelial cells. As a consequence hydrogen ions diffuse back across the mucus layer (Hazell et al., 1986). Ammonia resulting from *H.pylori* urease activity also increases antral pH and

stimulates gastrin release and causes acid secretion (Morris et al., 1987; Levi et al., 1989; Graham, 1991, Levi, 1991, Trieblich et al., 1991; Beardshall, 1992) potentially leading to hyperacidity (Levi, 1989). The finding of hypergastrinaemia due to increased pH in the antrum has not been confirmed by other observers (Chittajallu et al., 1991; Nujumi et al., 1991; Chittajallu et al., 1992).

Somatostatin, released from gastric D cells, has an inhibitory effect on gastric acid and gastrin secretion. Following infection of the stomach by *H.pylori*, gastric somatostatin secretion is diminished; consequently, the inhibitory function of somatostatin is insufficient to reduce gastric acid and gastrin secretion and this predisposes to DU. (Moss et al., 1992; Kaneko et al., 1992).

After the initial infection of the stomach with *H.pylori*, gastric acid secretion becomes lower than normal for a short period (Graham et al., 1989; Rademaker and Hunt, 1991). Hyperacidity leads to gastric metaplasia in the duodenal mucosa (Wyatt et al., 1987 and 1990; Wyatt, 1989; Dixon, 1991). In addition type B gastritis induced by *H.pylori* releases neurotransmitters which increase the motility of the upper gut, decrease duodenal buffering capacity and consequently lead to duodenal mucosal injury (fig. 1.6) and gastric metaplasia (Wyatt et al., 1990).

Although *H.pylori* is always associated with abnormal gastric mucosa, the organisms are unevenly distributed in the stomach (Goodwin et al., 1985). *H.pylori* is present in

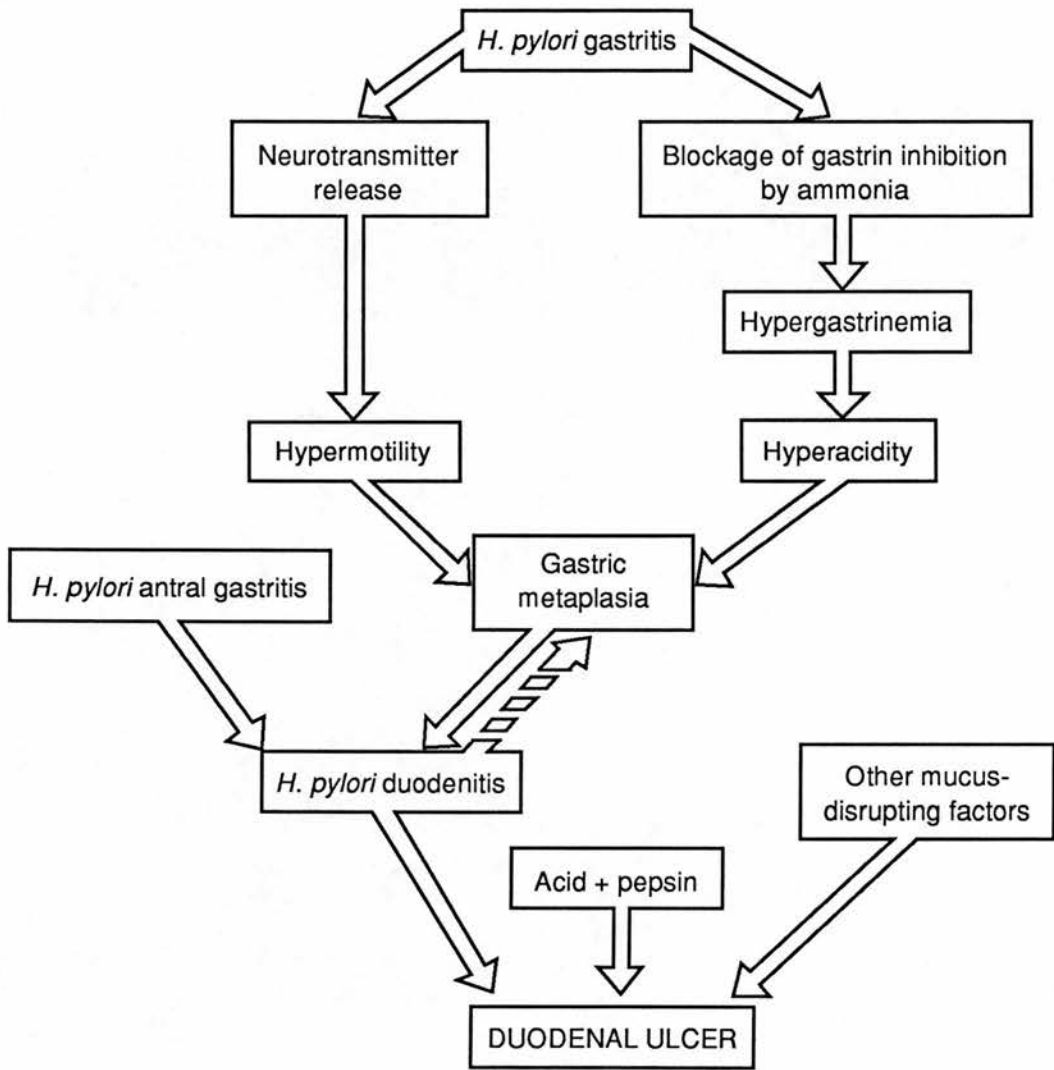


Fig. 1.6 Pathogenic mechanisms of *H. pylori* leading to duodenal ulcer

the duodenum only in association with gastric metaplasia and has never yet been found on normal duodenal mucosa (Rollason et al., 1984; Rathbone et al., 1986; Shabib, 1992). Conversely in the antrum the organisms are never found in areas of intestinal metaplasia but overlie the inflamed gastric mucosa surrounding the metaplastic tissue. This could reflect the presence of specific receptors for *H.pylori* in gastric cells that are absent from the cells of the small intestine, although the existence of these receptors has yet to be confirmed (Tytgat, 1989). If there is gastric metaplasia in the duodenum, *H.pylori* from antral type B gastritis colonises this area (Price, 1988); and, this is ultimately followed by duodenitis and DU (Levi et al., 1989; Dixon, 1991; Madsen et al., 1991). Different strains of *H.pylori* might have different pathogenic effects. Organisms which provoke a gastric IgA antibody response are more likely to be ulcerogenic (Crabtree et al., 1991; Holcombe, 1992).

These pathogenic processes also induce degranulation of mast cells (McGovern et al., 1991) and activate the classic complement pathway (Blaser, 1990).

The mechanisms by which *H.pylori* antigens elicit an inflammatory response has been extensively studied (Wyatt and Rathbone, 1988). A soluble bacterial component attracts phagocytic cells (neutrophils and macrophages) into the gastric glands and lamina propria leading to gastritis (Craig et al., 1989; Craig et al., 1992). Inflammatory reactions induced by *H.pylori* also contribute to tissue

damage and a breakdown of normal defence mechanisms (Rathbone et al., 1988; Blaser and Brown, 1989). The inflammatory responses might be due to *H.pylori* urease absorption at the mucosal surface eliciting chemotactic activity for leukocytes (Heatley, 1991; Wallace, 1991; Mooney et al., 1991; Mai et al., 1992).

In the affected area there are many plasma cells which produce specific IgG and IgA. Complement activation occurs in the presence of IgG and *H.pylori* antigens. Complement activation also occurs via the alternate pathway and enhances neutrophil chemotactic activity (Das et al., 1986). *H.pylori* infection also leads to the accumulation of suppressor T lymphocytes.

Histologically active chronic gastritis is characterised by spotty epithelial necrosis and microerosions, depletion of gastric mucus, polymorphonuclear leucocytosis permeating the epithelial layer, glandular abscess, and acute and chronic inflammation in the lamina propria (Lee and Hazell, 1988). Ultrastructurally the surface epithelial cells show oedema, mucosal bulging, degranulation or depletion of mucus granules. Surface microvilli are sparse or absent (Goodwin et al., 1986; Goodwin, 1988).

The evidence linking *H.pylori* to peptic ulceration (Malfertheiner, 1988; Tytgat, 1989; Megraud and Lamouliatte, 1992) can be summarised as follows. There is a very strong association between *H.pylori* infection, type B gastritis and peptic ulcer disease. This cause and effect relationship has been confirmed in volunteer

studies. Type B gastritis, the histological change associated with *H.pylori* infection, is unique to infection with this organism. There is evidence of person to person spread of gastritis and DU and for clustering in families. There are high ulcer relapse rates after healing with H₂ receptor antagonists if *H.pylori* persists in the stomach and H₂ receptor antagonists by themselves do not relieve gastritis. Bismuth subcitrate and antibiotic treatment heals DU and the ulcer recurrence is less likely after healing with antibiotics (amoxycillin / tetracycline, metronidazole) than after healing with H₂ receptor antagonist drugs. Ulcer healing after treatment with antibacterials effective against *H.pylori* is associated not only with disappearance of the organism but also with the restoration of normal gastric mucosa. The majority (85-100%) of *H.pylori* infected cases are associated with DU. *H.pylori* is associated with early DU recurrence. Ammonia, produced by the organisms' specific urease enzyme acting on urea, neutralises acid and helps to create an ideal environment for the organism in the gastric mucosa. *H.pylori* adheres to specific target mucosal surface cells in the antrum and metaplastic antral type cells in the duodenum. *H.pylori* penetrates into the intracellular spaces and, in some cases, invades the epithelial cells themselves. There is induction of local and systemic immunoresponses to *H.pylori*. *H.pylori* is absent in patients with type A gastritis and in histologically normal mucosa.

Evidence against the role of *H.Pylori* in the pathogenesis of duodenal ulcers is less convincing; however not all *H.pylori* infected persons develop DU. Although relapse is associated with the continued presence of *H.pylori*, H₂ antagonists with no antibacterial activity effectively heal ulcers. DU might be present when *H.pylori* is not (although this is rare).

1.3 BILE ACIDS AND *H.pylori*

Duodenogastric reflux which has been postulated to disrupt the mucosal defence barrier of the stomach leading to bile specific gastritis and ulcer. *H.pylori* are not found in presence of biliary gastritis (O'Connor et al., 1986b). In contrast others have reported that *H.pylori* can colonise the gastric remnant following gastric surgery and cause gastritis (Loffeld et al., 1988).

1.4 *H. pylori* AND GASTRIC CARCINOMA

In addition to the clear association between the presence of *H.pylori* and type B gastritis, (McKinlay et al., 1990; Peterson, 1991) a link has been proposed between the organism and gastric cancer (fig. 1.7). It has been postulated that type B gastritis due to *H.pylori* leads to intestinal metaplasia, atrophic gastritis, achlorhydria and finally gastric cancer. While this idea is attractive, this sequence is largely unproven. Dooley (1991) reported that *H.pylori* might not cause gastric carcinoma, but

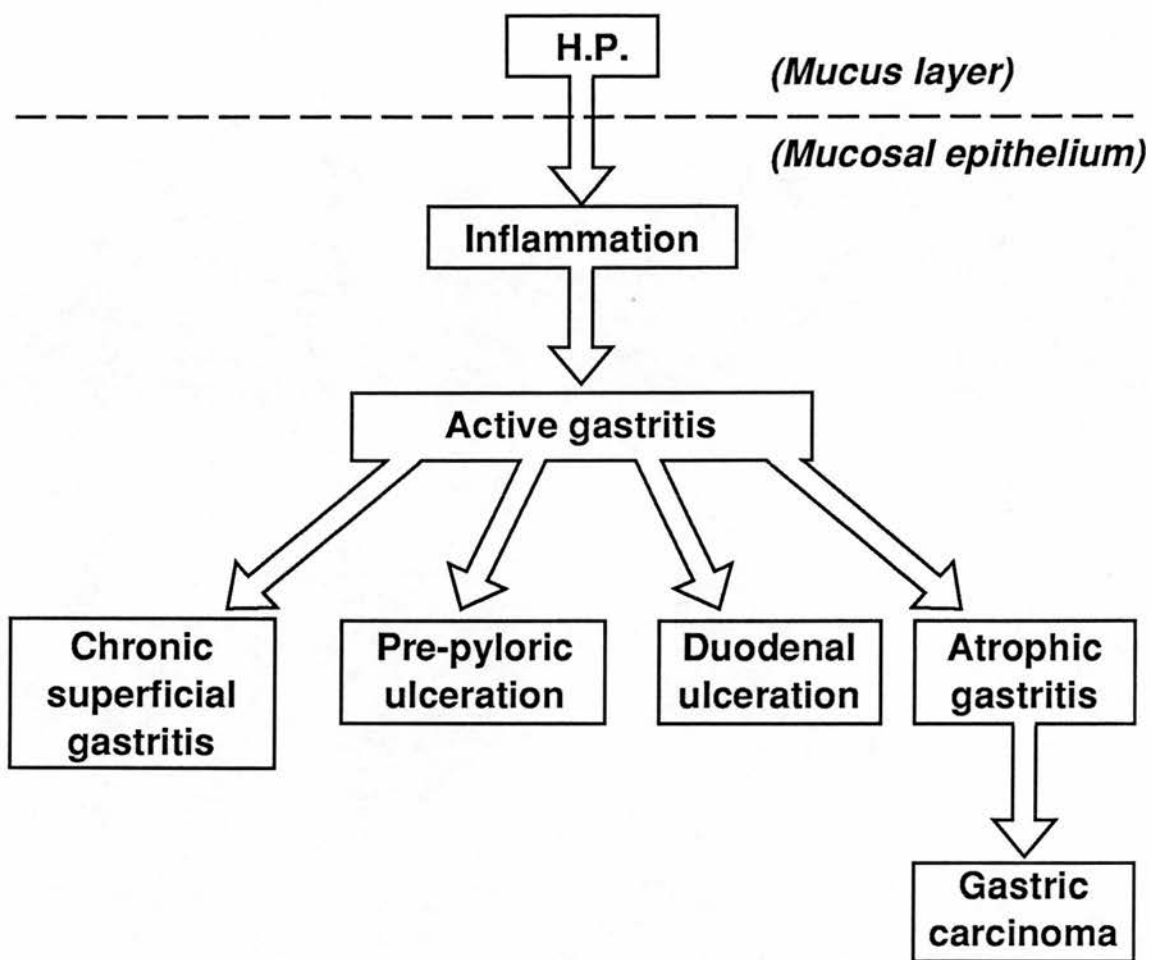


Fig. 1.7 Association of *Helicobacter pylori* with various pathologic lesions of the upper gastrointestinal tract.

gastritis induced by this organism could act as the fertile soil upon which other environmental variables come to bear. In favour of the proposed link between *H.pylori* and gastric cancer, Loffeld et al. (1990) reported the frequent presence of *H.pylori* in biopsies taken from mucosa not involved by gastric cancer. Forman et al. (1990; 1991) found that antibodies to the organism are present in the majority of gastric cancer patients. It has also been suggested that there is an increased risk of gastric cancer in *H.pylori* positive patients in America (Parsonnet et al., 1991) and a similar conclusion has been reached about the Japanese population living in Hawaii (Nomura et al., 1991). In contrast, two studies failed to show a relationship between the presence of *H.pylori* antibody and the presence of gastric cancer in indigenous Japanese (Fukuda et al., 1992; Igarashi et al., 1992), and this was also the conclusion drawn from multi-centre studies in Italy (Miglio et al., 1992; Farinati et al., 1992). In another study, *H.pylori* infection was rather uncommon in the presence of either atrophic gastritis or intestinal metaplasia, both of which are accepted as pre-cancerous lesions (Testoni et al., 1992).

Part of this thesis describes attempts made to clarify the relationship between the presence of *H.pylori* and gastric cancer by examining histologically stomachs resected from patients with benign or malignant gastric diseases.

1.5 ANTIBIOTIC TREATMENT OF *H.pylori* INFECTION

The discovery of *H.pylori* and its relationship to type B gastritis has not only brought new insights to the pathogenesis of gastritis and peptic ulcer disease but also has necessitated critical review of the treatment of these conditions. The efficacy of antiulcer treatment is measured by its capacity to provide symptomatic relief, to induce ulcer healing, to prevent ulcer relapse, and to avoid the complications of peptic ulcer disease. Data from many sources confirm that the prevalence of *H.pylori* in patients with DU is between 85 and 100%, compared with approximately 40% in an unselected Western population (Shallcross et al., 1989; Axon, 1991). Relapse of DU is 20 times more likely if *H.pylori* persists in the gastroduodenal mucosa or reinfects it than if it is absent (Axon, 1991). To treat DU disease and to prevent its relapse, attempts must be made to eradicate *H.pylori* from the stomach and at the same time to prevent it from becoming reinfecting. As an extension of this idea, if there is a relationship between *H.pylori* infection and gastric cancer, eradication of the organisms from the stomach might prevent gastric cancer.

During the last few years different antimicrobials have been tried alone or in combination in different doses and for different durations to try to eradicate *H.pylori* from the gastroduodenal mucosa.

Triple therapy with bismuth, amoxicillin and metronidazole was considerably more effective than bismuth alone in achieving *H.pylori* eradication at one month and

resulted in a significantly reduced rate of DU relapse over the next 12 months (Rauws and Tytgat, 1990). Triple therapy with bismuth, tetracycline, and metronidazole achieved more rapid DU healing than ranitidine alone (Graham and Borsch, 1990). Bismuth with metronidazole and either amoxicillin or tetracycline eradicate *H.pylori* in 80-90% of individuals (Axon, 1991). Recent trials which have been reviewed by Axon (1991) studied patients whose ulcers have been healed by a variety of medicaments and who have then been followed for both *H.pylori* status and DU recurrence. Six studies assessed *H.pylori* status one month after the end of treatment. When *H.pylori* was still present, 96 of 114 patients (84%) subsequently relapsed, compared with only 20 of 179 patients (11%) when it had been eradicated. Ten studies have assessed *H.pylori* status at the time of relapse. The combined data from the eight studies which followed patients for longer than nine months show that ulcer recurred in only six of 217 patients (3%) in whom *H.pylori* was absent, compared with 116 of 155 patients (74%) in whom it was identified. The relapse rate of 11% following eradication is considerably less than either the 82% found after treatment with H₂ receptor antagonists or the 53% following treatment with bismuth (Coghlan et al., 1989). Similar findings were reported by Marshall et al. (1988). They showed that if *H.pylori* persisted in the stomach, 61% of DU healed and 84% relapsed. When *H.pylori* was cleared, 92% of ulcers healed and only 21% relapsed during a 12-month follow-up period.



1.6 DIAGNOSIS OF *H.pylori* INFECTION

Before starting to treat *H.pylori* infection, it must be reliably diagnosed (Rauws et al., 1988). Routine diagnostic methods include: culture of biopsy material, direct microscopy, histological examination, urease test of material from antral biopsy, detection of antibody against the organism and the ^{14}C -urea or ^{13}C -urea breath test. In addition the technique of chromosomal DNA fingerprinting can be applied to confirm the identity of the organism.

1.6.1 CHROMOSOMAL DNA FINGERPRINT

Owen (1989) has reviewed the value of chromosomal DNA fingerprinting methods for identifying species and strains of microbial pathogens. DNA-probe technology has enormous potential in clinical diagnostic microbiology offering faster and cheaper identification of fastidious pathogens. DNA fingerprints are highly sensitive to minor genomic variations in nucleotide sequences for characterising species and for identifying individual strains of closely related bacteria when more traditional typing methods are unsuitable or unavailable.

1.6.2 CHROMOSOMAL DIGEST ANALYSIS

Analysis of genomic DNA by base composition (guanine + cytosine mol%) estimation and DNA-DNA hybridisation have provided an invaluable basis for defining closely related groups of microbial strains. Methods of identifying

strains include plasmid and whole cell protein electrophoretic profiles. The most precise and most widely used is bacterial restriction-endonuclease digest-analysis (BRENDA). BRENDA is a sensitive means of directly detecting minor genomic differences between micro-organisms. Restriction endonucleases specifically cleave DNA into different lengths, depending on the number and position of the individual recognition sequences, consisting in most cases of four or six nucleotides arranged in a specific order.

DNA polymorphism can result from a change in the size of restriction fragment. If a change occurs in the sequences of the genome DNA - even a single nucleotide-base mutation - this can delete a site or create a new recognition site. The change results in the generation of a restriction fragment-length polymorphism (RFLP), new DNA polymorphisms most often represent neutral mutations and do not cause any phenotype change.

DNA fragments generated by restriction-enzyme digestion are separated according to size by electrophoresis in agarose gels to give a pattern of bands. BRENDA has been used to distinguish between strains of various bacteria. The usefulness of such patterns as diagnostic tools is limited by their complexity because they can comprise 50 or more bands of various sizes depending on the cutting frequency of the restriction endonuclease used and the genome size of the organism. It is difficult to identify minor, but possibly significant, restriction

fragment-length polymorphisms (RFLPs) in complex multiband patterns of closely related strains.

When the separated DNA fragments are transferred by capillary blotting on to nitrocellulose or nylon membranes (Southern, 1975), the location on the membrane of a restriction fragment containing a particular gene or nucleotide sequence can be determined by hybridisation with a specific labelled nucleic acid probe. The resultant hybrid-band pattern provides a unique and reproducible fingerprint. The DNA-hybridisation reaction consists of the probe, the target DNA and a reporter molecule on the probe. The key component in Southern-blot hybridisation is the probe which is a sequence of single-stranded nucleotides containing a reporter molecule that can search out and hybridise to stretches of the target DNA- or RNA-containing complementary sequences. Nucleic acid probes are used in Southern-blot hybridisation analyses with the purpose of highlighting specific DNA restriction-site heterogeneities, which in turn can be used to detect strain differences both within and between species. The advantages of DNA fingerprinting by probe hybridisation are: patterns are relatively simple (maximum 10 bands) to facilitate comparisons between strains; applicability to any micro-organism; reproducibility; wide range of restriction enzymes available; commercially available rRNA can be used as a broad spectrum probe; biotin is an excellent reporter molecule in probes; high sensitivity to minor genome variations; and pattern matching can be computerized. The disadvantages are: loci detected by probe might not be

representative of a genome; and the method is relatively time consuming. Among various probes used for this purpose, ribosomal (r)RNA sequence probes are most widely used. In bacteria many of the rRNA-cistron sequences appear to have changed little during evolution and so DNA probes specific for these sequences can detect a wide range of bacteria containing similar sequences.

DNA fingerprinting is relatively laborious and not suitable for routine use in the clinical laboratory and is mostly used in epidemiological studies. Ribopatterns provide a precise fingerprint for individual strain identification and are clearly more discriminatory than the phenotypic differences between strains of *H.pylori*.

In the present study, DNA fingerprinting was used to examine *H.pylori* isolated from the pyloric antrum and from tooth plaque of the same individual.

1.6.3 UREASE TEST

H.pylori produces a powerful urea-splitting enzyme and tests for this urease are widely used to identify *H.pylori* infection. A urease test is usually done by putting antral biopsy material into the well of a commercially prepared strip which also holds a gel pellet containing urea, phenol red (a pH indicator), and sodium azide. During manufacture, the gel is buffered to an acid pH which gives it a bright yellow colour. The colour of the gel changes to pink if the pH rises above 6. This colour change should occur only when urea in the gel is hydrolysed to release

ammonia, i.e., urease is present. The time needed for a clear positive reaction is usually 20 min to 1 hour. The urease test is widely used to identify *H.pylori* infection and is considered valid because of its simplicity, rapidity, low cost, ease of execution, high sensitivity and specificity (Marshall et al., 1987; Hazell et al., 1987; Ameglio et al., 1991). The reported sensitivity and specificity of the urease test varies from author to author. The sensitivity has been variously reported as 62% (Coudron et al., 1989), 90% (Borromeo et al., 1987), 91% (Hazell et al., 1987; Morris et al., 1986a; Marshall et al., 1987), and 100% (Conti-Nibali et al., 1990). Vaira et al. (1988a) stated that 62% of positive cases are detected compared with other tests. The specificity was 87.5% (Conti-Nibali et al., 1990) and 100% (Hazell et al., 1987).

At present (late 1992) the urease test costs one pound sterling. In developing countries such a cost while modest by Western standards is of considerable importance to less robust economies. If the cost of this test and the time to carry it out could be reduced significantly this would be helpful for developing countries.

