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**STUDY OF NITROSATING POTENTIAL
AT THE
GASTRO-OESOPHAGEAL JUNCTION
AND
IN BARRETT'S OESOPHAGUS ©**

by

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Submitted for the degree of M.D.

Faculty of Medicine

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January 2006

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DECLARATION

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been submitted to previously for a higher degree.

This research was carried out in the Department of Medicine and Therapeutics, University of Glasgow, under the supervision of Professor K.E.L. McColl.

Hisaharu Suzuki

January 2006

<u>LIST OF CONTENTS</u>	<u>PAGE</u>
LIST OF FIGURES	i
LIST OF TABLES	v
SUMMARY OF THESIS	vi
<u>CHAPTER ONE</u>	
ANATOMY	
1.1 ANATOMY OF THE OESOPHAGUS	2
1.2 ANATOMY OF THE STOMACH	3
1.3 PHYSIOLOGICAL FUNCTIONS OF GASTRIC ACID	4
<u>CHAPTER TWO</u>	
EPIDEMIOLOGY OF CARDIA AND OESOPHAGEAL ADENOCARCINOMA	
2.1 INTRODUCTION	7
2.2 INCIDENCE OF ADENOCARCINOMA OF THE CARDIA	7
2.3 INCIDENCE OF ADENOCARCINOMA OF THE OESOPHAGUS	8
2.4 SEX DISTRIBUTION OF NON-CARDIA GASTRIC CANCER, ADENOCARCINOMA OF THE CARDIA AND OESOPHAGUS.	11
2.5 RACE AND ADENOCARCINOMA OF THE CARDIA AND OESOPHAGUS	11
2.6 SOCIOECONOMIC STATUS AND CANCERS OF THE UPPER GASTRO-INTESTINAL TRACT	12
<u>CHAPTER THREE</u>	
AETIOLOGY OF ADENOCARCINOMA OF THE CARDIA AND OESOPHAGUS	
3.1 INTRODUCTION	15
3.2 SMOKING	15
3.3 BODY SIZE	16
3.4 GASTRO-OESOPHAGEAL REFLUX	17

<u>LIST OF CONTENTS</u>		PAGE
3.5	<i>HELICOBACTER PYLORI</i> AND CANCERS OF THE UPPER GASTROINTESTINAL TRACT.	17
3.6	DIET AND ADENOCARCINOMA OF THE UPPER GASTROINTESTINAL TRACT	19
3.6.1	SUMMARY OF DIET AND UPPER GI CANCER	22

CHAPTER FOUR

BARRETT'S OESOPHAGUS

4.1	DEFINITION	25
4.2	EPIDEMIOLOGY	25
4.3	HISTOLOGICAL FEATURES	26
4.4	MALIGNANT TRANSFORMATION IN BARRETT'S OESOPHAGUS	27
4.5	DYSPLASIA IN BARRETT'S OESOPHAGUS	28
4.6	PATHOGENESIS OF BARRETT'S OESOPHAGUS	32
4.6.1	GASTRO-OESOPHAGEAL REFLUX AND BARRETT'S OESOPHAGUS	32
4.6.2	HIATUS HERNIA	33
4.6.3	ACID, PEPSIN AND BILE REFLUX	34
4.7	INTESTINAL METAPLASIA OF THE CARDIA AND CARDITIS	37

CHAPTER FIVE

NITROSAMINES IN CANCER

5.1	INTRODUCTION	41
5.2	NITROSO COMPOUNDS. WHAT ARE THEY?	41
5.3	NITROSAMINES IN ANIMAL MODELS OF OESOPHAGEAL ADENOCARCINOMA	44
5.4	<i>N</i> -NITROSO COMPOUNDS AND HUMAN CANCER	45
5.5	NITROSATION CHEMISTRY	46
5.5.1	ACID-CATALYSED NITROSATION	46
5.5.2	BACTERIALLY CATALYSED NITROSATION	47

LIST OF CONTENTS	PAGE
5.5.3 NITROSATION BY MACROPHAGES AND NEUTROPHILS: THE ROLE OF NITRIC OXIDE.	48
5.6 ASCORBIC ACID AS AN INHIBITOR OF NITROSATION	49
5.6.1 ASCORBIC ACID INHIBITS ACID-CATALYSED NITROSATION	49
5.6.2 ASCORBIC ACID INHIBITS BACTERIA- CATALYSED NITROSATION	50
5.6.3 ASCORBIC ACID INHIBITS N-NITROSATION BY ACTIVATED MACROPHAGES AND NEUTROPHILS	50
5.7 ACID-CATALYSED NITROSATION IN THE HUMAN STOMACH	51
5.7.1 INTRODUCTION	51
5.7.2 ENTEROSALIVARY CIRCULATION OF NITRATE AND THE FORMATION OF SALIVARY NITRITE	52
5.7.3 ASCORBIC ACID SECRETION IN THE HUMAN STOMACH	54
5.8 EVIDENCE FOR INTRAGASTRIC NITROSATION IN THE ACID-SECRETING HUMAN STOMACH.	54