Part of this thesis describes the preparation and use of an "in-house" urea solution which minimises the cost of the urease test for *H.pylori*, maintains satisfactory specificity and provides a result faster than the commercially available urease test.

The ^{14}C -urea or ^{13}C -urea breath test was not used in the work described in this thesis.

CHAPTER 2

SUMMARY OF OBJECTIVES

Experiments were conducted on patients recruited from outpatient clinics, endoscopy list and hospital wards. The number of patients was dependent upon availability from these sources. The same patients were studied in chapter 3 and 4. Different groups of patients were studied in chapters 5,6,8 and 9.

CHAPTER 3

Duodenal ulcer and its relationship to fatty acid in the diet and in adipose tissue, cigarette smoking and age

This chapter is devoted to the relationship between linoleic acid and DU formation. Previous work had established low adipose tissue linoleic acid content in DU patients, suggesting low dietary linoleic acid intake among these individuals. Dietary linoleic acid influences gastric PG output; and, this may, therefore, be of importance in the aetiology of DU. In this chapter dietary total fat, linoleic acid intake, and the adipose fatty acid content were examined in DU and matched non-ulcer dyspepsia control subjects. The influence of smoking was also examined.

The purpose of these studies was to establish whether the low adipose linoleic acid fraction was due to dietary deficiency, abnormal linoleic acid metabolism or smoking.

CHAPTER 4

***Helicobacter pylori* and Duodenal Ulcer related to fatty acid in the diet and in adipose tissue and to history of cigarette smoking and age**

The relationship between adipose percentage linoleic acid (and other factors studied in chapter 3) and *H.pylori* status is examined in this chapter. Because low adipose linoleic acid content in DU patients could be due to linoleic acid metabolism by *H.pylori*, the ability of *H.pylori* to hydrogenate linoleic acid using a simple *in vitro* culture techniques was assessed. Two possibilities are that the organism digests dietary linoleic acid or less plausibly, that *H.pylori* metabolises the linoleic acid present within the cell membranes of gastric epithelia resulting in mobilisation from fat stores. The latter seems unlikely since linoleic acid levels in the stomach and duodenal mucosa are normal.

CHAPTER 5

Culture of *H. pylori* from Antral Biopsy, Dental Plaque or material obtained by tooth picking

The possibility that dental plaque acts as a reservoir for *H.pylori* re-infection of the stomach was studied in this chapter. Plaque and gastric mucosa were examined for *H.pylori* by enzymatic, microscopic and DNA ribotyping. In

five patients homology of *H.pylori* in plaque and gastric mucosa was determined using DNA ribotyping.

CHAPTER 6

Presence of *H.pylori* in the Mouth of Hospital Inpatients

The epidemiology of *H.pylori* infection in dental plaque was examined in separate populations of hospital inpatients admitted for reasons other than DU. The influence of previous antibiotic therapy upon the presence of the organism in plaque was sought. This appears important because it is possible that systemic antibiotic therapy may not disinfect the teeth and re-infection of the stomach after triple therapy (bismuth subcitrate, amoxycillin and metronidazole) may be a consequence of this failure.

CHAPTER 7

The Effect of Bile Acids on *H.pylori* Growth *In Vitro*

Biliary gastritis and type B gastritis are different entities, but the role of *H.pylori* in the pathogenesis of biliary gastritis is unknown. It is possible that bile inhibits the growth of *H.pylori* and if so the influence of the organism in biliary gastritis will be negligible. To examine this, the effect of bile acids upon the culture of *H.pylori* was examined.

CHAPTER 8

***H.pylori* and Gastric Carcinoma**

It has been suggested that *H.pylori* gastritis predisposes to gastric cancer. The sequence of type B gastritis, gastric atrophy, intestinal metaplasia and gastric cancer has been postulated but not proven. To examine the relationship between *H.pylori* (at the time of surgery) and gastric cancer, histological evidence of the organism was sought in gastrectomy specimens from gastric cancer and a control group of gastrectomies performed for peptic ulcer.

CHAPTER 9

**The Development of a Cheap and Sensitive Urea Solution
For use in Urease tests**

The commercially available *Campylobacter pylori* like organism (CLO) test for detecting *H.pylori* is relatively expensive. A cheaper version with potential for use in developing countries was developed.

CHAPTER 3

DUODENAL ULCER

AND ITS RELATIONSHIP TO
FATTY ACID IN THE DIET
AND IN ADIPOSE TISSUE,
CIGARETTE SMOKING AND AGE

3.1 SUMMARY

To examine the relationship between dietary linoleic acid, adipose tissue linoleic acid, smoking, age and DU, a detailed prospective dietary history was obtained from 30 DU patients and 26 matched non-ulcer dyspepsia controls of similar age. The fatty acid profile in adipose tissue was measured chromatographically in biopsies taken from each individual. Dietary fatty acid intake was also quantitated.

Mean total dietary fat intake and linoleic acid intake were similar in DU and non-ulcer dyspepsia control subjects. In contrast, mean % adipose tissue linoleic acid was significantly lower in DU patients than control subjects ($p < 0.002$). Mean adipose % linoleic acid was significantly lower in smokers than non-smokers in both DU patients and control subjects ($p < 0.012$) after adjustment for their age.

3.2 INTRODUCTION

Previous studies showed that DU patients tend to have a reduced adipose tissue linoleic acid content compared to age matched control subjects (Grant et al., 1988). This has potential importance since the same group had also shown that dietary linoleic acid influenced gastric PG secretion (Grant et al., 1988). Wood et al. (1987) had previously suggested that adipose fatty acid contents closely reflects dietary intake and Grant et al. therefore postulated that relative dietary linoleic acid deficiency may be aetiologically important in DU. The association between smoking and DU is well established (Eastwood, 1988; Hudson et al., 1990) and smokers have reduced adipose linoleic acid content (Wood et al., 1987).

In this chapter the relationship between dietary linoleic acid, adipose linoleic acid, smoking, age and DU is re-examined. The hypotheses that low adipose % linoleic acid is due to 1) reduced dietary intake; 2) smoking; 3) altered linoleic acid metabolism; 4) age, were examined.

3.3 MATERIALS AND METHODS

3.3.1 SUBJECTS

Thirty subjects comprising 22 males and 8 females, ranging in age from 30 to 80 years (mean 54 years), all native Scots and all with endoscopically proven DU, were randomly selected and studied. All had long histories of

dyspepsia with signs and symptoms of DU, but none had recent acute gastrointestinal haemorrhage, a past history of gastric surgery, gastric malignancy, reflux oesophagitis nor treatment with drugs such as H₂ receptor antagonists, antibiotics, bismuth subcitrate ("De-Nol"), non-steroidal anti-inflammatory drugs (NSAIDs), steroids or anticoagulants at any time during the four weeks before the study began. Ten were cigarette smokers.

DU was diagnosed at endoscopy if at least one deep ulcer crater greater than 0.5 cm in diameter within the duodenal cap was present.

Controls

Subjects with DU were matched with 26 controls of similar age, sex, and smoking history. All efforts to find a further four controls failed. The control subjects were patients who had symptoms suggestive of peptic ulcer but who at endoscopy did not have an ulcer. Nineteen were males and 7 females ranging in age from 30 to 80 years (mean 55 years). Eight were cigarette smokers. The smokers in both groups were matched for their daily consumption of cigarettes. Non-smokers were defined as either those who had never smoked or who had stopped smoking for at least 6 months before the study. The same criteria were used to exclude subjects from the control group as from the group with ulcer.

It had been hoped to match the two groups by social class; however many of the participants were either retired, unemployed or housewives. The two groups were

similar in the number of such poorly defined groups , but these are not categories upon which previous epidemiological studies were based; therefore social class was not used in the analysis of the results.

3.3.2 METHODS

3.3.2.1 Study Design

The design of the study was explained to all the patients at their first visit to the gastrointestinal clinic. When they understood and agreed with the details of the study, they signed a consent form. At the same time they were asked to provide details of their diet, with special reference to fats, by completing a questionnaire designed by a senior hospital dietitian. Their smoking habits and physical complaints were also recorded. Seven to ten days later, during their second visit, the completed questionnaires were collected and samples of blood and biopsies of subcutaneous adipose tissue (see below) were obtained. The serum from the blood samples was separated and stored at - 70° C before analysis within three months. Antral biopsies were not obtained from this group of patients.

3.3.2.2 Adipose tissue biopsies

These were obtained by a surgical colleague, Mr.C.Rajgopal FRCS (Edin), as follows. With strict aseptic care, 2-3 ml of 2% lignocaine were injected subcutaneously

into the anterior abdominal wall, preferably in the right iliac fossa. A small, 1 to 1.5 cm incision was made through the skin and using scissors, a surgical scalpel and forceps, a small amount (20-30 mg) of fatty tissue was collected. The incision was closed using "Steristrips" or, rarely, by a stitch. The fatty tissue was rinsed in 0.9% (w/v) saline solution, dried on absorbent paper and stored in an Eppendorf container at -70° C before analysis.

3.3.2.3 Laboratory analysis of adipose tissue for fatty acids

3.3.2.3a Materials

The following equipment and chemicals were used: 1) Elvjehem glass potter homogenisers; 2) QF 15 ml tubes 19/26, MF24/2 ("Quickfit" BDH); 3) Saline solution- 0.9% NaCl (w/v), (Baxter Health Care Ltd); 4) Isopropanol (BDH) and Heptane (Sigma) as a mixture of 4:1 (v/v); 5) Heptane; 6) KOH, 0.5% aqueous (w/v), (BDH); 7) Washing solution consisting of Isopropanol/ Heptane /KOH (0.5%), 4:1:3 (v/v/v), from which the upper (heptane) layer had been aspirated and discarded; 8) Toluene ("Aristar", BDH); 9) Sodium methoxide, 0.5 M in dry methanol (BDH); 10) Acetic acid ("Glacial" BDH); 11) Hexane, (HPLC grade, Rathburn Chemical); 12) Standard fats: tripalmitin, tripalmitolein, tristearin, triolein and trilinolein (Sigma), dissolved in isopropanol and heptane as at 4 above; 13) Chloroform ("Analar" grade, BDH); 14) GLC Pye chromatograph 104 (see below).

Sample preparation

Fat standards containing tripalmitin, tripalmitolein, tristearin, triolein and trilinolein were analysed at concentrations of 0.2, 0.4, 0.6, 0.8, 1, 2 and 3 mg per ml of fatty acid in isopropanol/heptane solution to which 2 mg of an internal standard was added (see below). The correlation co-efficient for the standard curves was greater than 0.99.

To analyse patients' samples, a composite fat standard containing 1 mg palmitic, 0.5 mg palmitoleic, 0.5 mg stearic, 1 mg oleic and 1 mg linoleic acid per ml was used. As an internal standard, triheptadecanoin was used at a concentration equivalent to 2 mg per ml of heptodecanoic acid.

3.3.2.3b Methods

The adipose tissue was thawed; 10 mg were rinsed in saline and homogenised in 3ml of isopropanol/heptane solution in a QF tube. One ml of the internal standard was added. Two ml of the composite standard was taken into a QF tube and one ml of the internal standard and two ml of isopropanol/heptane mixture added. Adipose tissue and standards were analysed in duplicate. To each tube, 3 ml of heptane and 3 ml of KOH solution were added. The tubes were then shaken for 5 min and then left undisturbed for 10 min at room temperature; the top layer was transferred to a second QF tube. To this, 8 ml of washing solution was added. The tube was shaken for 5 min and centrifuged for 5

min at 1000-1300 rpm. The top heptane phase containing triglyceride free from free fatty acid (FFA) and phospholipids was transferred to a third QF tube and evaporated to dryness on a sand bath using oxygen-free nitrogen. One ml of toluene and two ml of sodium methoxide solution were added and transmethylation (of lipids) carried out at 50°C in a water bath for 10 min. Acetic acid (0.1 ml), five ml of water and five ml of hexane were added, mixed for five min and again centrifuged for five min. The top layer was transferred to a fourth QF tube and evaporated to dryness on a sand bath using oxygen-free nitrogen. Chloroform (0.5 ml), was added to redissolve fatty acid methyl esters (FAME) before injecting the samples into the gas liquid chromatograph (GLC). The injection volume was 3 μ L (Wood and Rimersma, 1987).

Gas-liquid Chromatography

The GLC (Pye chromatograph 104) was fitted with a 2m/4mm column, packed with 10% GP-DEGS on 80/100 mesh supelcoport (Supelco Co LTD). The FAMES were separated at an oven temperature of 150°C using as the carrier gas oxygen-free nitrogen (OFN) at a flow rate of 40 ml/min and flame ionisation detection with output to a "Vitaron" chart recorder (fig. 3.1).

In order to measure the absolute amounts of individual fatty acids in adipose tissue, internal standard in a concentration of 2 mg per ml, was added to both external standard and samples. This was done to correct for any minor variations in pipetting during the various extraction stages in the preparation of the fatty acid methyl esters

- P = Palmitic**
- Po = Palmitoleic**
- HD = Heptadecanoic (int. standard)**
- St = Stearic**
- O = Oleic**
- L = Linoeic**

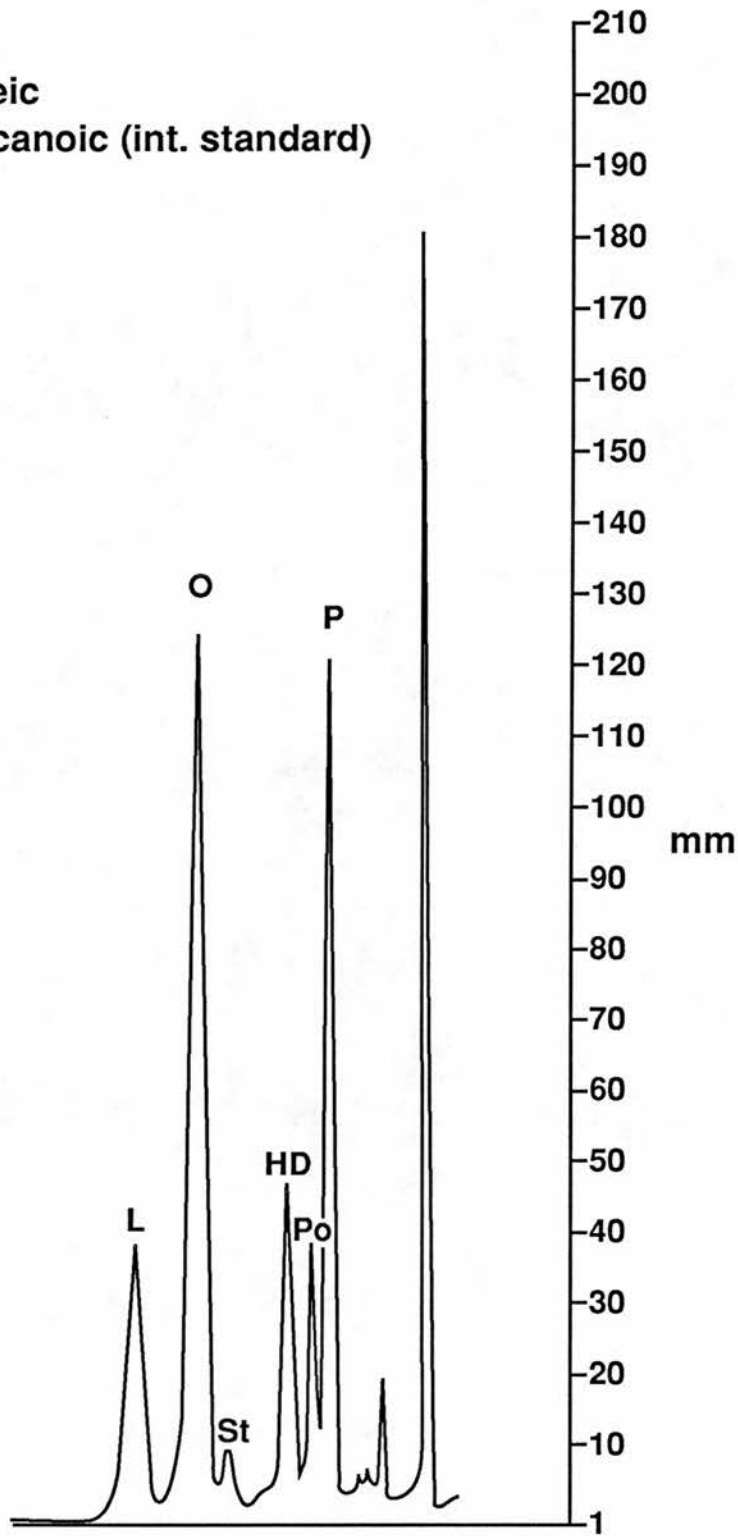


Fig. 3.1 Example of chromatography: fatty acid profile from adipose tissue biopsies

and also to correct for differences in injection volumes on to the GLC.

The individual fatty acids were quantified by comparison with a composite standard using ratios with the internal standard. Internal standard per se was not measured.

Mathematical calculation of analysed fatty acids

From the chromatograph trace the height of individual fatty acids and internal standard were measured in millimetres, and the ratio of individual fatty acid and internal standard peak height determined. This ratio was further divided by the ratio of the standard fatty acid peak heights to the internal standard peak height and then multiplied by the standard value in milligrams for the individual fatty acids. Finally the results in milligrams of fatty acid was divided by the amount of tissue/test sample (in milligrams) and multiplied by 100 to express the results as a percentage.

For example, peak height of linoleic acid in sample = 50 mm, peak height of internal standard in sample = 46 mm, peak height of linoleic acid as a standard = 60 mm, peak height of internal standard used in standard = 52 mm, concentration of linoleic acid used as standard = 1 mg per sample extracted, adipose tissue analysed in sample = 10 mg.
 $(50 \div 46) \div (60 \div 52) = 1.08 \div 1.15 = 0.93 \times 1 = 0.93 \div 10 = 0.093 \times 100 = 9.3 \% \text{ (linoleic acid).}$

Peak height was measured to the nearest millimetre. On average 1 millimetre was equivalent to a fatty acid weight of 0.02 mg per sample, indicating a satisfactory sensitive

method. For each chromatogram reading , the peak height of the internal standard was between 40 to 55 mm. Peaks less than 5 mm were not measured. This means that the lower limit of sensitivity was 0.1 mg per sample for each fatty acid.

The coefficient of variation for assay of the individual fatty acids was between 4% and 6%, indicating a proper and satisfactory separation of individual fatty acids.

3.3.2.4 Statistical methods

Quantitative variables were compared between groups by student's "t" test or Wilcoxon tests depending on distribution of data. Spearman rank correlation was used to test for association between quantitative variables. Multiple linear regression was used to test whether the various factors predicted to affect adipose tissue linoleic acid levels when adjusted for one another.

3.3.3 ANALYSIS OF DIETARY CONSTITUENTS

Each subject was asked to keep details of their diet for seven consecutive days. This involved their recording in a diary every item of food or drink consumed. The average daily intake during the seven day period was then calculated and the relative preponderance of different dietary constituents was determined using a computer model based on the tables of the composition of different foods

published by McCance and Widdowson (Holland et al., 1991). Particular attention was paid to the daily intake of saturated and unsaturated fats. (Dietary data were quantified by a senior dietitian, Alice Michie).

The accuracy of the assessment of dietary intakes is dependent on each individual's recording and reliability of their food measurement.

An example of a dietary diary and food frequency questionnaires and the way in which diet was assessed from diaries is shown in appendix 1.

3.4 RESULTS

There was no significant difference in the mean daily total fatty acid intake of DU patients and control subjects, whether smokers or non-smokers (table 3.1). In the case of smokers with DU, mean fatty acid intake tended to be less (67.69 gm) than in controls who also smoked (87.18 gm).

The mean % of linoleic acid in adipose tissue was significantly ($p < 0.002$) lower in the ulcer group ($7.73\% \pm 2.3$) than in controls ($10.04\% \pm 3.1$) and this was attributable to differences in non-smokers (see below). The mean % of other fatty acids did not differ significantly between the two groups (table 3.2).

The intake of linoleic acid as a percentage of total fatty acids was similar in the subjects with DU and in controls (9.9% and 9.7% respectively). The mean % linoleic acid content of adipose tissue fatty acids in control subjects was similar to the percentage in their

diets (10% and 9.7% respectively). The mean % of linoleic acid in the adipose tissue of DU subjects was significantly lower than that in their diet ($p < 0.05$; table 3.3).

The mean % linoleic acid in the diet of smokers and non-smokers was similar in both DU patients and control subjects. Among non-smokers, the mean % adipose tissue linoleic acid was lower ($p < 0.001$) in those with DU (7.85 ± 1.95) than in the controls (11.98 ± 2.41) but was similar in DU patients whether they smoked or not (table 3.4).

In addition among non-smokers, the mean % of monounsaturated fatty acids (MUSFA) in adipose tissue was significantly lower ($p < 0.05$) in the DU group (40.92 ± 4.82) than in the controls (44.32 ± 5.59).

There was no significant difference in the weight or height of DU and control subjects in relation to smoking (table 3.5). The body mass index of DU patients and control subjects was not statistically significantly different.

The mean % linoleic acid in adipose tissue was significantly lower ($p < 0.001$) in smokers ($7.6\% \pm 1.8$) than in non-smokers ($9.9\% \pm 2.2$). The distribution of linoleic acid among smokers was similar in both DU and control subjects (figure 3.2).

Results of statistical analysis

The mean % linoleic acid in adipose tissue was significantly lower in the DU group than in controls after adjustment for age ($p < 0.002$).

Table 3.1 The mean daily total dietary fatty acids* intake in smokers and non-smokers with and without duodenal ulcer.

	n	Total dietary fatty acid intake in gm (SD)
Duodenal ulcer	30	68.76 (23.33)
Smokers	10	67.69 (23.07)
Non-smokers	20	69.79 (22.55)
control	26	75.09 (26.88)
Smokers	8	87.18 (31.11)
Non-smokers	18	69.04 (21.84)

*(Saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids).

Table 3.2 Mean values of adipose tissue fatty acids (as % of total fatty acids) in duodenal ulcer and control subjects.

Fatty acids	Duodenal ulcer	Control
	n = 30	n = 26
	mean % (SD)	mean % (SD)
Palmitic acid (16:0)	18.21(2.43)	18.59(2.39)
Palmitoleic acid(16:1)	4.28(1.36)	5.1(1.26)
Stearic acid (18:0)	4.09(0.8)	4.35(1.03)
Oleic acid (18:1)	37.4(4.49)	39.39(4.47)
Linoleic acid (18:2)	7.73(2.3)*	10.04(3.1)*

* p < 0.002.

Table 3.3 Mean linoleic acid content of diet and adipose tissue (as % of total fatty acids) in subjects with duodenal ulcer and controls.

		%	
		(Linoleic acid)	
		Adipose tissue	Dietary
	n	Mean (SD)	Mean (SD)
Duodenal ulcer	30	7.7(2.3)*	9.9(3.1)*
Control	26	10.04 (3.1)	9.68(2.6)

* $p < 0.05$.

Table 3.4 Mean linoleic acid (LA) as % of total fatty acids in the adipose tissue and in the diet of smokers and non-smokers with and without duodenal ulcer.

		%	
		(Linoleic acid)	
		Adipose tissue	Dietary
	n	Mean (SD)	Mean (SD)
Duodenal ulcer	30	7.7(2.3)	9.9(3.1)
Smokers	10	7.52(1.76)	9.69(2.33)
Non-smokers	20	7.85(1.95)*	10.29(2.97)
Control	26	10(3.1)	9.68(2.6)
Smokers	8	7.97(2.01)	9.08(1.96)
Non-smokers	18	11.98(2.41)*	10.58(2.76)

* p<0.001

Table 3.5 The mean height and weight of duodenal ulcer patients and controls in relation to smoking.

	n	Weight in kgs (SD)	Height in cms (SD)
Duodenal ulcer	30	73.02 (12.9)	174.16 (12.19)
Smokers	10	72.68 (12.49)	168.65 (10.66)
Non-smokers	20	74.09 (13.51)	176.91 (11.96)
Control	26	68.17 (12.84)	170.96 (10.36)
Smokers	8	70.45 (12.85)	170.81 (8)
Non-smokers	18	67.09 (12.69)	171.01 (11.3)
Smokers (duodenal ulcer and control)	18	70.68 (12.38)	169.59 (9.9)
Non-smokers (duodenal ulcer and controls)	38	70.77 (13.42)	174.11 (11.88)

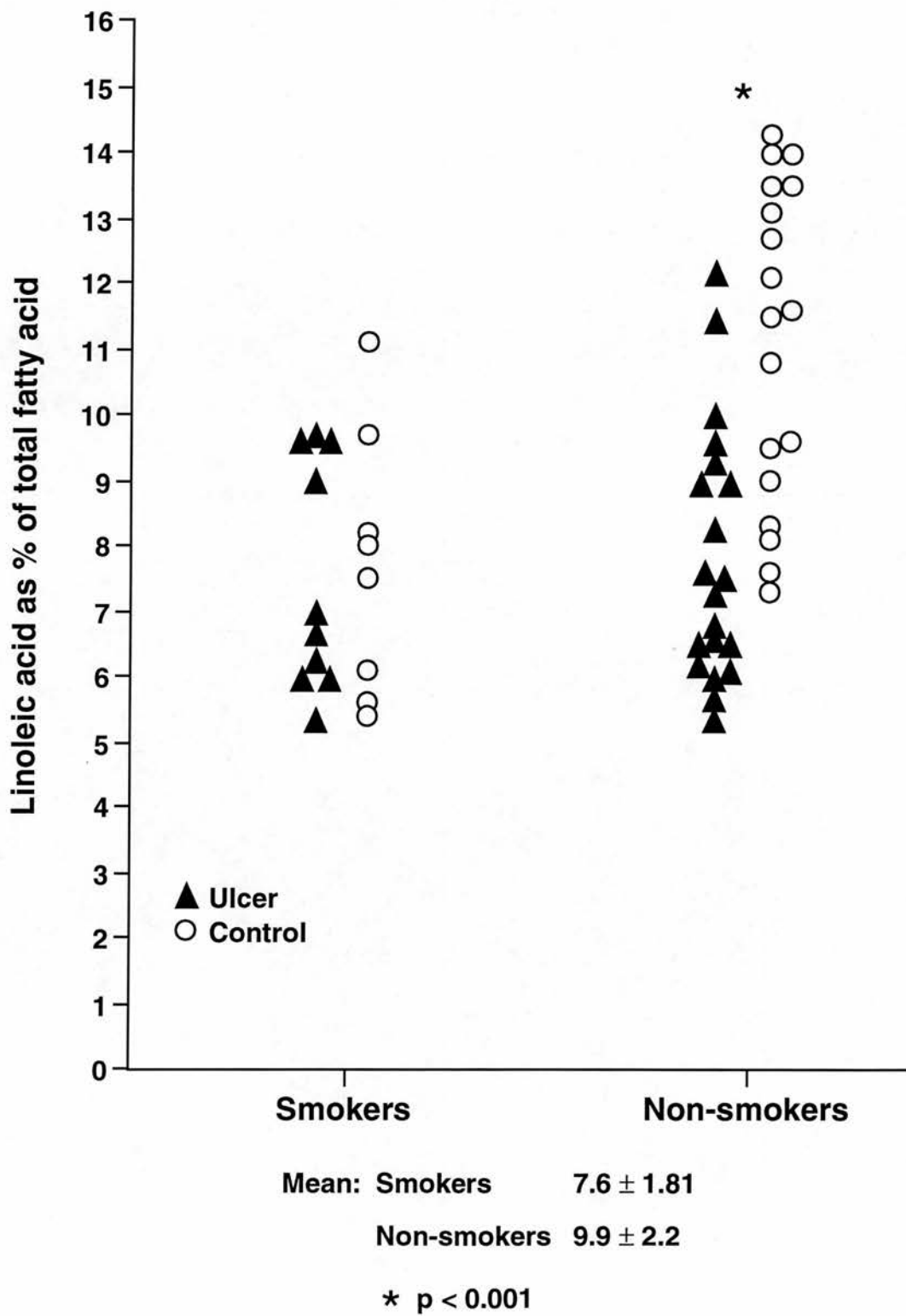


Fig. 3.2 Linoleic acid as percentage of total fatty acid in the adipose tissue of smokers and non-smokers with and without duodenal ulcer.

The mean adipose tissue % linoleic acid was significantly lower ($p < 0.012$) in smokers both in DU and control subjects than non-smokers after adjustment for age.

The mean % linoleic acid in adipose tissue of DU patients was significantly lower than that in their diet ($p < 0.05$).

Dietary linoleic acid did not correlate significantly with age.

Multiple regression analysis showed no significant interactions between dietary linoleic acid and any of the other factors (DU or controls, smokers or non-smokers and age) as predictors of adipose tissue linoleic acid.

3.5 DISCUSSION

The discussion of this chapter is combined with the discussion of the next chapter.

CHAPTER 4

Helicobacter pylori AND DUODENAL ULCER
RELATED TO FATTY ACID IN THE
DIET AND IN ADIPOSE TISSUE,
AND TO HISTORY OF CIGARETTE
SMOKING AND AGE

4.1 SUMMARY

In same group of patients studied in chapter 3, *H.pylori* antibody was measured by ELISA. *H.pylori* was cultured in the presence of olive oil using an *in vitro* assay.

Antibody titres were significantly higher in the elderly. This was true for both DU patients and control subjects. There was no correlations between age, antibody titre, dietary linoleic acid, or % adipose tissue linoleic acid. *H.pylori* did not metabolise linoleic acid *in vitro*.

4.2 INTRODUCTION

This chapter examines whether the factors studied in chapter 3 have any bearing on antral infection with *H.pylori*. The patients studied in chapter 3 were evaluated using a ELISA test for *H.pylori*. The object of the study being to see if a deficiency of linoleic acid in the adipose tissue, cigarette smoking or age were factors in the propensity of an individual to become infected with *H.pylori*.

A factor in the reduced tissue linoleic acid content could be a failure of absorption. There is no evidence which suggests that this is a reasonable hypothesis; however, unsaturated fatty acids are known to be metabolised by colonic bacteria. It is therefore possible

that *H.pylori* could also metabolise linoleic acid and hence reduce the linoleic acid available for absorption. This has been studied using simple culture techniques.

4.3 MATERIALS AND METHODS

Antibodies to *H.pylori* were measured in the DU and control patients as described in chapter 3. The study design is identical to that described in chapter 3. Antral biopsies were not obtained from patients described in chapters 3 and 4.

4.3.1 ENZYME LINKED IMMUNO-SORBENT ASSAY (ELISA) FOR ANTIBODY TO *H.pylori*.

4.3.1.1 Principles

This method involved the absorption of *H.pylori* antigen onto the plastic surface of a 96-well plate. Excess antigen was removed by washing and diluted test serum was incubated with the immobilised antigen. Unbound human antibodies were removed by washing. Thereafter any bound human antibodies usually IgG antibodies, were detected by incubation with anti-human immunoglobulin, conjugated with the enzyme peroxidase. The enzyme indirectly bound to the plate was then detected using a chromogenic substrate. The enzyme reaction was stopped and the colour product in each well measured using a plate reader. The assay is quantitative, the optical density of the colour product being directly proportional to the amount of bound antibody.

4.3.1.2 Materials

Serum antibody was assayed using a commercially available ELISA kit, "Helico-G", (Porton Cambridge). This contained: 1) 12 strips each of 8 microwells coated with *H.pylori* antigen stored in a sealed pouch containing desiccant; 2) two calibrator sera already diluted and ready for use. Calibrator 1 contained 10 and Calibrator 2 200 units per ml of specific anti IgG antibodies to *H.pylori*; 3) Peroxidase-conjugated Fab2 fragment (Goat Anti-human IgG). This was prepared for use by diluting 1 in 20 with wash fluid (0.25 ml of conjugate to 4.75 ml of wash fluid); 4) 250 ml concentrated wash/diluent fluid (ten fold concentrated); 5) Substrate chromogen (tetra methyl benzidine and urea peroxide) stabilised and ready for use; 6) Reaction stopping solution (2 M sulphuric acid).

Additional materials used were: 1) tubes and vials for sample dilution; 2) accurate and properly maintained pipettes for delivering volumes of 5, 10, 20, 50, 100, 200, 400 and 1000 μ l; 3) an eight-channel automatic pipette with disposable tips (Labsystem group Ltd); 4) multichannel plate photometer with 450 nm filter (Dynatech Laboratories Ltd); 5) deionised distilled water; 6) incubator or water bath at 37°C; 7) microtitration plate shaker (Denley Instruments Ltd).

4.3.1.3 Methods

The kit was warmed to room temperature. Before analysing a patient's serum, four standards containing

respectively 5, 20, 50 and 100 units per ml of antibody were prepared and analysed by dilution from the standard calibrator sera containing 10 and 200 units per ml respectively. Washing fluid (500 ml) was prepared by diluting 50 ml of concentrated fluid with 450 ml of deionised water. Before assay each patient's serum was diluted 200 fold by adding 995 μ l washing fluid to 5 μ l of serum.

Of the first column of wells, the first two were left vacant and from the third to eighth row 100 μ l of each of six different concentrations of the prepared standard antisera were added in order of increasing concentration. In the second and subsequent columns 100 μ l of each patient's serum diluted 1/200 were placed in adjacent wells. The plates were then incubated at 37° C in a moist atmosphere for 1 hr. Thereafter the wells were washed three times with diluted wash fluid and 100 μ l of peroxidase-conjugate was added. The plates were again incubated at 37°C in a moist atmosphere for 30 minutes. The wells were then washed three times with diluted wash fluid and 100 μ l of substrate added. The plates were shaken mechanically at room temperature for 10 minutes. Thereafter the reaction was stopped by adding 50 μ l of 2M sulphuric acid to each well and shaking the plates to ensure mixing. The underside of the strip wells were wiped and thoroughly cleaned and the intensity of any reaction (optical density) measured at 450nm in the photometer within 30 minutes of adding sulphuric acid. A test was considered positive if the reading was greater than or equal to that obtained with the

standard containing 10 units per ml. Serum concentrations were determined from a calibration curve constructed from the standard included with the kit.

This method is being used in different centres with satisfactory results (Newell et al., 1988; Wyatt and Rathbone, 1989). In this study the coefficient of variation of the method was 8%.

4.3.2 THE EFFECT OF *H.pylori* ON POLYUNSATURATED FATTY ACIDS

Experiments were performed to determine whether linoleic acid was metabolised by *H.pylori* to monounsaturated fatty acids. *H.pylori* was inoculated in presence of olive oil in Skirrow's medium and from the medium linoleic acid was measured (from both medium with or without inoculation of *H.pylori*).

4.3.2.1 Methods

Highly refined olive oil (Sigma) was homogenised in Skirrow's medium at concentrations of 0.1% and 0.5%. In some experiments 1% Tween 80 (polyoxyethylene-sorbitan mono-oleate, Sigma) was added as emulsifier to Skirrow's medium containing 0.5% olive oil. *Campylobacter* broth (Gibco) with supplements (Prolab Diagnostic) and 10% defibrinated horse blood was also prepared with the same concentrations of olive oil. Tween 80 was added as above to similar preparations of the broth.

The solid media and broths prepared with olive oil were

inoculated with *H.pylori* from colonies grown in Skirrow's medium from endoscopic antral biopsies. They were then incubated at 37° C in micro-aerophilic conditions (nitrogen plus 5% carbon dioxide and 5% oxygen) and in 95-100% humidity for 5 days. Two different controls were used and examined in parallel and in the same manner as the tests. The first consisted of 0.1% and 0.5% olive oil (with and without Tween 80) in Skirrow's medium and broth but uninoculated with *H.pylori*. The second control was Skirrow's medium and broth without added olive oil inoculated with *H.pylori*. Quantitative or semiquantitative assessment of numbers colonies were not done for *H.pylori* observed in the culture plates. All test samples and controls were analysed in duplicate for fatty acids using the same methods as for adipose tissue analysis.

4.3.3 Statistical methods

Quantitative variables were compared between groups by student's "t" test or Wilcoxon tests depending on the distribution of data. Spearman rank correlation was used to test for association between quantitative variables. Multiple linear regression was used to test whether the various factors predicted adipose tissue linoleic acid levels when adjusted for one another.

4.4 RESULTS

The mean % linoleic acid in the diet of the antibody positive and antibody negative groups did not differ significantly ($p>0.2$). The mean % linoleic acid in the adipose tissue of the antibody positive and antibody negative groups was similar (table 4.1). Antibody for *H.pylori* was positive in 27(90%) of DU patients and in 4(15%) of control subjects. The antibody titres were highest in older DU patients (fig. 4.1 a and b). As anticipated, antibody titres increase with increasing age in both DU and control subjects.

Results of statistical analysis

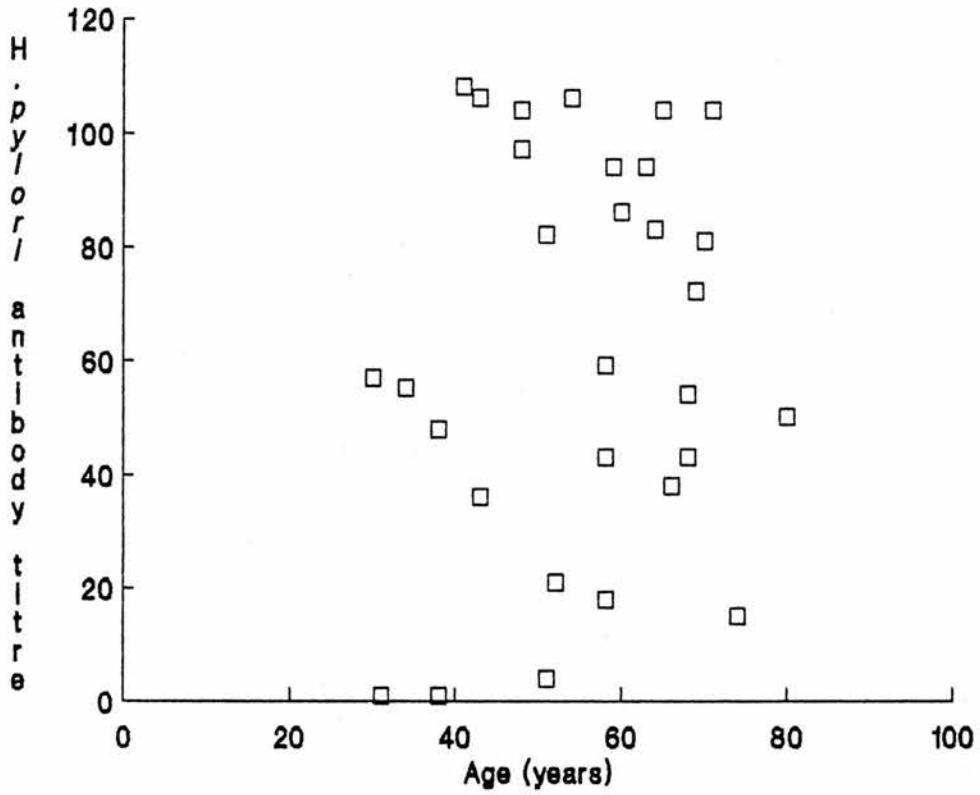
Quantitative serum ELISA antibody concentrations did not correlate significantly with either % adipose tissue or dietary linoleic acid. There was a positive correlation with age ($p<0.05$) and the concentrations were significantly higher in DU patients than in controls ($p< 0.001$).

Multiple regression showed no significant interactions between dietary linoleic acid and any of the other factors (DU or controls, smokers or non-smokers, age, quantitative ELISA value) as predictors of adipose tissue linoleic acid (table 4.2).

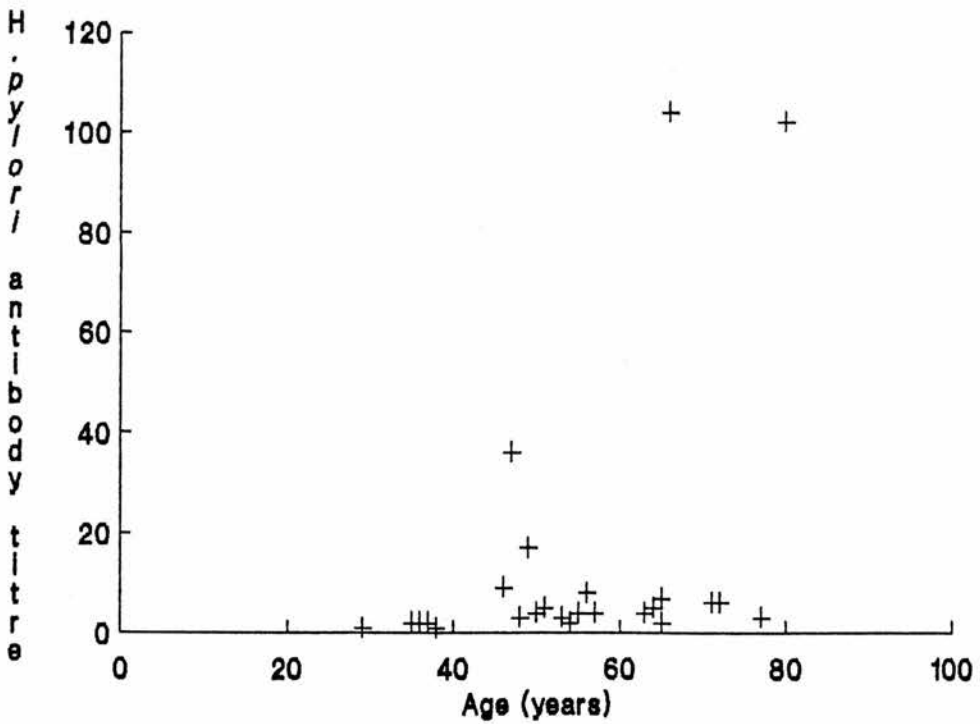
Table 4.1 Mean linoleic acid as % of total fatty acids in the diet and adipose tissue of duodenal ulcer and control subjects related to the presence or absence in their serum of antibodies to *H.pylori*.

	Antibody present n = 31 mean % (SD)	Antibody absent n = 25 mean % (SD)
Diet	10.7%(2.8)*	9.6%(2.7)*
Adipose tissue	8.3%(2.5)*	9.4%(2.7)*

* NS, $p > 0.05$



a) 30 duodenal ulcer patients



b) 26 control patients

Fig. 4.1 Antibody titre in relation to age

Table 4.2 Multiple regression coefficients for prediction of adipose tissue linoleic acid (LA) from dietary LA and other factors (DU / control, quantitative ELISA levels, smoking and age).

Factors	Coefficient (Standard error)	
Dietary LA	0.34	(0.38)
DU/control	-1.27	(2.41)
Interaction	-0.11	(0.22)
Dietary LA	0.14	(0.17)
ELISA titre	-0.02	(0.03)
Interaction	0.000	(0.003)
Dietary LA	0.10	(0.14)
Smoking	-0.61	(3.02)
Interaction	-0.11	(0.30)
Dietary LA	0.28	(0.51)
Age	0.03	(0.11)
Interaction	-0.003	(0.010)

No significant interactions demonstrated.

The linoleic acid content (as a percent of total fatty acids of olive oil) of Skirrow's medium containing homogenised olive oil was the same (3.85%) whether the culture medium had been inoculated with *H.pylori* or not and incubated, i.e., growth of *H.pylori* on the medium did not reduce the linoleic acid content.

4.5 DISCUSSION

Whilst the identification of *H.pylori* in the antrum of patients with peptic ulceration introduces an important new aetiological factor in chronic gastritis and DU, it is still not clear why *H.pylori* infects some individuals but not others. Cigarette smokers and patients with DU have a reduced adipose tissue linoleic acid content.

Dietary linoleic acid supplementation increases gastric PGE output and decreases gastric acid secretion. It has been suggested that the falling incidence and virulence of DU is related to increased dietary polyunsaturated essential fatty acid (linoleic acid) intake. The adipose tissue fatty acid content is said to closely reflect its dietary intake.

In the studies described in chapters 3 and 4, the DU patients and control subjects (smokers and non-smokers) were similar in both weight and height. The mean dietary intake of linoleic acid as a % of total fatty acid was identical in the subjects with DU and non-ulcer dyspepsia (9.9% and 9.7% respectively). The mean % linoleic acid

content in adipose tissue of the control subjects was identical to the mean % linoleic acid in their diets (10% and 9.7% respectively). The mean daily total dietary intake of fatty acids by patients with DU who were smokers was less than that of control smokers (67.7gm and 87.2 gm respectively), but multiple regression showed that the dietary intake of linoleic acid did not differ significantly when comparing DU patients with controls, smokers with non-smokers or subjects with or without antibody against *H.pylori* when controlled for age. Kearney et al. (1989) showed a lower intake of linoleic acid and a significantly lower intake of dietary fibre in both dyspeptic and peptic ulcer patients compared with healthy controls. They suggested that this difference might be due to rejection or acceptance of particular food items by dyspeptic and peptic ulcer patients. In contrast, the results of the studies reported here show that linoleic acid intake is not low in DU patients thus rejecting the suggestion of Kearney et al.. The protocol followed in this study was not designed to assess the dietary fibre in the diet.

Quantitative ELISA values did not correlate significantly with either adipose tissue linoleic acid or dietary linoleic acid. But elderly ($p < 0.05$) and DU patients had higher antibody levels than controls ($p < 0.001$). These findings are similar to published epidemiological studies (Perez-Perez et al., 1988; Kosunen et al., 1989; Sitas et al., 1991).

The mean % linoleic acid in the adipose tissue of DU

patients was significantly lower than in their diet ($p < 0.05$); however, there was no statistically significant difference in these levels when comparing DU patients and controls, smokers and non-smokers or subjects with or without antibody or antibody titre to *H.pylori* when controlled for age. This finding might be due to different numbers of DU patients and controls. In a large study Fulton et al. (1988) showed that cigarette smokers had a significantly lower intake of polyunsaturated fatty acid (PUSFA) and fibre (but they did not measure the adipose PUSFA).

The mean % of linoleic acid but not of the other fatty acids in adipose tissue was significantly lower in the ulcer group than the controls ($p < 0.002$). This finding suggests that linoleic acid levels are indeed important in DU development, either due to primary deficiency in the diet (although no such deficiency was found in the present study) or due to secondary defective metabolism during PG synthesis. This finding is similar to those of Kearney et al. (1989) and Grant et al. (1990). Riemersma et al. (1986) found the lowest proportion of linoleic acid in the adipose tissue of men from north Kerala (India), and Finland; the highest was in Italy and levels in Scottish men were intermediate. In a similar way, regional differences in peptic ulcer prevalence might be related to the proportion of linoleic acid in adipose tissue. Langman (1979) stated that in the UK, the prevalence of DU increases the further north is the population. It might be,

therefore, that the increased prevalence of peptic ulcer in the Scottish population is due to their having less adipose tissue linoleic acid than the English. In this case dietary intake might not be the sole variable; the difference might be related also to differences in cigarette smoking (Wald et al., 1988).

In addition, the mean % of linoleic acid as a % of total fatty acid in the adipose tissue in relation to age of the smokers (7.6 ± 1.8) was significantly lower than that of non-smokers (9.9 ± 2.2) with or without DU ($p < 0.012$). The distribution of % adipose linoleic acid among smokers was similar in both DU and control subjects. By contrast, among non-smokers, the mean % adipose linoleic acid was lower in ulcer patients (7.85 ± 1.94) than in controls (11.98 ± 2.41) ($p < 0.001$). Among non-smokers, the mean % of monounsaturated fatty acids in adipose tissue was lower in the DU group (40.92 ± 4.82) than in controls (44.32 ± 5.59) ($p < 0.05$).

The findings in chapter 3 raise certain possibilities which might account for the lower % of linoleic acid in the adipose tissue of smokers and DU patients. The first (but unlikely) possibility is that smokers are less able to provide a reliable dietary history than non-smokers. Secondly, *H. pylori* which is present in the stomachs of DU patients might metabolise or hydrogenate linoleic acid to other saturated fatty acids; thus, the linoleic acid is utilised by the stomach with a consequent lower % of linoleic acid in adipose tissue. Watson (1965) showed that PUSFA are hydrogenated by bacterial activity in the

colon to saturated fatty acids products. The % of linoleic acid in the diet and in the adipose tissue of individuals with or without antibody to *H.pylori* or antibody levels, age, smokers or non-smokers, DU or controls did not differ statistically, nor in addition, did the experiments in which *H.pylori* was incubated with olive oil show breakdown of linoleic acid in this commonly ingested form. There is no evidence from these experiments that *H.pylori* is responsible for the lower % of linoleic acid in adipose tissue in DU patients. At the present moment it is not reasonable to believe that *H.pylori* is capable of metabolising sufficient linoleic acid to affect levels in the fat stores.