CHAPTER SIX

HYPOTHESIS

6.1 NITRATE UTILISATION	57
6.2 SOCIAL CLASS AND SALIVARY NITRATE/NITRITE: A POSSIBLE LINK BETWEEN CARDIO-OESOPHAGEAL ADENOCARCINOMA.	57
6.3 THE PATHOPHYSIOLOGICAL PROCESS LEADING TO ADENOCARCINOMA OF THE OESOPHAGUS AND CARDIA OCCURS IN THE LUMEN OF THE DISTAL OESOPHAGUS AND THE CARDIA.	58
6.4 AIMS OF THE THESIS	59

CHAPTER SEVEN**GENERAL METHODS**

7.1	INTRODUCTION	63
7.2	MICRODIALYSIS	63
7.2.1	MICRODIALYSIS PROBES	64
7.2.2	TERMINOLOGY	64
7.2.3	SAMPLING PARAMETER CONSIDERATIONS	65
7.2.4	SETTING UP THE MICRODIALYSIS PROBE	66
7.2.5	COLLECTING THE MICRODIALYSIS PRODUCTS	70
7.3	ANALYSIS OF SAMPLES COLLECTED BY MICRODIALYSIS	70
7.3.1	DETERMINATION OF ASCORBIC ACID AND TOTAL VITAMIN C BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY	71
7.3.2	DETERMINATION OF NITRITE	72
7.3.3	DETERMINATION OF THIOCYANATE	75
7.3.4	DETERMINATION OF NITRATE	78

CHAPTER EIGHT**IN-VITRO STUDIES: VALIDATION OF MICRODIALYSIS PROBES**

8.1	INTRODUCTION	82
8.2	EFFECT OF PERFUSION RATE ON RECOVERIES OF NITRITE, THIOCYANATE, ASCORBIC ACID AND TOTAL VITAMIN C	82
8.2.1	METHODS	82
8.2.2	DATA ANALYSIS	83
8.2.3	RESULTS	83
8.2.4	CONCLUSION	83
8.3	EFFECT OF PH OF THE TEST SOLUTION BEING EXAMINED ON THE PH OF THE DIALYSATE	85

LIST OF CONTENTS		PAGE
8.3.1	METHODS	85
8.3.2	RESULT	85
8.3.3	CONCLUSIONS	86
8.4	EFFECT OF PH OF THE TEST SOLUTION ON THE RECOVERY OF VARIOUS CHEMICALS	86
8.4.1	METHODS	86
8.4.2	DATA ANALYSIS	87
8.4.3	RESULTS	87
8.4.4	CONCLUSIONS	88
8.5	ELUCIDATING THE CAUSE FOR REDUCED NITRITE RECOVERY AT PH 1.5 AND 2.5. PART I: PERMEABILITY OF THE MICRODIALYSIS TUBING TO NITRIC OXIDE	89
8.5.1	METHOD	89
8.5.2	RESULTS	90
8.5.3	CONCLUSIONS	90
8.6	ELUCIDATING THE CAUSE FOR REDUCED NITRITE RECOVERY AT PH 1.5 AND 2.5. PART II: FORMATION OF NITRIC OXIDE AND LOSS OF NITRITE AT PH 1.5 AND 2.5	93
8.6.1	METHOD	93
8.6.2	DATA ANALYSIS	93
8.6.3	RESULTS	94
8.6.4	CONCLUSION	94
8.7	DYNAMIC STUDIES SIMULATING THE INTERACTION BETWEEN SALIVARY NITRITE AND GASTRIC JUICE ASCORBIC ACID IN ACID STOMACH	96
8.7.1	INTRODUCTION	96
8.7.2	METHODS	97
8.7.3	DATA ANALYSIS	97
8.7.4	RESULTS	98
8.7.5	CONCLUSIONS	101

CHAPTER NINE

PROBE FOR HUMAN USE

9.1	ASSEMBLY	103
9.2	VALIDATION OF THE ASSEMBLED PROBES FOR USE IN THE HUMAN UPPER GASTROINTESTINAL TRACT	104
9.2.1	METHOD	104
9.2.2	DATA ANALYSIS	106
9.2.3	RESULTS	106
9.2.4	CONCLUSIONS	106
9.3	DISINFECTION OF THE MICRODIALYSIS PROBES	107
9.3.1	METHODS AND RESULTS	107

CHAPTER TEN

HUMAN VOLUNTEER STUDIES

10.1	INTRODUCTION	109
10.2	METHOD	110
10.2.1	SUBJECT SELECTION AND MICRODIALYSIS PROCEDURE	110
10.2.2	CHEMICAL ANALYSES	114
10.3	STATISTICAL ANALYSES	115
10.4	ETHICS	115
10.5	RESULTS	115
10.5.1	BLOOD	116
10.5.2	SALIVA	116
10.5.3	OESOPHAGUS	119
10.5.4	STOMACH	120
10.6	DISCUSSION	131