The data are confounded and might simply reflect the differences in social class. If social class could be measured accurately the effect of diet and smoking might have been clearer.

The remaining possibility is that there might be abnormal tissue metabolism of linoleic acid in DU patients who smoke.

Robert (1979) and Miller (1983) showed that PGs particularly PG_2 , and PGI_2 protect the gastric mucosa. Lam et al. (1986) showed that PGE_1 overcomes the adverse effects of smoking in chronic DU healing. On the other hand, both PGF_2 alpha, a cyclo-oxygenase product with vascular effect on the submucosal microcirculation (Whittle et al., 1985) and thromboxane A_2 ($Tx A_2$) a potent platelet aggregator and vasoconstrictor formed by thromboxane

synthetase (Whittle et al., 1981; Bennet, 1983; Whittle et al., 1985) might be responsible for gastric injury. McCready et al., (1985) showed that cigarette smoking decreases PGE levels and causes peptic ulceration. Fedi et al. (1990) showed that cigarette smoking decreased PGE₂ and increased PGF₂ alpha, TxB₂ and consequently predispose to peptic ulceration.

This study has shown that there is a lower adipose tissue content of linoleic acid in smokers regardless of whether or not they have DU. Non-smoking patients with DU had a reduced adipose tissue linoleic acid content compared to non-ulcer patients.

Comparison between dietary intake of linoleic acid and adipose tissue concentration suggest that there is in smokers some factor which influences the metabolism of linoleic acid. Zijlstra et al. (1992) showed that smokers had a significantly higher concentration of eicosanoids in bronchoalveolar lavage than non-smokers. It is not unreasonable to suppose that the major influence of smoking on linoleic acid metabolism is through modulation of cyclo-oxygenase and lipoxygenase derivatives.

The major quantitative differences in the eicosanoids are TxB₂, PGF₂ alpha (cyclo-oxygenase derivatives), 12-hydroxy-eicosa-tetraenoic acid (HETE) and 15-HETE (both are lipoxygenase products). It is the products of the cyclo-oxygenase pathway of arachidonic acid metabolism which are important in the gastric mucosa. In a similar way, in the gastric mucosa there might be more PGF₂ alpha and TxB₂ due to smoking, with consequent damage to the gastroduodenal

mucosa and subsequent peptic ulceration.

PGs can protect the gastric mucosa through many mechanisms (Robert, 1981 and 1984; Miller, 1983; Hollander and Tarnawski, 1986; Rask-Madsen and Lauritsen, 1987; Hawkey, 1989; Tovey et al., 1989; Northfield and Goggin, 1991), these include: the maintenance of an intact mucosal barrier; increasing gastroduodenal bicarbonate secretion (Feldman, 1983; Isenberg et al., 1986); decreased acid secretion when given orally or parentally (Horton et al., 1968; Classen et al., 1971; Befrits and Johansson, 1985). This may come about by PGs blocking histamine induced formation of cyclic AMP (Puurunen, 1983). Some PGs protect gastric mucosa against injurious agents such as alcohol (Konturek, 1985), possibly by acting not at the cell surface but on the microcirculation of the gastric mucosa or more centrally (Guth et al., 1984).

If gastric mucosal integrity is diminished by smoking and reduced linoleic acid concentration in the body tissues, this does not appear to be mediated through an increased propensity for *H.pylori* infection of the antrum. The mechanism may well be through the cytoprotective elements of the gastric mucosa. There could be a loss of linoleic acid from the body through the bronchial secretions or there may be alteration in eicosanoid metabolism.

DU is associated with changes in linoleic acid metabolism, probably a decrease in cyclo-oxygenase activity, and this is accentuated by smoking.

CHAPTER 5

CULTURE OF *H.pylori*

FROM GASTRIC ANTRAL BIOPSY,

DENTAL PLAQUE OR MATERIAL

OBTAINED BY TOOTH PICKING

5.1 SUMMARY

In order to identify the reservoir of *H.pylori* infection, tooth plaque cultures were performed in 60 dentulous DU patients, from the dental plates of 3 edentulous DU patients and in 30 age matched non-ulcer dyspepsia control subjects. Gastric antral biopsies were subjected to the urease test, histology and culture. Nucleic acid analysis of *H.pylori* was done on five pairs of strains isolated by culture from DU patients.

Fifty nine (94%) DU patients had *H.pylori* associated gastritis and *H.pylori* was demonstrated by histology in 53(84%). *H.pylori* was cultured in Skirrow's medium from tooth plaque in 12 patients (19%) and in 56(89%) from antral biopsies. In DU patients the urease test was positive in 57% of tooth plaques and 79% of antral biopsies. *H.pylori* isolated from tooth plaques and antral biopsies had an identical nucleic acid structure. Seven control subjects had *H.pylori* gastritis, *H.pylori* was demonstrated histologically in four, and two of these had a positive culture from tooth plaque. *H.pylori* was not cultured from the dental plates of edentulous individuals nor was it isolated from saliva or the tooth plaque of individuals who did not exhibit gastritis.

5.2 INTRODUCTION

When *H.pylori* is eradicated from the gastric antrum using triple therapy there is a more prolonged remission of DU than if the ulcer is healed by ranitidine. Relapse is associated with colonisation of the gastric antrum by *H.pylori*. The source of the *H.pylori* could be an external one, but the regularity of the re-infection suggests a nidus of infection within the host. The mouth, possibly dental plaque or saliva were considered to be prime possibilities. Saliva and dental plaque were cultured and the *H.pylori* identified by enzymatic, microscopic, and ribotyping methods.

5.3 MATERIALS AND METHODS

5.3.1 Subjects

Sixty three subjects, 48 males and 15 females, ranging in age from 20 to 83 years (mean 50 years) with endoscopically proven DU (at least one deep ulcer crater 0.5cm in diameter within the duodenal bulb) were studied. Three were edentulous. All had a long history of dyspepsia with signs and symptoms of DU but none had recent acute gastrointestinal haemorrhage, past history of gastric surgery, malignancy, reflux oesophagitis nor treatment with drugs such as H₂ receptor antagonists, antibiotics, bismuth subcitrate ("De-Nol"), non-steroidal anti-

inflammatory drugs (NSAIDs), steroids or anticoagulants at any time during the previous four weeks before the study.

Thirty control subjects of similar age and sex comprised a control group. Initially the ulcer and control groups each consisted of 30 subjects, but after initial success in culturing *H.pylori* from the dental plaque of DU patients, this group was extended to 63.

The control patients were those who had symptoms suggestive of peptic ulcer but in whom an ulcer was not seen at endoscopy. They comprised 18 males and 12 females, aged 20 to 81 years (mean 52 years). The same criteria were used to exclude patients from the control group as from the group with ulcers. All of the control patients had at least some of their own teeth.

Antibody against *H.pylori* was not measured in these patients. Smoking and social class were not recorded.

The patients were endoscoped in the normal manner. A mouth guard was placed in the mouth between the teeth prior to inserting the endoscope. The endoscope was directed straight into the pharynx to protect the instrument. The mouth guard is oval, measuring 3x4 cms in size and has a cusp 5 cms by 4 cms at the front which sits across the teeth. The important function of the guard is to protect the endoscope from the teeth. By the same token the endoscope will be quite remote from contamination by dental plaque. Contamination of the teeth with *H.pylori* during endoscopy is therefore extremely unlikely.

5.3.2 Methods

Tests for *H.pylori*

At endoscopy, macroscopic findings were documented and three antral biopsies were taken. One was examined histologically after being stained with haematoxylin and eosin to determine if type B gastritis was present and to detect *H.pylori*. The second was used in a *Campylobacter*-like organism (CLO) test for urease. The sensitivity of the CLO test using an unspecified bacterial load in antral biopsies is 95% at three hours after incubation (Morris et al., 1986a; Marshall et al., 1987). The third biopsy was examined for live *H.pylori* by transporting it immediately in 0.5-1 ml of 0.9% saline solution in a sterile "Universal" container to the laboratory where within thirty minutes to one hour it was cultured on Skirrow's medium (Skirrow, 1977). This contained: Columbia agar base (Gibco Life Technology Ltd) containing special peptone, 23 gm/L; starch, 1 gm/L; NaCl, 5 gm/L; agar, 10 gm/L. These ingredients were dissolved in one litre of distilled water and sterilised by autoclaving at 121° C for 15 minutes. The pH was adjusted to 7.3. Defibrinated 10% horse blood (Scottish Biotechnology Instrumentation) 100 ml/L and selective (Skirrow's) supplements (Prolab Diagnostic) consisting of vancomycin 10 mg/L, polymixin B 2500 IU/L and trimethoprim lactate 5 mg/L were added to the cooled but molten base medium.

Inoculated plates were incubated for 4-6 days at 37°C in 95-100% humidity and an atmosphere containing nitrogen, oxygen and carbon dioxide in ratios of 90:5:5. Colonies resembling *H.pylori*, 1 mm in diameter, shiny, convex, rounded and weakly haemolytic were further examined by standard tests (Cowan, 1974) for urease, catalase and oxidase activity and after staining by Gram's method for typical S - shaped, curved or spiral Gram-negative bacilli.

Attempts were also made to culture *H.pylori* from saliva and tooth pickings. In the endoscopy recovery room patients were asked to spit saliva into a sterile "Universal" container. A minimum of 4-5 ml was collected. This was centrifuged for 15 minutes at 1000 rpm using a Centaur 1 (M.S.E) centrifuge and the deposit was spread on Skirrow's medium.

Tooth pickings (plaque) were collected after endoscopy. In the recovery room patients were asked to scrape between their teeth with a plastic tooth pick ("Pick plak", HWC Supplies). The material produced was transported to the laboratory in a sterile "Universal" container and was spread within 15-30 minutes on Skirrow's medium as described above. From these plates which always produced mixed bacterial and fungal growth, suspect colonies of *H.pylori* were subcultured 2-4 times on Skirrow's medium until a pure culture was obtained. The colonies were identified and confirmed as *H.pylori* by their morphology, enzyme reactions and nucleic acid analysis.

Identification and typing of strain by nucleic acid analysis

This was done by Dr. R.J. Owen, London. Ribotyping was carried out on five pairs of strains of *H.pylori*, each pair consisting of strains cultured from the antral biopsy and tooth plaque of one individual with DU and on an eleventh mouth strain from a patient whose antral strain did not survive after repeated subculture. Confirmation of their identity as *H.pylori* was sought by Southern blotting of *HindIII* digest probed with a cloned random DNA sequence (HP7) specific for *H.pylori* (Owen, 1989; Owen et al., 1992a). The relatedness (homology) of paired strains from the mouth and antrum of single patients was examined by i) Southern blotting of *HaeIII* digest probed with a 16+23S and a 16S rRNA gene probe (Owen et al., 1992b); ii) *HindIII* digest probed and in the same way iii) by Southern blots of *HaeIII* digest probed with a specific 16S rRNA gene probe obtained by the polymerase chain reaction.

DNA isolation, restriction digestion, and ribosomal RNA gene probe hybridisation

Chromosomal DNA was isolated and purified using the guanidium thiocyanate reagent method (Owen, 1989; Owen et al., 1992a and 1992b). All DNA samples (5 μ g) were digested for four hours at 37°C with *HaeIII* and *HindIII* (2-3 U/ μ g of DNA). The digested DNA was electrophoresed at 25 V for 16 hours using a horizontal 0.7% (w/v) agarose gel in a buffer containing 89 mM Tris hydrochloride, 89 mM boric acid, and 2 mM disodium ethylenediaminetetra-acetic acid

(EDTA) (pH 8.3). After electrophoresis, the DNA fragments were transferred to Hybond-N nylon membranes by vacuum transfer blotting. The membranes were then hybridised by standard procedure for 18 hours at 42°C with a biotinylated cDNA probe prepared from a 16S and 23S rRNA mixture of *H.pylori* NCTC 11638 using Moloney mouse leukaemia virus reverse transcriptase. Biotinylation was achieved by the incorporation of biotin-16-dUTP. The membranes were washed after hybridisation and the hybridised probe detected colorimetrically using a non radioactive detection kit-BlueGENE. The pattern of bands obtained was designated the ribopattern.

Examination of strains by electron microscopy

Some of the strains of *H.pylori* recovered from antral biopsy and tooth plaque were also examined by electron microscopy. (This was done by Mr. F. Donnelly, Edinburgh).

For this, a rectangle of Skirrow's medium carrying a dense growth of *H.pylori* from an antral biopsy or from tooth pick plaque was cut from a culture plate and fixed in 3% glutaraldehyde (Merck-Darmstadt Ltd)/0.1M sodium cacodylate HCL (Merck, Darmstadt Ltd) buffer pH 7.2-7.4 for 45 minutes at 4-8° C and washed three times (each time for 10 minutes) gently in 0.1M sodium cacodylate HCL buffer. Post fixation (for lipids) was in 1% aqueous osmium tetroxide (Johnson Matthey) in 0.1M sodium cacodylate HCL buffer for 30 minutes at 4-8° C in a fume cupboard. The samples were washed three times (each time for 5 minutes)

with deionised water. Thereafter the culture block was dehydrated in gradually increasing concentrations of ethanol (50%,70% and 90%) for 10 minutes in each. Three exposures for 10 minutes in 100% ethanol finally dehydrated the preparation. The culture block was submerged in a linking agent (propylene oxide, Merck Darmstadt) three times (each time for 10 minutes in fresh solution) and then impregnated with araldite resin (Agar Scientific) overnight. The culture block was then imbedded (with the colonies face upwards) in freshly prepared resin and "cured" for 48 hrs at 60° C. Sections 90 nm thick were cut on an ultratome (Reichert OM42) using a triangular glass knife, picked up on 300 mesh copper grids, dried for 1 hr, then stained with heavy metal salts (uranyl acetate with lead citrate, Merck, Darmstadt) by Reynolf's method. Sections were washed in deionised water and dried for 10 minutes before viewing in a JEOL 100 CXii (JEOL) transmission microscope- operated at 60 KV. Photographs were taken and processed using Ilford film, paper and chemicals.

Histological examination

Standard paraffin sections prepared from endoscopic antral biopsies were stained with haematoxylin and eosin, or whenever necessary by Giemsa stain, and examined for the presence of bacilli morphologically resembling *H.pylori* and the presence of type B-gastritis.

5.3.3 Statistical methods

DU and control patients were compared for presence of binary factors after adjustment for age difference by multiple logistic regression. Chi-squared test also used for presence or absence of *H.pylori* in both groups.

5.4 RESULTS

Fifty nine (94%) of the patients with DU had histologically demonstrable antral gastritis of varying degree and in 53 (84%) the presence of *H. pylori* also could be demonstrated histologically (table 5.1).

In the control group, seven (23%) had some form of gastritis and *H. pylori* was seen in four (13%). *H.pylori* was cultured from 56(89%) of 63 antral biopsies (fig. 5.1a) and from samples of tooth plaque of 12(19%) patients with DU (fig. 5.1b). In the controls, *H.pylori* was cultured from the antral biopsies of four (13%) and from the tooth plaque of two (7%). *H.pylori* was not cultured from the dental plates of three edentulous patients with DU nor from the centrifuged saliva of any of the patients in either group. The CLO test for urease was positive with the antral biopsies in 50(79%) of the DU patients and from three (10%) of the controls. Tooth plaque urease (CLO) tests were positive in 19(57%) of 33 DU patients.

Electron microscopic examination showed that *H.pylori* isolated from antrum (fig. 5.2a) and tooth plaque (fig. 5.2b) were similar.

Table 5.1 Antral biopsies and tooth pick plaque culture, urease test and histology of duodenal ulcer and control (non-ulcer dyspepsia) group of patients.

	Duodenal ulcer group n=63 (%)	Control group n=30 (%)
Gastritis present histologically	59 (94%) *	7 (23%) *
<i>Helicobacter pylori</i> present histologically	53 (84%) **	4 (13%) **
Antral biopsy culture positive	56 (89%) ***	4 (13%) ***
Antral biopsy urease (CLO) test positive	50 (79%) +	3 (10%) +
Tooth pick plaque culture positive	12 (19%)	2 (7%)
Tooth pick plaque urease (CLO) test positive	n=33 (%) 19 (57%)	-----

----- = no experiments made

*; **; ***; + : p<0.001

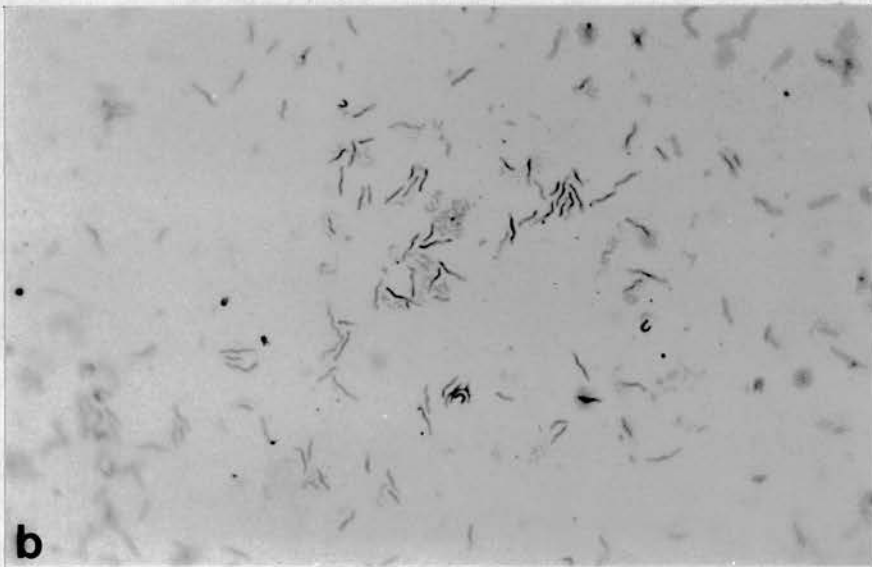
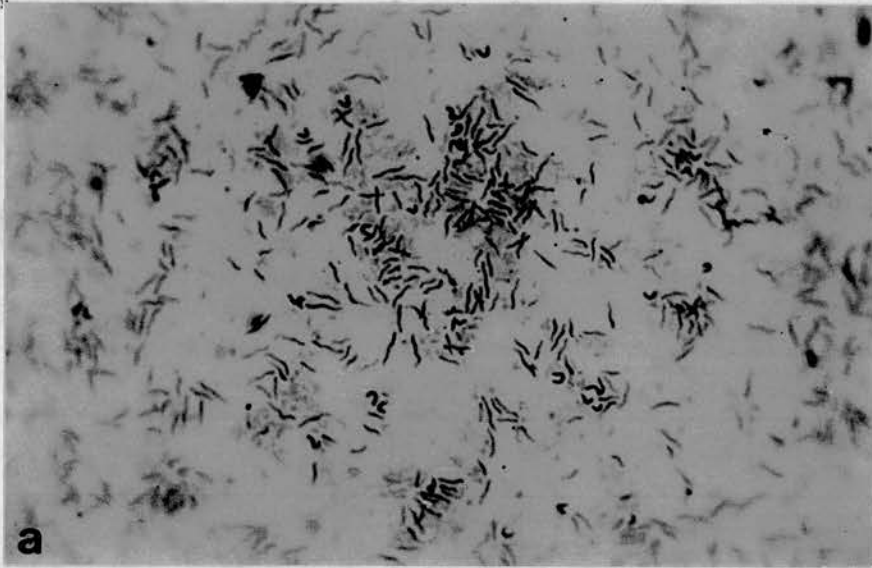


Fig. 5.1 Light microscopic pictures of *H. pylori*
(total magnification 800)
a) Isolated from gastric antrum
b) Isolated from tooth plaque

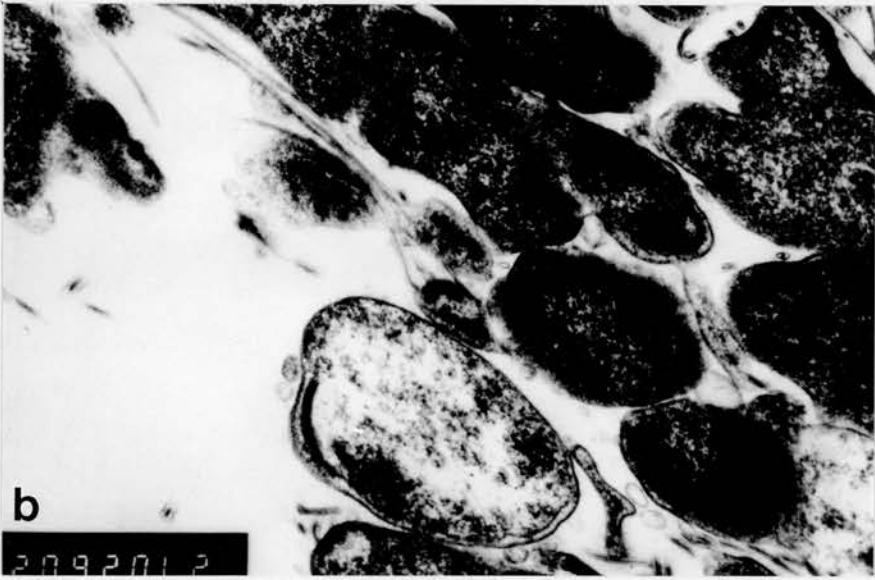


Fig. 5.2 Electron microscopic pictures of *H. pylori*
a) Isolated from gastric antrum
(total magnification 76108)
b) Isolated from tooth plaque
(total magnification 53568)

In 12 DU patients, *H.pylori* was cultured from both the gastric antrum and tooth plaque. No patient from this group who was negative for *H.pylori* culture from the stomach had *H.pylori* isolated from dental plaque (table 5.2).

In contrast, (table 5.3) only 2 control subjects were found to have *H.pylori* isolated from both the gastric antrum and tooth plaque. All control and ulcer patients who were negative for *H.pylori* culture from plaque were also negative for culture from the stomach.

In 48 DU patients, *H.pylori* was demonstrated histologically and all of them had positive urease test. Five patients had negative urease test though histologically *H.pylori* was identified. One patient had positive urease test but histologically *H.pylori* was not identified (table 5.4).

In two control subjects, urease test was positive and *H.pylori* was also demonstrated histologically. Two other control subjects had negative urease test but *H.pylori* was identified histologically. One subject had urease test positive but histologically *H.pylori* was not identified (table 5.5).

Relatedness of paired strains isolated from the mouth and antrum of five patients were determined by ribotyping. A mouth strain from a sixth patient was not analysed because the antral strain did not survive for further analysis. The identity of the five strains was confirmed as *H.pylori* by DNA probing. Ribotyping showed that the oral and antral isolates from each individual were identical. The difference between the strains from patient C were

minor and the strains were almost certainly the same (table 5.6).

In figures 5.3a and 5.3b, 5.4 and 5.5 strains were ordered according to patient (A-E); in each pair the first specimen is from tooth plaque and the second one from antrum. It was confirmed that pair strains of individual are same by the following: rRNA gene patterns (ribopattern) for *Hae III* and *Hind III* digest of genomic DNA from *H.pylori* (fig. 5.3); Southern blots of *Hae III* digest probe with a specific 16S rRNA gene probe obtained by polymerase chain reaction (fig. 5.4); and recombinant DNA probe (HP7) profiles of genomic Southern blots (*Hind III* digest) of *H.pylori* which is specific for the organism (fig. 5.5).

Results of statistical analysis:

After adjustment for age, significant differences ($p < 0.001$) were found between DU and control patients for presence of *H.pylori* in histology, antral biopsy culture and antral biopsy urease test but the effect of tooth plaque culture was not significant. Age did not differ significantly between *H.pylori* positive and negative group in tooth plaque culture or by urease test.

Table 5.2 Comparative findings of antral biopsy and tooth plaque culture for *H.pylori* from duodenal ulcer patients.

Duodenal ulcer n=63		
	Tooth plaque culture positive	Tooth plaque culture negative
Antral biopsy culture positive	12	45
Antral biopsy culture negative	0	6

Table 5.3 Comparative findings of antral biopsy and tooth plaque culture for *H.pylori* from control subjects.

	Control n=30	
	Tooth plaque culture positive	Tooth plaque culture negative
Antral biopsy culture positive	2	3
Antral biopsy culture negative	0	25

Table 5.4 Comparative findings of histological presence of *H.pylori* and urease test from antral biopsy in duodenal ulcer patients

	Duodenal ulcer n=63	
	Urease test positive	Urease test negative
<i>H.pylori</i> positive histologically	48	5
<i>H.pylori</i> negative histologically	1	9

Table 5.5 Comparative findings of histological presence of *H.pylori* and urease test from antral biopsy in control subjects.

	Control n=30	
	Urease test positive	Urease test negative
<i>H.pylori</i> positive histologically	2	2
<i>H.pylori</i> negative histologically	1	25

Table 5.6 Ribotypes of *H.pylori* strains isolated and their relation to each other.

Patient	Source	DNA type
A	Teeth	1a
	Antrum	1a
B	Teeth	2
	Antrum	2
C	Teeth	1b
	Antrum	1a
D	Teeth	3
	Antrum	3
E	Teeth	4
	Antrum	4
F	Teeth	5

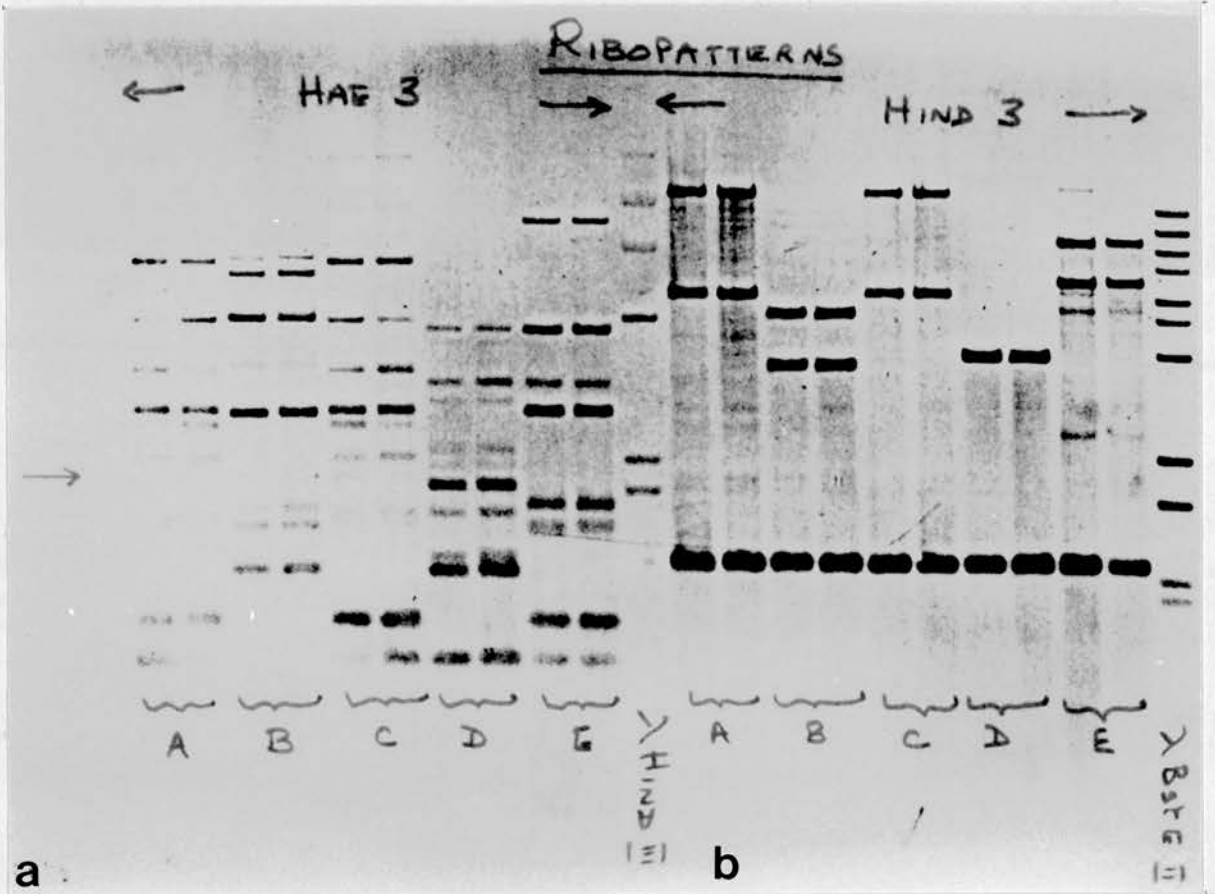


Figure 5.3 The rRNA gene patterns (ribopattern) for a) *Hae III* and b) *Hind III* digest of genomic DNA from *H. pylori* are illustrated here. Size marker is *Bst E II* digest of bacteriophage Lambda DNA. The strains were ordered according to patient (A-E) and with each pair were arranged by the site of isolation *i.e.*, first one from tooth plaque and the second one from antrum. Strains isolated from each patients were same except minor variation in patient C.

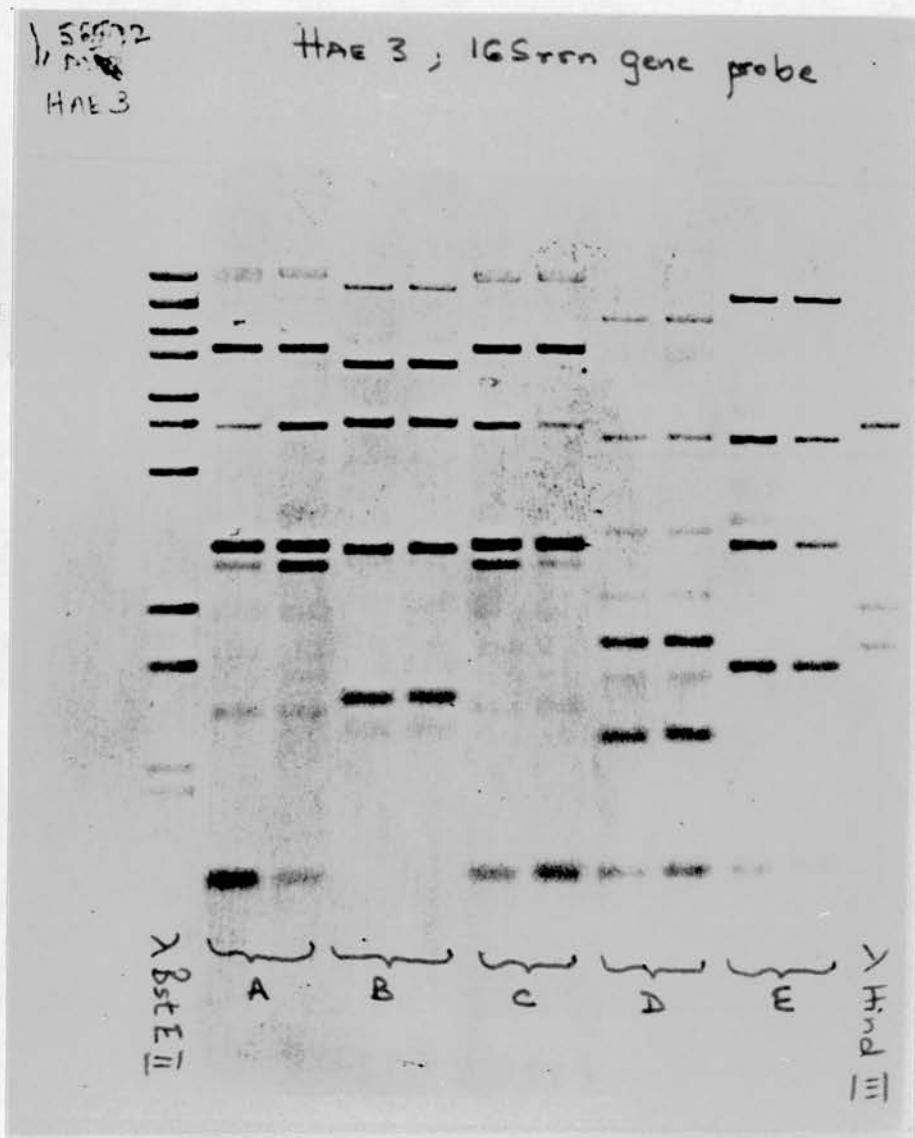


Figure 5.4 The Southern blots of *Hae III* digest probe with a specific 16S rRNA gene probe obtained by polymerase chain reaction. Size marker is *Bst E II* digest of bacteriophage Lambda DNA. The strains were ordered according to patients (A-E) and within each pair were arranged by the site of isolation *i.e.*, first one from the tooth plaque and the second one from antrum. Strains isolated from each patients were same except minor variation in patient C.

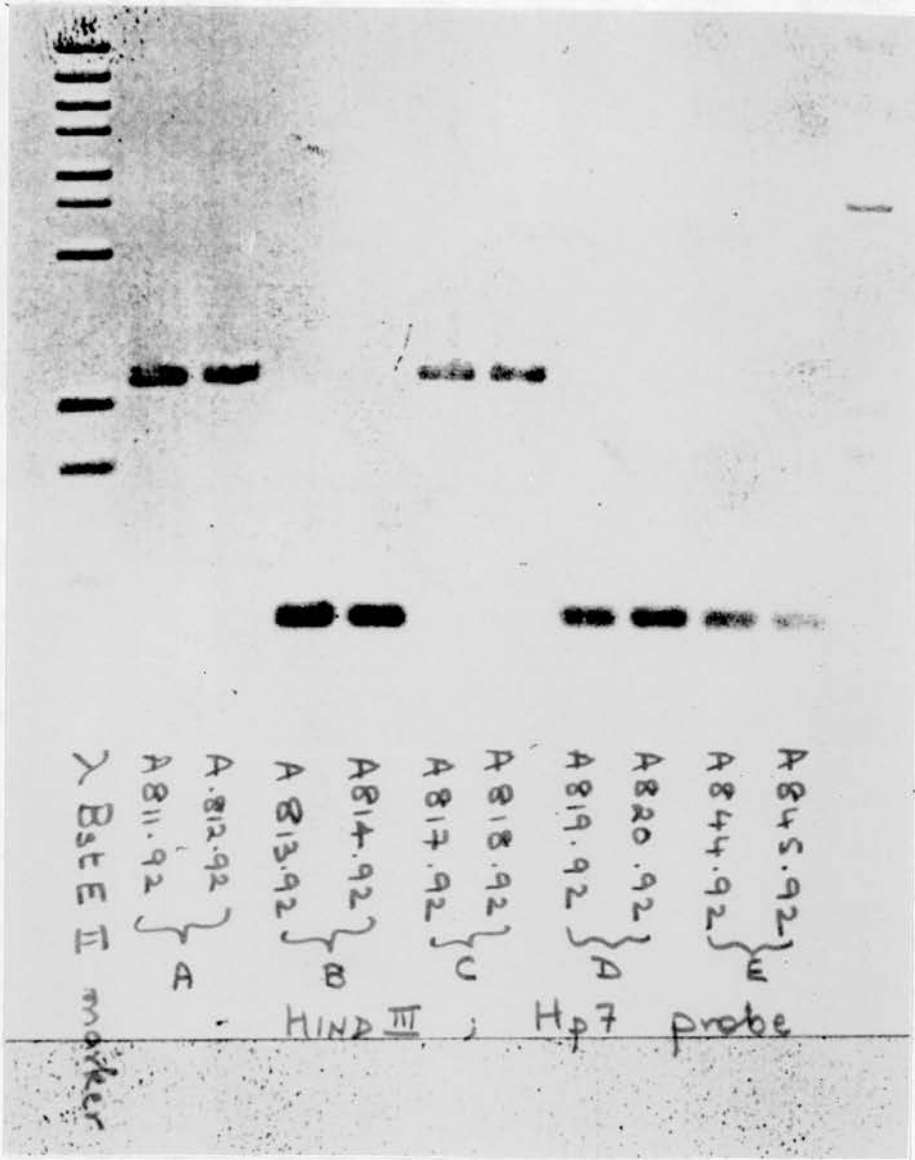


Figure 5.5 Recombinant DNA probe (HP7) profiles of genomic Southern blots (*Hind* III digests) of *H. pylori* are shown above. This is *H. pylori* specific. Size marker is *Bst* E II digest of bacteriophage Lambda DNA. The strains were ordered according to patients (A-E) and within each pair were arranged by the site of isolation *i.e.*, first one from tooth plaque and the second one from antrum. Strains isolated from each patients were same except minor variation in patients C.

5.5 DISCUSSION

H.pylori is now accepted as a major contributor to the development of chronic type B gastritis and subsequent peptic ulcer formation in man; and eradication of *H.pylori* from the stomach is an important requirement for long term remission from peptic ulceration. It is still not clear where the primary infection or re-infection originates. An important start to such a study is a method which unequivocally identifies *H.pylori* infection. Tests for specific antibody have demonstrated that *H. pylori* is widespread in the general population, the prevalence increasing with age. The demonstration of the extracellular enzyme activities of *H.pylori*, i.e., urease , catalase and oxidase, enables further identification, as do the histological and electron microscopic appearances.

H.pylori HindIII ribopatterns were used to substantiate the identification of cultures of the organism. Whilst lacking the absolute precision of DNA restriction endonuclease digest patterns, the ribopatterns based on rRNA gene restriction offer greater potential for typing isolates of *H.pylori*. A wide range of different strains of *H. pylori* have been identified in human infections with no single strain being predominant in the aetiology of peptic ulceration. A majority of *H.pylori* infected persons develop DU, but many others do not. This might be due to host

resistance, varying virulence of the organism or infection with an ulcerogenic strain (Figura et al., 1989; Marshall, 1991).

In this study, antral biopsies from 94% of DU subjects showed histological gastritis, 84% were *H.pylori* positive on histological examination and 89% were positive for *H.pylori* by culture. In contrast, the respective figures in control subjects were 23%, 13% and 13%. All these differences are statistically significant in relation to age, supporting again the close association of *H.pylori* with DU (Malfertheiner, 1988; Tytgat, 1989; Axon 1991). There was no correlation found between age and *H.pylori* positivity by any of the tests. To diagnose *H.pylori* infection no single test is perfect (Megraud, 1988). In this study *H.pylori* was shown to be present by culture or histology in 89% and 84% respectively of DU patients. Thus both tests equally reliably diagnose *H.pylori* infection, an observation similar to that of Little et al. (1991).

H.pylori was cultured from tooth pickings in 12(19%) of 63 DU patients, and from 2(7%) of 30 control subjects. This difference is not statistically significant. This may be due to the different numbers of subjects in each group or may reflect the common difficulty of isolating *H.pylori* in the laboratory from culture plates carrying a heavy mixed mouth flora.

It is possible that there is contamination of the oral flora by antral contents containing *H.pylori*. The careful separation of the endoscope from the teeth by the quite large mouth guard, and the front piece of the guard over

the teeth make the chances of such contamination very remote. Furthermore the plaques were removed from teeth in the anterior mouth which unlike saliva is not readily contaminated. No organism was cultured from the mouth of edentulous individuals.

The urease test carried out on tooth pickings was positive in 19(57%) of samples from 33 DU patients. *H.pylori* was isolated from tooth pickings from 5 of these patients and from tooth pickings from 7 of 30 further DU patients on whose tooth pickings urease tests were not done. Urease tests were positive with antral biopsies from 79% of DU patients but from only 10% of control subjects. This difference is statistically significant. In DU patients, the antral biopsy urease test is positive almost as often as histological examination or culture. As the urease test is simple and cheap, it could be used for preliminary screening, in the field. It is, therefore, a sensible alternative to the other tests, especially in developing countries where test costs are paramount and if its cost can be minimised.

An important potential source for re-infection of the stomach by *H.pylori*, might be the polymicrobial microaerobic environment of dental plaque or saliva. In this study *H.pylori* was not grown from saliva. Krajden et al. (1989) made a similar observation but were able to grow *H.pylori* from the dental plaque of one patient out of a study group of 71 patients with antral gastritis, 29 of whose antral biopsies were culture-positive for *H.pylori*. Shames et al.

(1989), in a complementary study to that of Krajden et al. (1989), by using restriction endonuclease analysis with *HindIII*, *HaeIII* and *BglIII* endonucleases showed three distinct strains of *H.pylori* in the mouth. Only one was found in the antrum. Using a less precise identification method, Desai et al., (1991) were able to demonstrate CLO-test-positive organisms in both the antrum (83 %) and dental plaques (99 %) of 43 patients with DU. Plaque was obtained before endoscopic examination. Majumder et al. (1990) isolated *H.pylori* from the dental plaque of all of 40 subjects (endoscopy was not done in this study), identifying it by urease tests, direct microscopy after Warthin-Stary staining and by culture. All three tests were positive in all 40 cases. The fact that two of the five reports of successful culture of *H.pylori* from the mouth are from a developing country (i.e., India) might simply reflect either the high prevalence of infection in that population or the fact that it is not yet fashionable to look for *H.pylori* in the mouth. Desai et al. (1991) were able to eradicate *H.pylori* from the pyloric antrum, but not from dental plaque using triple drug therapy consisting of bismuth subcitrate, amoxicillin and metronidazole. This is an important observation because it suggests that dental plaque could be a persistent reservoir for antral re-infection and subsequent recurrent ulceration.

This is the first report from the United Kingdom of the isolation of *H.pylori* from dental plaque. Among the pairs of strains from six patients (from antrum and dental plaque) the precision of the ribotyping was able

unequivocally to relate mouth and antral strains to each other. In four of five patients the strains were exactly the same. The strains from patient C showed minor differences and they were almost certainly virtually identical. Normally the differences between each RNA "house keeping geno-type" which were measured in this methodology are very marked. Type 1a and 1b is the presence or absence of one or two minor bands which are possibly due to one or two base substitutes. Otherwise 1a and 1b are same strain. The results also confirm the experiences of others (Owen et al., 1992b) i.e., each patient would be expected to carry a unique ribotype. Mouth carriage may be more complex however; Shames et al., (1989), described three distinct strains in dental plaque in one patient but one only strain from the antrum.

Different individuals have been found to have the same *H.pylori* strain if they live in the same household or have very close contact with another household (Malaty et al., 1991; Oderda et al., 1991; Vaira et al., 1991).

Subjects A and C carried similar DNA types they were investigated at different times remote by several months and had no contact with each other.

The mouth carries a heavy and extremely varied microflora. Some bacteria might protect others against harmful influences e.g., beta-lactamase producers can protect benzylpenicillin from eradicating otherwise penicillin-sensitive organisms such as *Strep.pyogenes*. A similar mechanism might protect oral *H.pylori* from the

effects of ampicillin so that they survive triple therapy with bismuth subcitrate, amoxycillin, and metronidazole and remain available for reinfection of the antrum.

Complete eradication of *H.pylori* from dental plaque might be a prerequisite for successful long term remission of peptic ulcer. Further studies should be undertaken to confirm this proposition. It is not clear whether the mouth colonises the stomach or the stomach the mouth. Nor is it clear from where the original infection arises, though it is evident that there is an enhanced risk of infection from close contact grouping such as within families (Malaty et al., 1991; Oderda et al., 1991; Vira et al., 1991). Recently, Thomas et al. (1992) have reported for the first time the isolation of *H.pylori* from stool of 10 of 24 Gambian subjects. Faecal-oral transmission of *H.pylori* must now be considered a strong possibility and this method of transmission would explain much of the epidemiology of the organism, particularly its increased prevalence in developing countries where this route of transmission is common.

CHAPTER 6

PRESENCE OF *H.pylori* IN THE
MOUTH OF HOSPITAL INPATIENTS

6.1 SUMMARY

The presence of *H.pylori* in the teeth of 100 dentulous inpatients was sought by culture, the urease test and by measuring *H.pylori* antibodies. From 30 edentulous inpatients *H.pylori* antibodies was also measured. A history of antibiotic therapy on admission or at any time in preceding 3 to 6 months was recorded.

Elderly patients were more often seropositive than younger subjects. Among dentulous patients tooth plaque urease test positivity was significantly associated with increasing *H.pylori* antibody titre ($p < 0.001$). Urease positivity was significantly correlated with age and antibody titre in hepatic disease and ulcerative colitis. A history of antibiotic consumption did not apparently influence positivity.

6.2 INTRODUCTION

Having found *H.pylori* in the dental plaques of DU patients, the presence of *H. pylori* in dental plaques of hospital inpatients who had no evidence of DU was sought. The effects of various antibiotic therapies upon *H.pylori* in dental plaque were also examined.

6.3 MATERIALS AND METHODS

6.3.1 Subjects

One hundred patients, 55 males and 45 females, aged from 15 to 86 years (mean 50 years) were studied. All were patients in the gastrointestinal unit ward Western General Hospital between October 1991 to May 1992. Each had at least four adjacent remaining teeth. All were native born Scots. Their presenting complaints or diagnosis, past medical history, presence of DU or dyspepsia and history of antibiotics therapy on admission or at any time in the preceding 3-6 months were recorded.

Thirty edentulous subjects, 14 males and 16 females, age ranged from 48 to 87 years (mean 70 years) were studied as controls. All had attended the gastrointestinal unit, Western General Hospital and all were native born Scots. Their presenting complaints or diagnosis, past medical history, presence of DU or dyspepsia and antibiotic history were recorded using the same standard protocol.

6.3.2 Methods

From 100 dentulous subjects teeth were scraped with a plastic tooth pick ("Pick plak" HWC Supplies) and some of the material recovered was used to inoculate a CLO test strip to test for urease activity. A change of colour from yellow to dark pink within 20 minutes to 3 hours after inoculation would signify a positive test. The remaining material was used to inoculate a plate of Skirrow's medium within 15 to 30 minutes. This was subsequently incubated for 4-6 days. Thereafter, colonies morphologically resembling *H.pylori* were subcultured until pure. The organisms were identified as *H.pylori* by i) morphology in Gram's stained film; ii) positive tests for urease activity; iii) positive test for catalase and oxidase activity (Cowan and Steel, 1974).

A sample of blood was taken from both dentulous and edentulous subjects; the serum was separated and stored at - 70°C for subsequent testing for antibody to *H.pylori*.

6.3.3 Statistical methods

Age and antibody levels were compared in edentulous and dentulous patients, urease positive and negative, by Wilcoxon rank sum test. The antibody levels and age were correlated using the Spearman correlation coefficient. Multiple logistic regression was used to assess antibodies in dentulous and edentulous patients, adjusted for age. Kruskal-Wallis test was used to compare age and antibody levels and chi-squared test for urease.

6.4 RESULTS

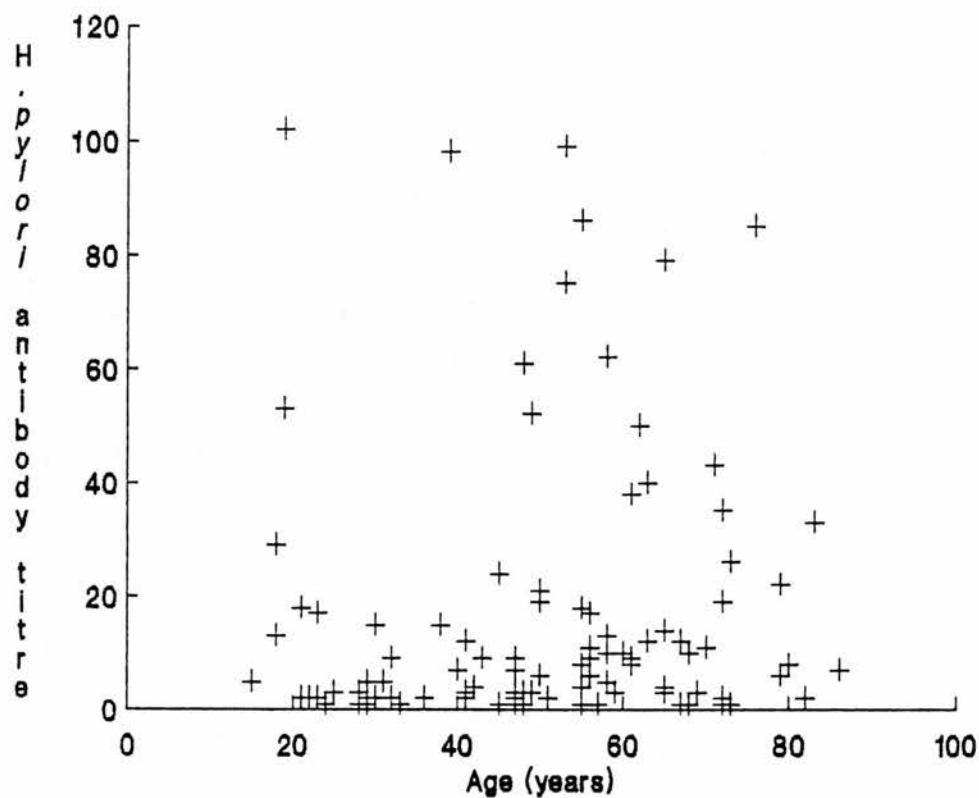
Forty one (41%) of 100 dentulous inpatients and 16 (53%) of edentulous inpatients had antibody to *H.pylori*. Tooth plaque urease (CLO) tests were positive in 30(30%) of 100 dentulous inpatients but *H.pylori* was isolated by tooth plaque culture in only three (3%) of these (table 6.1). Elderly patients had higher antibody levels whether dentulous or edentulous (fig. 6.1 a and b).

Among the hundred dentulous inpatients (table 6.2), 28 had ulcerative colitis or some form of colonic disorder; 11 of these had antibody to *H.pylori* and nine had tooth plaque urease (CLO) tests positive. Of 16 patients with Crohn's disease, six had antibody to *H.pylori* and one was tooth plaque urease test positive. *H.pylori* was cultured from the tooth plaque of one of 16 patients admitted with Crohn's disease. Of 27 patients admitted with liver disease or for endoscopic retrograde cholangiopancreatography, 14 had antibody to *H.pylori*, 14 had positive tooth plaque urease tests and *H.pylori* was cultured from the tooth plaque of two. Of seven patients with chronic pancreatitis, two had antibody to *H.pylori* and two had a positive urease tests. Eleven patients had nonspecific abdominal pain; of these three patients had antibody to *H.pylori*. Of six patients with melaena, four had antibody to *H.pylori* and three had a positive tooth plaque urease tests. 5 patients had iron deficiency anaemia and malabsorption syndrome; of these, one had antibody to *H.pylori* and another

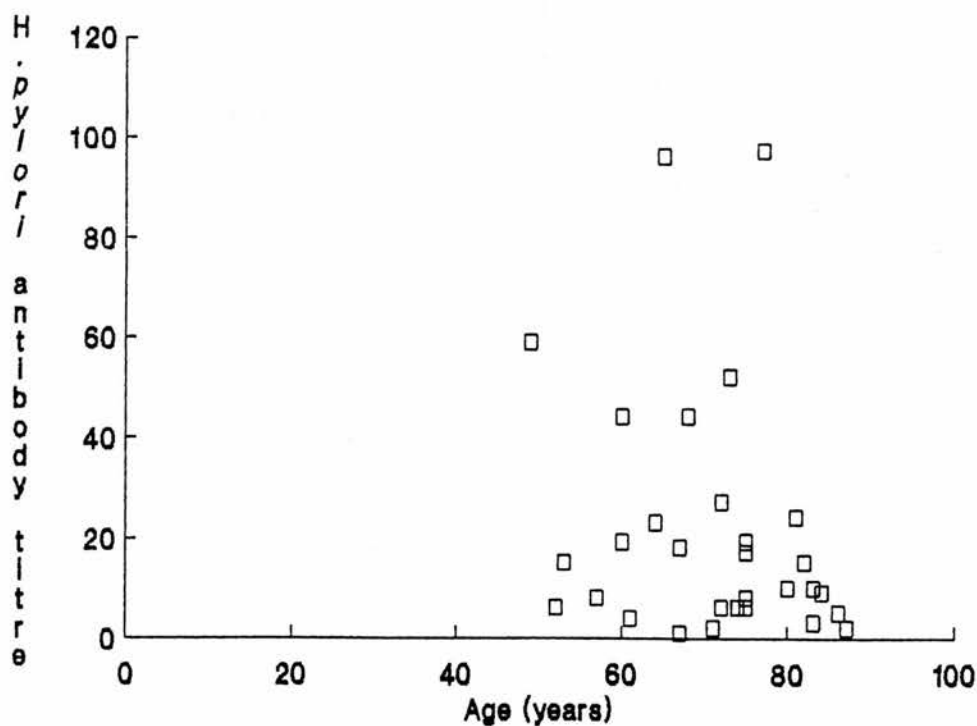
Table 6.1 The presence of antibody to *H.pylori* in inpatients with teeth and edentulous inpatients compared with the results of tooth plaque urease (CLO) tests and culture in inpatients with teeth.

	With teeth n = 100	Edentulous n = 30
Test	number (%)	number (%)
Antibody present	41 (41)*	16 (53)*
Tooth plaque urease (CLO) test positive	30 (30)	-
Tooth plaque culture positive for <i>H. pylori</i>	3 (3)	-

* p > 0.2.



a) 100 inpatients with teeth



b) 30 Inpatients without teeth

Fig. 6.1 Antibody titre (units/ml) in relation to age

Table 6.2 Disease pattern or diagnosis, related to the presence of antibody to *H.pylori*, urease test results and culture of *H.pylori* from tooth plaque in 100 inpatients with teeth.

Diagnosis on admission	n	Antibody to <i>H.pylori</i> (present)	Tooth plaque urease test positive	Tooth plaque culture positive
Ulcerative colitis and other colonic disorder	28	11	9	0
Crohn's disease	16	6	1	1
Hepatic disease	27	14	14	2
Chronic pancreatitis	7	2	2	0
Nonspecific abdominal pain	11	3	0	0
Melaena	6	4	3	0
Anaemia/mal-absorption syndrome	5	1	1	0
Total	100	41	30	3

patient had a positive tooth plaque urease test.

The subsets shown in table 6.3 are too small to allow any conclusions to be drawn about the relative prevalence of *H. pylori* infection in these different disease states.

Antibiotics had no effect on *H.pylori* antibody status whether dentulous or not. Among 5 CLO positive patients 3 had history of antibiotic consumption (table 6.4). The commonly used antibiotic was amoxycillin (table 6.5) and middle aged and elderly people consumed more antibiotics (table 6.6).

Results of statistical analysis

Although antibody levels were significantly higher in patients without teeth, this difference did not achieve statistical significance when values were adjusted for age.

Among patients with teeth, a positive urease test was significantly associated with age and antibody levels, both at $p < 0.001$.

The diagnostic group (table 6.2) did not differ significantly in either age or antibody levels, but did show a difference ($p < 0.05$) in positive urease test, which was more prevalent in those with hepatic disease and ulcerative colitis.

6.5 DISCUSSION

Epidemiological studies suggest that *H.pylori* infection increases with age both in developed and developing countries. Studies of the prevalence of *H.pylori* infection

Table 6.3 Disease pattern or diagnosis and presence of antibody to *H.pylori* in 30 edentulous control inpatients.

Diagnosis on admission	n	Antibody to <i>H. pylori</i>
Ulcerative colitis and other colonic disease	10	5
Crohn's disease	5	2
Hepatic disease	5	3
Oesophageal cancer	4	2
Coeliac disease	2	1
Nonspecific abdominal pain	2	1
Melaena	1	1
Deficiency anaemia	1	1
Total	30	16

Table 6.4 Antibody to *H.pylori* in inpatients with teeth and edentulous inpatients who had taken antibiotics related to the results of tooth plaque urease (CLO) tests and culture for *H.pylori*.

	With teeth n=100 number (%)	Edentulous n=30 number (%)
Antibiotics taken	24 (24)	8 (27)
Antibody to <i>H. pylori</i>	8 (8)	2 (7)
Tooth plaque urease test positive	5 (5)	-
Tooth plaque culture positive	0	-

Table 6.5 Antibiotics consumed and levels of antibody titre (units/ml) in 100 inpatients with teeth.

	n=100				
	0-25	26-50	51-75	76-100	101-125
	u/ml	u/ml	u/ml	u/ml	u/ml
No antibiotics used	64	2	5	4	1
Amoxycillin	13	2	0	0	0
Tetracycline	4	0	0	1	0
Nitrofurantoin	1	0	0	0	0
Metronidazole	1	0	0	0	0
Gentimicin	0	1	0	0	0
Ampicillin and metronidazole	1	0	0	0	0

Table 6.6 Antibiotics consumed and age distribution of 100 inpatients with teeth

	0-20 years	21-40 years	41-60 years	61-80 years	81 and over
No antibiotics used	4	16	33	20	3
Amoxycillin	1	4	6	3	1
Tetracycline	0	0	2	3	0
Nitrofurantoin	1	0	0	0	0
Metronidazole	1	0	0	0	0
Gentimicin	0	0	0	1	0
Ampicillin and Metronidazole	0	1	0	0	0

among hospital inpatients have not yet been reported. Many hospitalised patients (up to 40% in some studies) take antibiotics either singly or in combination before or during their hospital stay (Chodak and Plaut, 1977). Triple therapy (comprising bismuth subcitrate, and metronidazole with amoxycillin or tetracycline) can eradicate *H.pylori* from the stomachs of infected patients (Rauws and Tytgat, 1990; Axon, 1991). Desai et al. (1991) reported for the first time that while triple therapy eradicated *H.pylori* from the stomach, the organism persisted in dental plaque. At present it is difficult to comment on whether systemic antibiotics affect mouth carriage of *H.pylori*. The ease or otherwise of eradicating *H.pylori* with antibiotic treatment from its proposed reservoir of infection and reinfection in the mouth has implications for reinfection of the stomach and recurrence of DU.

A general ward population of patients with and without teeth was studied by bacterial culture of tooth plaque, by urease testing of tooth plaque and by measurement of plasma *H.pylori* antibody. The 100 patients with teeth studied had a mean age of 50 years while the 30 edentulous patients had a mean age of 70 years. The age difference stems directly from the presence or absence of teeth which were used as the principal selection criterion.

Antibody to *H.pylori* was present in 41% and 53% of patients with and without teeth respectively. This difference is not statistically significant. Elderly patients had higher antibody levels (fig. 6.1) and urease test positivity.

Antibody levels were significantly higher in edentulous patients but did not differ significantly after adjustment of age for the presence or absence of teeth. Elderly people were seropositive, a finding similar to other epidemiological studies (Jones et al., 1986; Morris et al., 1986b; Perez-Perez et al., 1988; Kosunen et al., 1989; Megraud et al., 1989). Taylor and Blaser (1991) reported that approximately half of all adults are colonised with *H.pylori* by the age of 60 years in developed countries. Jones et al. (1986) showed that in England 50% of those over the age of 50 years have antibody to *H.pylori*. The two latter findings are almost identical to those of the present study. At present *H.pylori* infection is inversely related to socioeconomic status in both developed and developing countries (Al-Moagel et al., 1990; Taylor and Blaser, 1991; Fiedorek et al., 1991; Sitas et al., 1991). It is tempting to speculate that those born in the United Kingdom or in developed countries in the first half of this century had in general, poorer socioeconomic status than those born later, thus explaining the higher percentage of elderly people with *H.pylori* antibody.

H.pylori was cultured from tooth plaque of 3(3%) patients; urease tests on tooth plaque were positive in 30(30%) patients and *H.pylori* antibody was detected in 41(41%) patients. The lower positivity by culture of tooth plaque probably reflects the difficulty of growing the organism and separating it from other microflora present in the tooth plaque material. It is not surprising that these

three tests gave different results. Culture of *H.pylori* is demanding; organisms other than *H.pylori* can produce urease and the presence of antibody relates as much to past as to current infection, the defence mechanisms clearing *H.pylori* but antibody persisting (Meyer et al., 1991). If the present study protocol had included antral biopsy culture in addition to tooth plaque culture, urease test and assessment of antibody status, stronger comments concerning the relative value of these tests would have been possible. Dooly et al. (1989) reported that culture of the antral biopsy was positive in 19.4% of asymptomatic patients but antibody to *H.pylori* was present in 33.7% of these. This is similar to the urease test and serological findings of the present study.

Of the 100 patients with teeth, antibody to *H.pylori* was found more often in patients with hepatic disease who have often been admitted for repeated endoscopic retrograde cholangiopancreatography, patients with ulcerative colitis, with Crohn's disease and in those admitted with haematemesis or melaena. Hepatic disease and ulcerative colitis patients had significant antibody levels and urease test positivity in relation to their age. The reason for this distribution is not clear. Langenberg et al. (1990) reported that *H.pylori* has occasionally been transmitted from person to person by improperly cleaned endoscopes. It is unlikely to be the explanation in the present study because modern endoscopic cleaning techniques are very stringent. In the present study, systemic antibiotics given for whatever reason had no effect on the rate of recovery of *H.pylori*

from tooth plaque. Desai et al., (1991) reported similar findings, i.e.; they were able to eradicate *H.pylori* from the pyloric antrum but not from dental plaque by triple therapy with bismuth subcitrate, amoxycillin / tetracycline and metronidazole.

It is very difficult to comment on the effect of antibiotic treatment on the presence of antibodies to *H.pylori*, because antibodies were not measured before antibiotic treatment started; however, antibody titres were similar whether antibiotics had been taken or not.

To treat peptic ulcer disease and prevent its recurrence it is probably necessary to eradicate *H.pylori* from the mouth as well from the stomach. Whether this can be done by high dose antibiotic therapy or local antiseptic dental pastes is a matter of future study.

CHAPTER 7

THE EFFECT OF BILE ACIDS

ON *H. pylori* GROWTH IN VITRO

7.1 SUMMARY

H.pylori was cultured in the presence of sodium taurocholate and human bile acids. No growth of *H.pylori* was observed when higher concentrations of sodium taurocholate (18mmol) and human bile acid (16mmol) were added in Skirrow's medium. It is, therefore, possible that *H.pylori* growth is suppressed or prevented in the presence of high concentrations of either sodium taurocholate or bile acids *in vitro*.

7.2 INTRODUCTION

H.pylori is not found in the stomach of patients with reflux biliary gastritis (O'Connor et al., 1986a; O'Connor et al., 1986b).

Biliary gastritis and Type B gastritis have a different aetiology. Patients with these diseases may also differ in regard to their *H.pylori* status and this could be because of the effects of bile upon growth of the organism. This was studied by culturing *H. pylori* in the presence of bile acids.

7.3 MATERIALS AND METHODS

Sodium taurocholate (Sigma) was incorporated into Skirrow's medium (Skirrow, 1977) in concentrations of 0,

4, 6, 8, 10, 12, 14, 16, 18 and 20 mmol. A single strain of *H.pylori* isolated from an antral biopsy was inoculated on to each plate. The plates were examined for growth after being incubated for 4-6 days at 37°C in 95-100% humidity and an atmosphere containing nitrogen, oxygen and carbon dioxide in ratios of 90:5:5. In a second series of experiments a single sample of human bile was collected after laparotomy and stored at -70° C. Within 15 days of collection bile acid was measured (see below); and then it was freeze-dried and its contained bile acids redissolved into Skirrow's medium in concentrations of 0, 4, 6, 8, 10, 12, 14, 16, 18 and 20 mmol. A single strain (one loopful) of *H.pylori* isolated from an antral biopsy but different from that described above was inoculated on to each plate. These plates were incubated and examined as above.

Only one strain of *H.pylori* was used in the experiment due to the restricted availability of bile.

Measurement of bile acid concentration

Bile acid concentration was measured using the Sterognost 3 alpha-pho kit for bile acid determination in intestinal contents (Nycomed Ltd, Birmingham, UK). Bile (20 μ l) was incubated for 15 minutes at room temperature with 1 ml test reagent containing 3-hydroxy-steroid dehydrogenase and nicotinamide adenine dinucleotide (NAD), and 0.25 ml methanol. NADH formed was measured at 340 nm in a spectrophotometer (Pye Unicam Ltd, Cambridge, UK. 8610). Blanks were set up omitting the enzyme reagent. A 2 mmol sodium taurocholate solution was used as standard. Bile was

measured with and without x10 dilution.

Test bile diluted 1 in 10 gave a mean optical density 340 nM of 0.263 which corresponded to 20.3 mmol/L on the standard curve produced with sodium taurocholate; 2 mmol/L standard gave a mean optical density 340 nM of 0.259. Analysis was carried out in duplicate.

7.4 RESULTS

The *H.pylori* growth was confluent when cultured in Skirrow's medium alone. When concentrations of 4 to 14 mmol of sodium taurocholate or human bile acids were added in the Skirrow's medium the *H.pylori* grew in numerous separate colonies. The growth of *H.pylori* was sparse when 16 mmol of sodium taurocholate was added, whereas no growth was observed at the same concentration with human bile acids; therefore, high concentrations of sodium taurocholate or human bile acids suppressed or prevented the growth of *H.pylori* in Skirrow's medium (table 7.1).

7.5 DISCUSSION

Bile acids have an adverse effect on the gastric mucosa which may lead to biliary gastritis. *H.pylori* infection is responsible for type B gastritis. The two types of gastritis are thought to be distinct (O'Connor et al., 1986a; Dixon et al., 1986).

In the present study, *H.pylori* was cultured in

Table 7.1 Growth of *H.pylori* cultured on Skirrow's medium with and without sodium-taurocholate or human bile acid.

	Skirrow's medium alone	With Na-tauro- cholate	With bile acid
Concentration			
0 mmol	+++	---	---
4, 6 and 8 mmol	---	++	++
10,12 and 14 mmol	---	++	++
16 mmol	---	+	NG
18 mmol	---	NG	NG
20 mmol	---	NG	NG

--- = No experiment made

NG = No growth of *H.pylori*

+++ = Confluent growth

++ = Numerous separate colonies

+ = sparse colonies of *H.pylori*.

Skirrow's medium in the presence of either human bile acids or Na-taurocholate. In the human bile duct, the concentration of bile acids is 16 to 35 mmol/L. In the presence of 16 mmol/L of Na-taurocholate or of 14 mmol/L of human bile acid *H.pylori* grew sparsely; however, at higher concentrations no growth of *H.pylori* was detected.

Normally intragastric bile acid concentration is less than 1 mmol/L (Dixon et al., 1986) and if duodenogastric reflux occurs, concentration varies according to the frequency and amount of bile refluxed. During gastroduodenal reflux, concentrations of bile acids of the order of 5 mmol/L have been measured (Dixon et al., 1986). Bile acid concentration beneath the mucus layer is not known although it is likely that this will be less than intraluminal concentrations.

There are, therefore, reasons for suspecting that *H.pylori* could exist in the stomach affected by biliary gastritis. However, O'Connor et al. (1986b) and Lamers et al. (1991) reported that patients who had had a Billroth I or Billroth II partial gastrectomy or truncal vagotomy and gastroenterostomy were significantly less likely to carry *H.pylori* than those who had had highly selective vagotomy or than controls. They suggested that reflux might disrupt the mucus layer leading to the death of *H.pylori* living beneath it, but did not identify the mechanism by which bacterial death comes about.

The results of the present study show that refluxed bile could not suppress the growth of *H.pylori*. It is clear that in patients with Billroth I or Billroth II

partial gastrectomy, truncal vagotomy and gastroenterostomy bile reflux is greater and more frequently than occurs often in highly selective vagotomy or gastrectomy with Roux-en-Y anastomosis. O'Connor et al. (1989) also reported that gastric remnants of patients with Roux-en-Y biliary diversion may be colonised by *H.pylori*. In contrast, Loffeld et al. (1988) reported that *Campylobacter* like organisms have a role in gastritis especially of the corpus after partial gastrectomy.

CHAPTER 8

H.pylori AND GASTRIC CARCINOMA

8.1 SUMMARY

Gastrectomy specimens obtained from 83 patients undergoing gastrectomy for primary gastric cancer and 34 controls who underwent gastric surgery for peptic ulcer were examined. The presence of *H.pylori* was determined using H and E stains. Multiple sections uninvolved by tumour or peptic ulcer were examined and the findings were related to a range of histological abnormalities.

H.pylori was identified in 26(76%) of peptic ulcer specimens. In contrast only 32(39%) gastrectomy specimens from gastric cancer patients had evidence of *H.pylori* ($p<0.001$). The presence of *H.pylori* was similar in tumours of intestinal and diffuse type. Seventy three gastric cancer patients had type B gastritis but only 27(37%) had *H.pylori*. There was no significant correlation between the presence of *H.pylori* and advancing age in intestinal type and diffuse types of gastric cancer. There was no significant association between the presence of *H.pylori* and gastric intestinal metaplasia, tumour extent or stage.

8.2 INTRODUCTION

A number of publications have suggested that *H.pylori* infection predisposes to the development of gastric cancer. These suggestions are not yet confirmed.

It is possible that chronic *H.pylori* infection might lead to atrophic gastritis and gastric metaplasia. As achlorhydria develops, *H.pylori* infection becomes less because of the altered microenvironment within the stomach and because of competition with other organisms which colonise the stomach and may be involved in the formation of carcinogenic chemicals. Cancer patients whose stomachs exhibit type B gastritis may have different degrees of *H.pylori* colonisation compared to patients with atrophic gastritis or gastric metaplasia.

8.3 MATERIALS AND METHODS

8.3.1 Subjects

Specimens from 83 subjects with gastric cancer, 48 males and 35 females ranging in age from 35 to 89 years (mean 69 years), were studied. All had undergone resectional surgery.

Peptic ulcer comparison group

Specimens from 34 subjects with peptic ulcer were studied. They comprised 19 males and 15 females ranging in age from 25 to 87 years (mean 58 years). All had undergone

resectional surgery between 1986 - 1990 for benign disease, principally gastric ulcer (in 31) or DU (in three).

8.3.2 Methods

The material studied consisted of tissue removed at operation, fixed and subsequently stored in the Pathology Department, Western General Hospital, Edinburgh. From the different parts of each specimen six to 10 standard paraffin sections were prepared and stained with haematoxylin and eosin. In some cases, further sections were stained with Giemsa stain. All were examined (by both a specialist histopathologist and the author) for the histological types of tumour present, for its stage and for associated or other histological abnormalities, especially gastritis and for the presence or absence of bacilli morphologically resembling *H.pylori*.

The tumour stage was noted, and all tumours were typed using the Lauren classification (Lauren, 1965).

The intestinal type of tumour was identified by the presence of glandular differentiation, large cells with abundant cytoplasm and hyperchromatic nuclei, intracellular mucus polarised to cell apex and extracellular mucous within gland lumen. This type of tumour grows with an expanding or pushing margin.

The diffuse type of tumour was identified as tumour cells in single or in small clumps, small cells with inconspicuous cytoplasm and pyknotic nuclei; signet-ring cells and extracellular mucus within the stroma were common features. This type of tumour diffusely infiltrates

to the adjacent area.

In addition, the presence and degree of gastritis in the adjacent gastric mucosa was assessed. Where possible this was assessed in both the body and antral mucosa. At the same time the presence of significant intestinal metaplasia was noted as indicating an atrophic gastritis. Special stains for mucin were not used, the diagnosis being based on haematoxylin and eosin stains only.

Semi-quantitative methods were used for the presence of *H.pylori* (small number = + ; moderate quantity = ++ ; numerous = +++).

8.3.3 Statistical methods

Statistical analysis of the results used the Wilcoxon rank sum test for paired comparison for age and histological presence of *H.pylori* in controls and in patients of different groups of histological findings. Multiple logistic regression was used to test for group differences in histological presence of *H.pylori* adjusted for age. Chi-squared test also used for histological presence or absence of *H.pylori*.

8.4 RESULTS

The patients with the intestinal type of adenocarcinoma were younger (mean age 66 years, range 35 to 87 years) than patients with diffuse type of adenocarcinoma (mean age 71 years, range 52 to 89 years).

In 32(39%) of the patients with gastric cancer there was histological evidence of *H.pylori* infection in areas not involved by tumour. By contrast 26 (76%) of the control patients with peptic ulcer had evidence of *H.pylori* (table 8.1). This difference is statistically significant ($p < 0.001$).

In 83 patients with gastric cancer, 73(88%) had type B gastritis, 48(58%) had intestinal metaplasia and/or atrophic gastritis, 33(39%) had an intestinal type of adenocarcinoma, 34(40%) had diffuse type of adenocarcinoma, and 16(19%) indeterminate type of tumour (table 8.2).

Further analysis (table 8.3) the relationship between *H.pylori* status, type B gastritis and gastric atrophy showed relatively low proportion of specimens which were non-atrophic with type B gastritis had histological evidence of *H.pylori* (50%). In contrast, the majority of those with gastric atrophy but without type B gastritis were colonised with *H.pylori* (80%). The numbers are however relatively small and statistical analysis is therefore inappropriate.

Histologically *H.pylori* was seen in 27(37%) of patients with gastritis (n=73), 16(33%) of patients with intestinal metaplasia and or atrophic gastritis (n=48), 11(33%) of patients with an intestinal type of adenocarcinoma (n=33), 15(44%) of patients with the diffuse type of adenocarcinoma (n=34) and in 6(37%) of patients with indeterminate types of tumour (n=16). None of these differences ($p > 0.5$) is statistically significant even after adjustment for age.

In the 34 control patients, *H.pylori* was present significantly more often ($p < 0.02$) when type B gastritis was present than if intestinal metaplasia or atrophic gastritis were present (table 8.4).

In 50(60%) of the 83 patients with gastric cancer, the tumour involved the full thickness of the stomach wall. In 33(40%), the involvement was superficial. *H.pylori* was demonstrated histologically in 19(38%) of the former and 12(36%) of the latter.

Of 52 tumours (63% of the total) found in the body of the stomach 20 were in the higher lesser curve, 5 in the greater curve, 16 in the anterior or posterior wall or both and 11 involved the whole of the stomach. Eight (9%) were in the fundus or cardia, 20 (24%) in the gastric antrum, 1(1%) in the pylorus and 2(2%) in anastomotic sites.

In 32 patients with gastric cancer in whom *H.pylori* was demonstrated histologically, the tumour was in the antrum in 13(42%) and in the body of stomach in 29(94%).

Of 34 ulcer patients with peptic ulcer disease, the ulcer involved the body of the stomach in 29 (85%). Of these, 24(83%) had histological evidence of *H.pylori*. In two (6%) of the control patients the ulcer was in the gastric antrum and *H.pylori* was present in both on histological examination. In three (9%) of the control patients the ulcer was in the duodenum; in none of these was there histological evidence of *H.pylori*.

Table 8.1 Histological presence of *H.pylori* in patients with gastric cancer and controls with benign disease.

	n	<i>H.pylori</i> positive(%)
Gastric cancer	83	32(39)*
Control	34	26(76)*

* p <0.001

Table 8.2 Additional histological findings in 83 patients with gastric cancer related to the presence of *H.pylori*.

		<i>H.pylori</i> present (%)
Additional histological findings		
Type B gastritis	73	27 (37)*
Atrophic gastritis and/or intestinal metaplasia	48	16 (33)*
Intestinal type of adenocarcinoma	33	11 (33)*
Diffuse type of adenocarcinoma	34	15 (44)*
Indeterminate type of tumour	16	6 (37)*

* $p > 0.5$. Not significant.

Table 8.3 *H.pylori* status in relation to the presence of gastric atrophy and type B gastritis in 83 cancer patients.

	<i>H.pylori</i> positive specimens	
	Atrophic	No-atrophic
	H.P +ve / total(%)	H.P +ve / total(%)
Type B gastritis	12 / 43(28)	15 / 30(50)
No type B gastritis	4 / 5(80)	1 / 5(20)

Table 8.4 Histological findings in 34 control patients related to the presence of *H.pylori*.

Additional histological findings		<i>H.pylori</i> present (%)
Type B gastritis	23	18 (78)*
Intestinal metaplasia and/or atrophic gastritis	9	6 (67)*

* $p < 0.02$.

Of 26 ulcer patients in whom *H.pylori* was demonstrated histologically, the ulcer involved the gastric body in 18 (69%) and the antrum in 15(58%).

Small numbers of *H.pylori* were present in 10(56%) and 5(33%) of patients with an ulcer in the gastric body or antrum respectively. *H.pylori* was numerous (+++) in 8(44%) of the former and 10(67%) of latter.

Results of statistical analysis

H.pylori was significantly more common in gastrectomy specimens from peptic ulcer than specimens from cancer patients ($p < 0.001$). There were no differences in the rate of *H.pylori* positivity between intestinal and diffuse type of cancer. Patients with the intestinal type of cancer and ulcer patients did not differ significantly in age, but those with the diffuse type of cancer were significantly older than both of these groups ($p < 0.05$ and $p < 0.01$ respectively). After adjustment for age differences, both the intestinal and diffuse cancer patients still differed significantly ($p < 0.01$) from that with ulcers in the histological identification of *H.pylori*; but, there remained no significant difference between the two cancer groups.

8.5 DISCUSSION

The findings of this study do not support (but do not contradict) a causal relationship between *H.pylori* infection and gastric cancer. As expected, this organism was usually present in stomachs resected for peptic ulcer; the prevalence of infection was rather low in the cancer cases and was similar to that expected for an age-matched disease-free group of Western individuals (Graham et al., 1991). *H.pylori* was not found more often in association with tumours of intestinal than of diffuse type, nor was it commonly found in association with intestinal metaplasia or chronic gastritis. These conclusions are similar to those reached by a group from Tokyo (Igarashi et al., 1992) who could not find serological evidence linking *H.pylori* infection and gastric cancer. Another Japanese group also failed to show increased prevalence of *H.pylori* infection in gastric cancer patients (Fukuda et al., 1992). A recent study from Singapore (Wee et al., 1992) failed to show a relationship between gastric tumour type and infection; and, although *H.pylori* was commonly found in cancer patients, the importance of this is unclear because a non-cancer control group was not studied. An Italian group has also failed to show any relationship between the presence of *H.pylori*, chronic atrophic gastritis and gastric metaplasia both of which are regarded as pre-malignant entities (Testoni et al., 1992).

It is not clear why *H.pylori* and type B gastritis

were not associated with intestinal metaplasia / atrophic gastritis in the stomachs of some of the cancer patients. These findings are similar to those of Igarashi et al. (1992) and Testoni et al. (1992). One possible explanation is that there is a patchy distribution of change.

Our data show much lower level of *H.pylori* in association with type B gastritis than those normally been recorded. Sub analysis showed that this was partly attributable to the high prevalence of atrophy may diminish *H.pylori* infection as the atrophy it induces progresses (Sipponen, 1991). However, it may also reflect the unreliability of routine reporting of *H.pylori*.

It is possible that pre-operative antibiotic therapy prescribed to cancer patients could have eradicated *H.pylori* or reduced the numbers of *H.pylori*. Questioning the surgeons operating on the patients during this period suggests that there was no pre-operative antibiotic policy at that time. This makes the possibility less likely. The results are more likely to be the consequences of gastric atrophy accompanying the gastric cancer.

The findings of the present study and those of the groups cited above are thus clearly at variance with other publications which suggest that *H.pylori* infection predisposes to the development of gastric cancer. For example, it has been reported from China that the frequency of gastric cancer correlates with the prevalence of *H.pylori* antibodies (Forman et al., 1990). Other populations with a high incidence of gastric cancer also

have a high prevalence of these antibodies and infection seems to be acquired at any early age in these individuals (Fox et al., 1989; Gastrointestinal physiology working group, 1990). This association cannot be assumed to be causal because other factors such as dietary habits, racial differences and exposure to other carcinogens might also be present in these high risk groups. Dooley (1991) suggested that *H.pylori* might not cause gastric carcinoma, but gastritis induced by the organism could act as the fertile soil upon which other environmental variables come to bear.

Those who support a causal role for *H.pylori* infection in the pathogenesis of gastric cancer suggest that chronic infection with the organism leads to atrophic gastritis and gastric metaplasia. As achlorhydria develops, *H.pylori* infection becomes less because of the altered microenvironment within the stomach and because of competition with other organisms which colonise the stomach and might be involved in the formation of carcinogenic chemicals. The evidence that *H.pylori* gastritis leads to atrophic gastritis is not compelling; and, in this study and those of others (Testoni et al., 1992; Wee et al., 1992), cancer patients whose stomachs did not exhibit atrophic gastritis or gastric metaplasia had no higher prevalence of *H.pylori* infection.

If *H.pylori* and gastric cancer were causally linked then it would be reasonable to expect this to be particularly true for the intestinal rather than diffuse type of tumour. This study, as well as others (Wee et al., 1992; Nomura et al., 1991), did not find this to be the case in contrast to

the study reported by Parsonnet et al. (1991).

The reasons for these discrepancies in the literature are not clear. Our study does not support a causal relationship between *H.pylori* and gastric cancer, but, because it is not prospective, does not provide critical evidence against the hypothesis.

CHAPTER 9

THE DEVELOPMENT OF A CHEAP
AND SENSITIVE UREA SOLUTION
FOR USE IN UREASE TESTS

9.1 SUMMARY

An "in-house" urea solution was compared with the commercially available urease (CLO) test. Three antral biopsies were taken from 53 patients who were investigated for upper gastrointestinal symptoms. One biopsy was examined by histology; the others were subjected to urease testing using CLO strips and the "in-house" urea solution. The "in-house" urea solution was compared with the CLO test using different bacterial dilutions and different buffer concentrations.

The "in-house" urea solution gave faster positive results than commercial CLO strips, using standard inoculation of *H.pylori*.

9.2 INTRODUCTION

As commercially available kits for *H.pylori* urease detection are too expensive for regular use in developing countries, an alternative urea solution was developed.

A commercial kit for urease detection *Campylobacter pylori* like organism (CLO) test and a freshly prepared "in-house" urea solution were compared. Reactivity to *H.pylori* urease was assessed in paired antral biopsies and compared with histological findings.

9.3 MATERIALS AND METHODS

9.3.1 Urea solution

An "in-house" urea solution was prepared by dissolving urea (Sigma) 2 g, phenol red (BDH) 0.05 g and sodium azide (Sigma) 0.02 g in 100 ml of 0.01M phosphate buffer (potassium di-hydrogen orthophosphate and di-sodium hydrogen orthophosphate) adjusted to pH 6.5 (Hazell et al., 1987). From this solution, 50 μ l was poured into the wells of a microtitre tray (Flow Laboratory Inc). Some further experiments were performed in parallel in which urea was dissolved in buffer solutions of 0.005M and 0.0025M concentration, to determine if a reduction in buffer strength increased or decreased the sensitivity of the test.

9.3.2 Subjects

Fifty three patients, all native born Scots, were studied. There were 23 males and 30 females, ranging in age from 14 to 84 years (mean 51 years); gastrointestinal endoscopy was carried out in each as part of an investigation of dyspepsia. Patients were excluded if they had acute gastroduodenal haemorrhage, gastric surgery, reflux oesophagitis or had taken drugs such as H2 receptor antagonists, antibiotics, bismuth subcitrate ("De-Nol"), nonsteroidal anti-inflammatory drugs (NSAIDs), steroids or anticoagulants at any time during the four weeks before the study. At endoscopy, macroscopic findings were documented and three pinch antral biopsies were obtained from an area no further than 5 cms proximal to the pylorus. Each biopsy was about 3 mm in diameter. One was fixed in neutral buffered formalin for subsequent histological examination to determine if *H.pylori* were present.

9.3.3 Methods

Tests for urease

The second biopsy was used in a CLO test for urease activity. The third was inoculated to the freshly prepared "in-house" urea solution in a microtitre tray well. This was sealed with adhesive transparent tape. The time from inoculation to a predetermined dark pink colour change was measured and the optical density was then determined. This was recorded and compared with the intensity of colour

change and the time to achieve it in the CLO strip. *H.pylori* was not quantified. A biopsy was considered to be urease negative if a significant colour change did not develop within 90 minutes. Uninoculated "in-house" urea solution in an adjacent well served as a negative control.

In preliminary experiments the sensitivity of a CLO strip and newly prepared "in-house" urea solution were compared with a suspension of *H.pylori*. This was prepared as follows. *H.pylori* was grown from an antral biopsy on Skirrow's medium and incubated for 4 to 6 days at 37° C in microaerophilic conditions with 95-100% humidity. One colony from this growth was suspended in 2 ml of 0.9% normal saline. Neither the bacterial count nor the cloudiness of the suspension were recorded. The objective of these experiments was to compare the new method with the established CLO test rather than explore sensitivity in terms of bacterial count. In a colony the average bacterial count is usually 10^5 to 10^6 . By dilution with normal saline, four further suspensions, dilutions of 1 in 10, 1 in 20, 1 in 50 and 1 in 100 were prepared. From each of the parent suspensions and the four dilutions, 25 μ l were used to test for urease activity in a CLO strip and in freshly prepared "in-house" urea solution as described above.

At the same time and in the same way, the sensitivity to urease of freshly prepared "in-house" urea solutions in 0.005M and 0.0025M phosphate buffer was determined.

Four sets of experiments were performed to allow comparison of the CLO strip with the "in-house" urea solution. The estimated time of positive reaction was the

same in all experiments.

The sensitivity of the commercially available CLO test is reported by the manufacturers by using an unspecified bacterial load in antral biopsies as 65% positivity at 20 min, 80% at 40 min, 85% at 60 min and 95% at 180 min. Maximum incubation period is 24 hours (Morris et al., 1986a; Marshall et al., 1987).

9.3.4 Statistical methods

Chi-squared test was used for comparison between the urease test and histological findings.

9.4 RESULTS

Both commercial and "in-house" urease tests demonstrated *H.pylori* urease in 12(23%) of 53 antral biopsies. Histological examination detected *H.pylori* in 16(30%) patients (table 9.1). These differences are not statistically significant.

The sensitivity and specificity of the "in-house" urea solution were 65.5% and 95% respectively. Positive and negative predictive values were 83% and 85% respectively.

"In-house" urea solution gave positive results for *H.pylori* urease activity with original bacterial suspension and 1:10, 1:20, 1:50 and 1:100 dilutions of it in 20, 25, 35, 48 and 60 minutes respectively. By contrast the commercial CLO test exposed to the same bacterial dilutions gave positive results in 20, 28, 45, 60 and 90 minutes

respectively, i.e., the "in-house" urea solution consistently gave faster results (table 9.2 and fig. 9.1).

Table 9.3 and fig. 9.2 show that lowering the buffer concentration speeds the already quick development of a positive test for urease in the "in-house" urea solution.

Results of statistical analysis

There were no significant differences in the sensitivity of urease test and histological findings (table 9.1).

9.5 DISCUSSION

To fulfil the need for a simpler, faster and cheaper way of diagnosing *H.pylori* infection, several modifications of the urease test have been attempted (McNulty, 1989). The specificity of this test is satisfactory though the sensitivity varies.

The sensitivity depends upon elapsed test time, size of biopsy, concentration of urea, phenol red (pH indicator), buffer in the gel or solution and temperature (Marshall et al., 1987; McNulty, 1989). Sensitivity might also depend on the viability of the organisms and urease activity (Chodos et al., 1988). Hazell et al. (1987) found 75% sensitivity within one hour, reaching 91% within 18 hours. The positive and negative predictive values were 96% and 73% respectively. Vaira et al. (1988a) found positive results in 62% of cases compared with other tests and

Table 9.1 Comparative detection of *H.pylori* in gastric antral biopsies by (a) urease demonstrated by commercial CLO test, (b) urease demonstrated by "in-house" urea solution and (c) by histological examination.

	Positive for <i>H.pylori</i>
	n = 53
	number (%)
Commercial CLO test	12 (23)
"In-house" urea solution	12 (23)
Histological examination	16 (30)

Table 9.2 Comparison of the reactivity of "in-house" urea solution and commercial CLO test with different dilutions of *H.pylori* suspension.

	Approximate number of <i>H.pylori</i>	Urea solution test positive in: min	Commercial CLO test positive in: min
Non-dilute	$5 \times 10^4 - 5 \times 10^5$	20	20
1:10	5,000-50,000	25	28
1:20	2,500-25,000	35	45
1:50	1,000-10,000	48	60
1:100	500-5,000	60	90

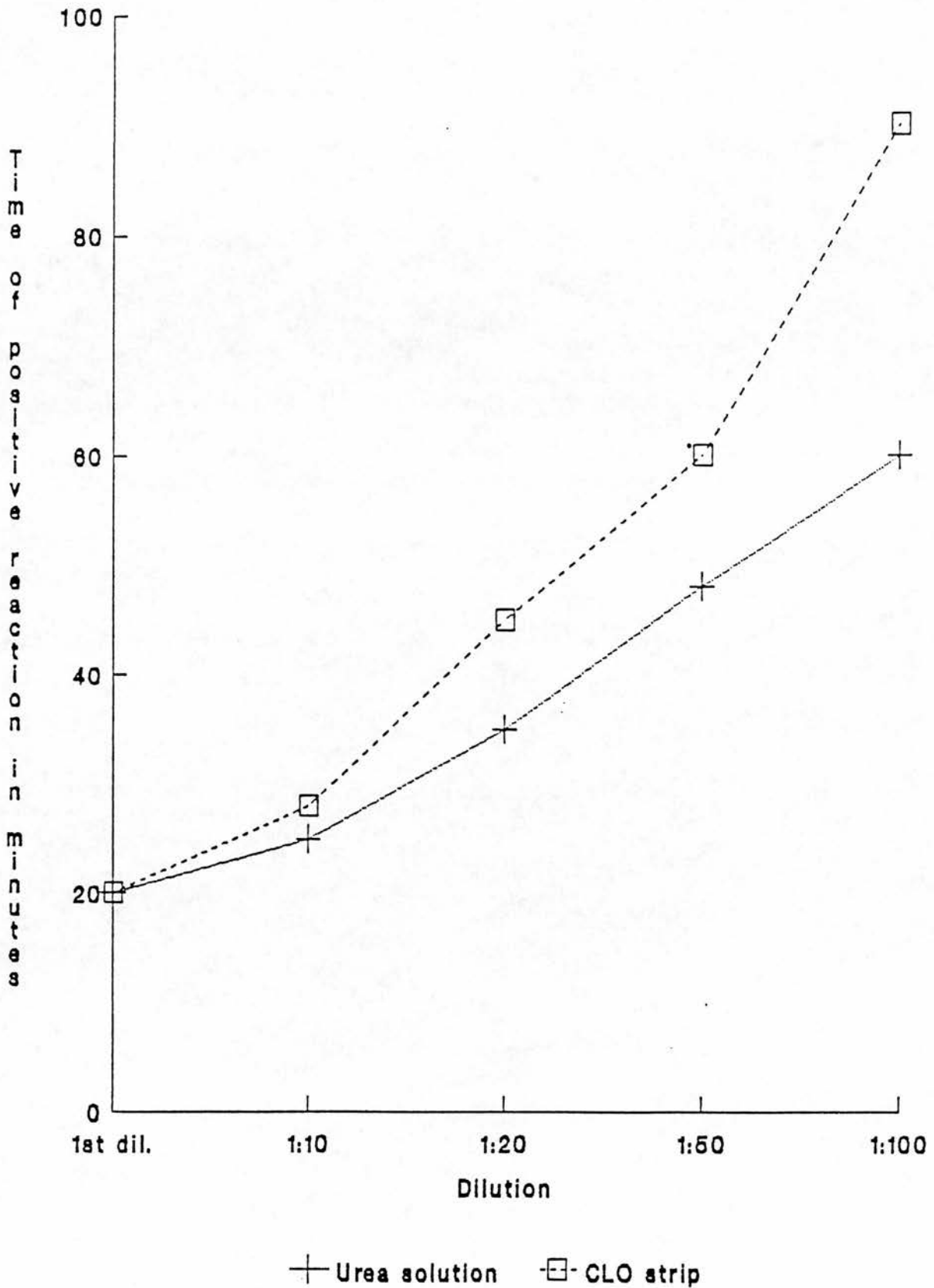


Fig. 9.1 Comparison of reactivity of 'in-house' urea solution and CLO test with different dilutions of *H. pylori* suspension

Table 9.3 The effects of phosphate buffer strength on the demonstration of *H.pylori* urease.

Strength of <i>H.pylori</i> suspension	Time (min) to a positive "in-house" urease test with phosphate buffer of strength:		
	0.01M	0.005M	0.0025M
Original suspension	20	16	12
1:10 dilution	25	20	17
1:20 dilution	35	30	26
1:50 dilution	48	44	39
1:100 dilution	60	56	51

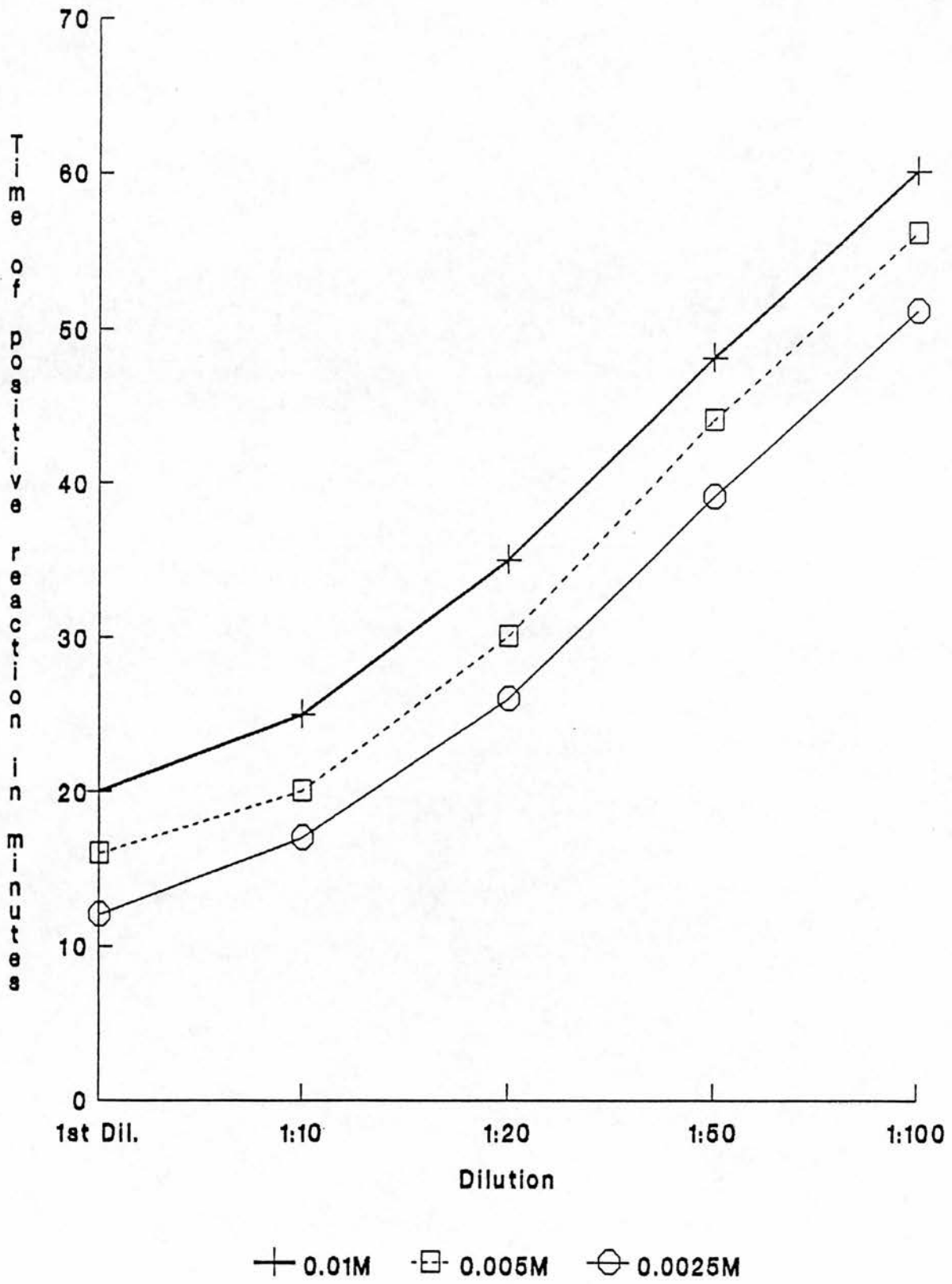


Fig. 9.2 Effects of phosphate buffer strength on the demonstration of *H.pylori* urease

Coudron and Kirby (1989) found 62% sensitivity. Megraud (1988) stated that compared with other tests, the urease test is insensitive, but specific, rapid, very simple and the cheapest way of diagnosing *H.pylori* infection.

In the present study, three antral biopsies from each of 53 patients were tested for the presence of *H.pylori* by histology and by two urease tests, one made with a commercial CLO strip and the second with the freshly prepared "in-house" urea solution. After 90 minutes, positive results were identical in both tests. The sensitivity and specificity (compared to histology) were 65.5% and 95% respectively and positive and negative predictive values were 83% and 85% respectively.

The slightly lower sensitivity of the urease test made with "in-house" urea solution in the present study might be due to the elapsed time (90 min), the temperature of the endoscopy room (30° C), the fact that uncrushed biopsy material was used or small numbers of organisms present in the particular biopsies which were used.

Though sensitivity can be affected by several variables, it must ultimately depend on the number of organisms present in each biopsy (Marshall et al., 1987).

To compare the sensitivity of the "in-house" urea solution with the commercial CLO urease test, bacterial suspensions of gradually increasing dilution of the original suspension were used (table 9.2 and fig. 9.1). With each dilution of bacterial suspension, the "in-house" urea solution gave a faster result than the commercial CLO

urease test. With the greatest dilution of bacteria tested (1:100, = 500 - 5,000 bacteria) the time required to produce positive results was 30 minutes less with the "in-house" urea solution than with the commercial CLO urease test.

Dilution of the "in-house" urea solution more than 1:100 was not tested because it was felt that very dilute solution might be unstable and time taken for a positive result would be unacceptably long.

Vaira et al. (1988b) described a rapid urease test using 6% urea and reading at one minute. Arvind et al. (1988) used 10% urea and 1% phenol red. In these tests buffer was not used, although Vaira et al. stated that a minimum concentration of buffer is needed to stabilise the test and improve its shelf-life.

In the present study, to improve the sensitivity of the test and to reduce its cost compared with the commercial CLO test, the buffer concentration was reduced. Table 9.3 and fig. 9.2 shows that lowering the concentration of buffer produced faster positive results even with gradually reducing bacterial numbers e.g., at (1:100) dilution with 0.0025M buffer, a positive result appeared 9 minutes earlier than with 0.01M buffer.

Each commercial CLO urease test costs one pound sterling. The "in-house" urea solution prepared in this study cost at most 10 pence for each test. McNulty (1989) has also found that an "in-house" test with 2% urea broth was cheaper costing only 5 pence for each test compared with two pounds sterling for each commercially produced test.

In spite of all the advantages of the urease test, there is some lack of concordance when it is compared with other tests, possibly due to sampling variation (Mobley et al., 1988). *H.pylori* infection can be patchy, the organism being present in one specimen but not others; however the *in vitro* studies suggest that as few as 500 bacteria might be detected. As might be expected, the urease test is less sensitive in patients who have received antimicrobial drugs or bismuth treatment (Deltenre et al., 1989).

As a field test, especially in developing countries where the cost of investigation is a major problem for diagnosis, the "in-house" urea preparation will help to provide faster and cheaper results.

CHAPTER 10

GENERAL DISCUSSION

The work reported in this thesis has addressed aspects of the role of *H.pylori* in gastroduodenal disease.

The relationship between linoleic acid consumption, peptic ulcer, smoking and *H.pylori* status was explored in chapters 3 and 4. Previous workers had suggested that low adipose percentage linoleic acid in DU was a consequence of low dietary intake but as discussed in the chapters mentioned; the finding of normal intake refuted this hypothesis. Individuals who smoked also exhibited low adipose percentage linoleic acid, despite a normal intake. This suggests abnormal linoleic acid metabolism in smokers and DU patients and it is tempting to speculate that this might in some way represent a mechanism by which smoking predisposes to DU. There was, however, no additive or synergistic effect in smokers with DU. Conceivably, abnormal PG metabolism is a consequence of smoking and this accentuates an ulcer diathesis. Other possible explanations including linoleic acid malabsorption seem unlikely. The relationship between low percentage linoleic acid in adipose tissue and *H.pylori* status is difficult to establish because *H.pylori* and DU are so strongly linked. It is conceivable that abnormalities in the microenvironment of the mucus layer are created by underlying alterations in linoleic acid and PG metabolism and this facilitates *H.pylori* colonisation. The experiments described in this thesis are unable to take this possibility further.

Whether it is possible to modulate linoleic acid and PG metabolism, thereby alleviating the DU tendency, is not clear. Workers have shown that increased dietary linoleic acid intake enhanced gastric PG output; but it is not known whether this affects either *H.pylori* status or the natural history of peptic ulcer disease.

The work in chapter 5 describes the isolation of strains of *H.pylori* from the mouth and antrum of DU patients. The similarity of these strains was determined by ribotyping, the most discriminatory typing method for *H.pylori* currently available. Not surprisingly, the strains from the mouth and stomach are indistinguishable. This supports the hypothesis that a reservoir of *H.pylori* for re-infection of the stomach lies in the mouth. The methods used in endoscopy make contamination from stomach unlikely, but does not eliminate this possibility.

It is now clear that DU can be successfully treated with antibiotics by eradicating *H.pylori* from the stomach but relapse is associated with re-infection. Whether triple therapy eliminates *H.pylori* from dental plaque is unclear, but if not, this might be a mechanism for re-infection of the stomach. This point should be clarified by further investigation, and it may be necessary to devise specific means to eliminate *H.pylori* from the mouth.

Chapter 6 describes the prevalence of serum antibody to *H.pylori*, and the presence of *H.pylori* in the mouth of hospital inpatients. This is the first time such a study has been reported in the UK. It confirms that

seropositivity and tooth plaque urease test increases with age - a result possibly due to poorer socio-economic conditions in the UK during the first half of this century or a phenomenon due to less emphasis upon oral hygiene during this period. Antibiotic therapy did not influence the prevalence of oral *H.pylori*. This suggests that triple therapy only eradicates infection in the antrum, leaving a nidus for re-infection in the mouth.

It has also been shown at a statistically significant level, that patients with hepatic disease and ulcerative colitis are more likely to have antibody titres to *H.pylori* and positive urease test. The reasons for this are not clear. Such patients are either more likely to be exposed to infection or less able to resist infection when exposed in the general population. Current knowledge does not allow further speculation.

The experiments described in chapter 7 show that concentrations of bile acid likely to be present in the stomach subjected to biliary reflux do not inhibit *H.pylori in vitro*. However, it is now recognised that *H.pylori* is infrequently found in the presence of biliary gastritis.

The work recorded in chapter 8 failed to show any direct connection between the presence of *H.pylori* in the stomach and the development of gastric cancer. This does not support the hypothesis that gastric cancer and *H.pylori* gastritis are associated, although the data do not refute the possibility. This adds to the body of evidence that, if *H.pylori* is linked to malignant disease of the stomach,

the link is an indirect one. If it is true that gastritis induced by *H.pylori* prepares the way for other carcinogenic influences to act (Dooley, 1991), eradication of *H.pylori* from the stomach and the mouth should lead to a reduced incidence of gastric carcinoma.

Given the extent and significance of *H.pylori* infection, rapid and cheap methods for its recognition are important. The urease test is widely used to identify the presence of *H.pylori* but the commercial kit is relatively expensive. Chapter 9 describes the preparation of a cheap alternative which is satisfactorily sensitive and specific and could be of great value for epidemiological studies in developing countries. Whilst the limits of sensitivity were not defined, the range of sensitivity is the same as the commercial kits. These results fulfil the requirements originally defined.

H.pylori infection is probably one of the common bacterial infection of man, but its reservoir and mode of transmission have not yet been clearly established. If the usual pattern of investigation of a newly discovered infection is followed in this case, research to produce an effective vaccine should develop in the near future.

SUGGESTIONS FOR FUTURE WORK

(1) Modification of linoleic acid levels by dietary manipulation to define effects upon gastric *H.pylori* status and the natural history of DU.

(2) Eradication of *H.pylori* from dental plaque using local or systemic means to determine the natural history of gastric re-infection by *H.pylori* following triple therapy.

(3) A longterm prospective study of *H.pylori* status by serological testing to define the role of the organism in gastric cancer.

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A P P E N D I X

1. An example of a dietary diary.
2. Names and addresses of suppliers of chemicals and equipment referred to.

Guidelines for diet history

Please record all food and drinks eaten over a 7 day period. The following guidelines will help you to give an accurate account of your diet.

1. Describe the type of food eg milk if skimmed, semi-skimmed full fat.
2. Indicate the amount of food. If unable to give a weight use household measures eg tablespoon, teaspoon, cups etc.
3. State cooking methods used eg fried, grilled or baked.
4. Foods not eaten regularly. Indicate where possible the number of times eaten per week and the quantity.
5. Remember to indicate drinks and any sugar or mixers added to them.

MONDAY [Dietary record]

24/12/90

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
fast →	Fresh grape fruit (unsweetened) Muesli (unsweetened) with semi-skimmed milk (toasted) Bread (highly white) with flax margarine and honey — " —	1/2 one fruit 4 table spoons + 200ml milk 1 1/2 rounds 2 teaspoon honey trace margarine
u →	Coffee with whole milk — " —	1 cup with 1 Tablespoon milk
cl →	Chicken and mushroom thick soup Brown bread roll with butter — " —	200 ml 1 roll 10g butter
u →	Tea with semi skimmed milk Fruit bread (toasted) with flax margarine Chocolate biscuit (penguin) — " —	1 cup 1 table spoon milk 1 round with trace margarine 1 biscuit.
ev →	Gin and tonic (ordinary tonic) Monk fish poached with tomatoes and onions Rice (steamed) Raspberry pavlova : raspberries with whipped cream* on meringue base (* made from whipping cream) which I think is vegetable based) Herb tea without milk or sugar	2 units gin 400 ml tonic 200g fish 1/3 tin tomatoes 1/3 onion 5 table spoons (cooked volume) 1 desert dish full (or about 2 table spoons) 1 cup

TUESDAY [Dietary record]

25/12/90

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
Breakfast →	Fresh grape fruit (unsweetened)	1/2 one fruit
	Muesli (unsweetened) with semi skinned milk	3 tablespoons 150ml milk
	Toast with flour margarine and honey	2 rounds bread trace margarine 4 teaspoons honey
	Tea with semi skinned milk	1 cup of tea 1 tablespoon milk
	—————"————"	
Break →	Coffee with semi skinned milk	1 cup 1 tablespoon milk
	—————"————"	
Lunch →	Tomatoe soup (Heinz)	200g (1/2 pint)
	Tomatoe rolls with salad cream	2 medium size rolls with flour margarine, 1 tomato on each with salad cream mixed
	Apple —————"————"	1 (small)
Tea →	Tea with semi skinned milk	1 cup + 1 tablesp. milk
	Fruit cake with marzipan and icing	1 small piece.
	—————"————"	
Dinner →	Smoked salmon with brown bread with flour margarine	26g salmon 1/2 round bread
	Turkey (roast)	150g 150g
	Potatoes (boiled)	2 pieces
	" (roast in olive oil)	2 pieces
	Carrots	1 tablespoon
	Brussel sprouts	3 "
	Swede	3 tablespoons
	Chopped date sausage	3 sausage
	Crang	2 tablespoon
	Christmas pudding	100g
	Mustard (with semi skinned milk)	3 tablespoons
	Sherry (dry)	2 small glasses
	White wine and port wine	5 glasses + 3 glass

WEDNESDAY [Dietary record]

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
Breakfast →	First grapefruit (unsweetened) Museli (unsweetened) with semi skimmed milk Bread with blue margarine and honey Coffee with semi skimmed milk	1/2 one fruit 4 tablespoons + 200 ml milk 2 round bread blue margarine 2 teaspoons honey 1 cup + 1 tablespoon milk
L →	———— Macaroni (baked) with sauce made from semi skimmed milk, margarine, corn flour and grated cheese Apple	5 tablespoons of composite dish 1
D →	Cold Turkey 2 small sausages Potatoes (fried in sunflower oil) Raw cabbage / celery / carrots Christmas pudding Custard made with semi skimmed milk Jasmine tea (without milk or sugar) 2 walnuts 4 hazelnuts Stillon cheese 2 oatcakes	4 oz 2 tablespoons 2 " 1 helping 1/4 pint 1 cup Very small portion (20g approx)

THURSDAY [Dietary record] 20/12.

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
breakfast →	Muesli (unsweetened) with whole milk	Approx 4 tables + 200ml milk
	Bread (mughty white with wheat + rye grains)	2 slices
	Flour	1 teaspoon
	Honey	4 "
→	Coffee (instant with powdered milk)	1 teaspoon (caf)
	"	1 " (mil)
→	Egg sandwich - one round of wholemeal bread with mayonaisse (description unknown)	2 slices of bread 1 egg approx
	Milk (whole) - 1 glass	1/2 pint.
	Apple	1
→	Tea + semi skinned milk	1 cup approx 1 table spo milk.
→	Pork casserole with tomatoes (tinned), celery and mushrooms (cooked with vegetable oil) plus mashed potatoes and cabbage	Approx 6 oz pot 3 table spoon potatoes mashed with milk + flour margarine 3 table spoon cabbage with 1/2 of flour margarin
	Yogurt Yogurt Whole milk unsweetened	5 oz. (130g)
→	Herb tea without milk n sugar	
	Fruit cake	1 slice (snack)
	Bread, flour margarine and honey	1 1/2 slices 2 teaspoon honey.

FRI DAY [Dietary record]

21/12.

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
fast →	Fresh grapefruit (unsweetened)	1/2 of 1 fruit
	Muesli (unsweetened) with	4 table spoons
	Milk (whole)	Approx 200ml
	Bread (light white)	2 slices
	Flour margarine on bread	small amount
	Flour margarine	2 teaspoons
→	Coffee + milk (full)	1 teaspoon ^{1/2 t.} coffee
	" "	1 table spoon
→	"Meat pie"	1
	Chips	1 scoop (1/2 pt)
	Apple	1
→	Tea with semi-skimmed milk	1 Mug
	Sultana short-cake	1 table spoon milk
		1 small piece (40 grams)
→	Lemonade (53g sugar per litre)	400 ml.
	Trout (baked with little flour margarine)	300g
	Potatoes fried in sunflower oil	180g
	Cabbage, celery and carrot (raw) mixed with salad cream + milk + pepper	Side plate full (3 table spoons)
	Black-current crumble made with whole meal flour, ^{and margarine} sweetened with sugar	
	Herb tea without milk or sugar	1 cup
	Roschip wine - very dry	1 small stem glass

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
Breakfast →	Fresh grapefruit without sugar	1/2 of 1 fruit
	Muesli (unsweetened)	4 table spoon
	Milk (semi skimmed)	Approx 200 ml
	Bread (wholly white)	2 slices
	Flora margarine	Small amount
	Honey	3 teaspoon
Tea →	Coffee with semi skimmed milk	1 Cup with
	" " "	1 1/2 Cup milk
Lunch →	Lemonade (53 g/l sugar)	250 ml.
	Soup - potatoes and leek (mutton stock)	1 dish
	Smoked haddock baked with a small quantity of sunflower oil	180 g fish 1 teaspoon oil
	Bread with flora margarine	2 slices
	Bread with " " + honey	1 slice 1 teaspoon hon
	Whole milk fruit yogurt	150 g
Tea →	Tea with semi skimmed milk	1 cup with 1 1/2 of milk
	Short-cake biscuits (26 g each)	2
Supper →	Lemonade (53 g/l sugar)	200 ml
	Beef casserole with onions cooked with vegetable oil and cornflower	150 g meat
	Potatoes (boiled)	4 table spoon
	Brussels sprouts (boiled)	3 "
	Red wine	3 glasses
	Cheese cake with Kiwi fruit, digestive biscuit / flora / honey misc.	1/4 of normal sized cake

SUNDAY [Dietary record] 23/12

MEAL/SNACK	FOOD DESCRIPTION	QUANTITY
breakfast →	Fresh grapefruit (unsweetened)	1/2 1 fruit
	Muesli (unsweetened) + semi-skimmed milk	4 tablespoons approx 200 ml
	Bread (mighty white) with flour margarine and honey	2 slices 2 teaspoons honey
	Coffee instant with semi-skimmed milk	1 cup
→	Lemonade (53 g/l)	1 1/2 cups approx 200 ml
	Soup potato and vegetable (muller stock)	1 dish
	Tomato sandwiches with Herby salad cream	4 slices slices of bread 2 tomatoes Trace of flour margarine 2 teaspoons salad cream
→	Chocolate biscuit	1 biscuit
	Currant bread with flour margarine	1 round of bread trace margarine
	Tee with semi-skimmed milk	1 cup 1 tablespoon milk
→	Tomato water (ordinary)	200 ml
	Lentil soup (with 1/2 bread roll spread with butter)	1 dish < 1/2 roll
	Roast-beef	80 g
	Boiled potatoes (plain)	6 pieces
	Carrots (boiled)	2 tablespoons
	Pumpkin (boiled)	2 tablespoons
	Brussels sprouts (boiled)	2 tablespoons
	Gravy	2 tablespoons
	Christmas pudding	1 small dish
	Single cream	2 teaspoons
	Coffee with skimmed milk	1 cup + 1 teaspoon milk
	Red wine	1 glass

Food Description	Amount Daily	Amount Weekly
k l fat		50 ml (in coffee)
i-skimmed		1500 ml
mmed		nil
ter/ garine e Almost-entirely from Margarine		250 g
getable		Nil
n Oil		Nil
d		Nil
er Rape or sunflower or ve oil		80 ml 80 ml
eese rd eg Cheddar		25g (as part part of bread up up dishes such as for pie + lasagne)
am		Nil
tage		Virtually nil (eaten or drunk like cheese cakes only)
reading		Nil
w Fat		Nil
eam ngle	Eaten only very occasionally on	Virtually nil
uble	deserts such as truffles and fruit	Nil
w Fat	Salads	Virtually nil
gs oking methods in very rarely other in prepared food as sponge cakes etc q.v.	—	One egg on average boiled, poached or occasionally Scrambled.

Food Description	Amount Daily	Amount Weekly	
Biscuits		300 - 400 g	
Low Fat		Nil	
Sweets/Cakes	100 g in the form of fruit or jam pies or tarts		
<u>Cried Foods</u>		<u>Fried</u>	<u>Grilled or roasted</u>
Chips		300 g	nil
Crisps		nil	nil
Fish		nil	nil
Potato		nil	virtually nil
Desserts		nil	virtually nil
Vegetables		nil	about 100g of potatoes & parsnips
<u>Cakes</u>			
Cream filled		Nil	
Long		200g (jam filled)	
Biscuit base		150g	
Biscuits		100g	
<u>Biscuits</u>			
Plain		Biscuits only - 6 per week	
Cheese		nil	
Cream filled		nil	
Chocolate		2 biscuits	

Food Description	Amount Daily	Amount Weekly
<u>Infectionery</u>		
Chocolate (Milk/Plain)		Nil
Coffee/Fudge		Nil
Cakes		100-150g as <u>museli</u>
Cream filled		Nil
<u>Desserts</u>		
Ice cream		50g
Milk Puddings		250g
Muffins/Sponge		200g
Mousse		nil
<u>Meat</u>		
Beef		
Pork		
Pig's ham		
Lamb		
Chicken/turkey		
<p>I eat some form of meat 5 days per week at an rate of approximately 150g/day or average</p>		<p>450g</p> <p>300g</p>
<u>Dairy Products</u>		
Cheeses		Approximately 2 standard size per week
Usage Rolls		nil
Lidies		nil
Eggs		50g or average
Beef Burgers		Nil

<u>Description</u>	<u>Amount Daily</u>	<u>Amount Weekly</u>
h sh		250g
ked		40g
Y		200g
ned		Tin of salmon 4 times per week
all fish type		
<u>Salads and Pickles</u>		
ad Cream	1 tablespoon	
onnaise		1 tablespoon
ench Dressing		2 tablespoons
mato Ketchup		4 tablespoons
<u>Alcohol</u>		
Beer - Spirits Wine		Nil, other than about 20 tm. of lager while on holiday in the summer One to two units per week $\frac{1}{2}$ bottle per week

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J. A. HEATHERINGTON 2/7/41

13-B-1991

NUTRIENT TOTALS DIVIDED BY 7 . THE CURRENT DIVISOR

3	Water	*	1338.09g	22			Copper	*	1.80mg
4	Alcohol	*	20.58g	23			Zinc	*	11.36mg
5	Solids	*	7.41g	24			Total sulphur	*	880.03mg
6	Lactose	*	15.28g	25			Chloride	*	5367.49mg
7	Sugars	*	74.18g	26			Iodine	*	605.11ug
8	Starch & Dextrin	*	143.53g	27			Carotene	*	2587.61ug
9	Fibre (Sulphate)	*	20.26g	28			Vitamin D	*	3.71ug
10	Total nitrogen	*	17.43g	29			Thiamin	*	1.50mg
11	Energy kcal	*	2583.11	30			Riboflavin	*	1.81mg
12	Energy kJ	*	10843.89	31			Nicotinic acid	*	20.11mg
13	Protein NRo.25	*	106.75g	32			Pot. nico. acid	*	21.55mg
14	Fat	*	106.55g	33			Vitamin C	*	77.59mg
15	Carbohydrate	*	280.09g	34			Vitamin E	*	7.54mg
16	Sodium	*	3437.81mg	35			Vitamin K6	*	1.59mg
17	Potassium	*	376.85mg	36			Vitamin B12	*	6.85ug
18	Calcium	*	1043.51mg	37			Free folic acid	*	97.38ug
19	Magnesium	*	402.40mg	38			Total folic acid	*	198.38ug
20	Phosphorus	*	1727.34mg	39			Pantothenic acid	*	4.37mg
21	Iron	*	15.84mg	40			Biotin	*	26.03ug

D.A. HETHERINGTON 2/7/41

13-B-1991

FATTY ACID TOTALS DIVIDED BY 7, THE CURRENT DIVISOR

Fatty Acid	Amount (mg)	Fatty Acid	Amount (mg)	Fatty Acid	Amount (mg)
4:0	636.79	14:1	788.26	19:3	0.00
6:0	397.43	15:1	164.86	20:3	2.57
8:0	232.93	16:1	1745.43	22:3	0.00
10:0	586.33	17:1	325.79	16:4	0.00
12:0	956.36	18:1	18026.91	18:4	17.29
13:0	0.00	20:1	796.22	20:4	273.64
14:0	3290.83	22:1	1117.72	22:4	19.71
15:0	269.53	24:1	1.36	20:5	492.64
16:0	13264.50	16:2	0.86	22:5	154.14
17:0	362.93	18:2	6608.95	22:6	276.93
18:0	6049.04	20:2	2.86	24:6	0.00
20:0	199.34	22:2	0.00	20:1 to 20:5	4.29
22:0	147.48	18:3	1039.83	22:5 & 22:6	172.14
24:0	4.81				

Total Saturated Fats :
 Total Mono-Unsaturated Fats :
 Total Poly-Unsaturated Fats :
 P:S Ratio :

26418.30mg
 22968.57mg
 9064.94mg
 0.34

Names and addresses of suppliers of chemicals and equipment referred to.

1. Agar Scientific Ltd., 66a Cambridge road, Stansted, Essex, UK.
2. Baxter Health Care Ltd., Thorpe Lea Manor, Egham, Surrey, UK.
3. BDH Ltd., Bruntfield Avenue, Thornly Bank, Glasgow, UK.
4. Delta West Ltd., 15 Bridie Hall Drive, Bentely, Western Australia.
5. Denley Instruments Ltd., Natts Lane, Billingshurst, West Sussex, UK
6. Dynatech Laboratories Ltd., Daux Road, Billingshurst, West Sussex, UK.
7. Flow Laboratories Ltd., Wood Cock Hill, Harefield Road, Rickmansworth, Hert, UK.
8. Gibco Life Technology Ltd., P.O. Box 36, Trident House, Renfrew Road, Paisley, Glasgow, UK.
9. HWC Supplies, 94 Priory Road, Cheam, Surrey, UK.
10. Jeol(UK) Ltd., Jeol House, Silver Court, Watchmead, Welwyn Garden City, Hertfordshire, UK.
11. Johnson Matthey Ltd., Orchard Road, Royston, Hertfordshire, UK.
12. Lab System UK Group Ltd., Unit 5, The Ringway Centre, Edison Road, Basingstoke, Hants, UK.
13. Merck-Darmstadt Ltd., Bruntfield Avenue, Thornly Bank. Glasgow, UK.
14. Prolab Diagnostic, 10a Croft Business Park, Bromborough, Wirral, Merseyside, UK.
15. Proton Cambridge, Proton House, Vanwall Road, Maidenhead, Berkshire, UK.
16. Rathburn Chemical, Caberston Road, Walkerburn, Peebleshire, UK.
17. Scottish Biotechnology Instrumentation, Blairgowrie Business Centre, 60 High Street, Blairgowrie, Perthshire, UK.
18. Sigma Chemical Co Ltd., Fancy Road, Poole, Dorset, UK.
19. Supelchem Co UK Ltd., Shire Hill, Saffron Walden, Essex, UK.