CHAPTER ELEVEN

**NITRITE CONCENTRATIONS IN UPPER AERO-DIGESTIVE
TRACT IN HEALTHY VOLUNTEERS**

11.1	INTRODUCTION	136
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<u>LIST OF CONTENTS</u>		PAGE
11.2	AIM	136
11.3	METHODS	137
11.4	DATA ANALYSES	139
11.5	RESULTS	140
	11.5.1 SERUM NITRATE	140
	11.5.2 SALIVA	141
	11.5.3 MICRODIALYSIS SAMPLES	142
	11.5.4 THIOCYANATE, NITRITE AND NITRATE CONCENTRATIONS IN THE PHARYNX AND THE OESOPHAGEAL LUMEN RELATIVE TO SALIVARY CONCENTRATIONS.	143
11.6	DISCUSSION	149

CHAPTER TWELVE

STUDY OF NITROSATION POTENTIAL IN BARRETT'S OESOPHAGUS

12.1	INTRODUCTION	155
12.2	METHODS	156
	12.2.1 DESIGN OF THE MICRODIALYSIS PROBES	156
	12.2.2 SUBJECTS AND METHODS	158
	12.2.3 CHEMICAL ANALYSES	159
12.3	STATISTICAL ANALYSES	159
12.4	RESULTS	160
	12.4.1 SERUM	160
	12.4.2 SALIVA	160
	12.4.3 MICRODIALYSIS SAMPLES	161
12.5	DISCUSSION	174

CHAPTER THIRTEEN

NITRIC OXIDE GENERATION IN BARRETT'S OESOPHAGUS

13.1	INTRODUCTION	179
13.2	AIMS	180

<u>LIST OF CONTENTS</u>	<u>PAGE</u>
13.3 SUBJECTS	180
13.4 METHODS	180
13.4.1 NITRIC OXIDE SENSOR	180
13.4.2 CALIBRATION OF NITRIC OXIDE PROBE <i>IN-VITRO</i>	181
13.4.3 CLINICAL PROCEDURE USING THE NITRIC OXIDE PROBE	182
13.5 STATISTICAL ANALYSES	183
13.6 RESULTS	183
13.6.1 SERUM	183
13.6.2 SALIVA	183
13.6.3 NITRIC OXIDE	184
13.7 DISCUSSION	195
<u>CHAPTER FOURTEEN</u>	
GENERAL DISCUSSIONS	
14.1 SUMMARY	202
14.2 CONCLUSIONS	207
PRESENTATIONS AND PUBLICATIONS CONTAINING THE WORK UNDERTAKEN FOR THIS THESIS	208
REFERENCES	210

CHAPTER ONE

- Figure 1.1 Anatomy of the stomach 5

CHAPTER FIVE

- Figure 5.1: Bioactivation of *N*-nitroso compounds. 43
Figure 5.2: Enterosalivary recirculation of dietary nitrate in man. 53

CHAPTER SIX

- Figure 6.1: Nitrogenous fertiliser use in Western Europe and the age-standardised incidence rate per 100 000 person-years at risk (European standard population) for adenocarcinoma of the cardia and oesophagus in Scottish male. 60
Figure 6.2: Nitrosation chemistry in the upper gastrointestinal tract. 61

CHAPTER SEVEN

- Figure 7.1: The flexible CMA microdialysis probe. 67
Figure 7.2: The concentric microdialysis probe tip. 68
Figure 7.3: Univentor 864 microdialysis syringe pump. 69
Figure 7.4: Standard curve for nitrite assay using the Greiss reagent. 74
Figure 7.5: Standard curve for thiocyanate . 77

CHAPTER EIGHT

- Figure 8.1: Recovery of nitrite, thiocyanate, ascorbic acid and total vitamin C at different rates of perfusion. 84
Figure 8.2: Mean nitrite recoveries in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C. 91
Figure 8.3: Mean thiocyanate recoveries in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C. 91
Figure 8.4: Mean ascorbic acid recovery in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C. 92
Figure 8.5: Mean total vitamin C recovery in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C. 92

<u>LIST OF FIGURES</u>	<u>PAGE</u>
Figure 8.6: Mean nitrite recovery at pH 1.5 at different rates of perfusion in aqueous solutions and gastric juice.	95
Figure 8.7: Artificial stomach used in the dynamic experiments.	100
<u>CHAPTER NINE</u>	
Figure 9.1: Assembled Microdialysis Probes For Human Use.	105
<u>CHAPTER TEN</u>	
Figure 10.1: Schematic diagram of the positions of the microdialysis probes and the pH sensors.	113
Figure 10.2: Median serum nitrate before and after nitrate administration.	117
Figure 10.3: Median salivary nitrite before and after a nitrate administration.	118
Figure 10.4: Individual salivary and oesophageal nitrite concentrations before and after nitrate administration.	121
Figure 10.5: Individual salivary and oesophageal thiocyanate (SCN ⁻) concentrations before and after nitrate administration.	122
Figure 10.6: Concentrations of nitrite in the stomach before and after nitrate administration.	125
Figure 10.7: Concentrations of SCN ⁻ in the stomach before and after nitrate meal.	126
Figure 10.8: Concentrations of ascorbic acid in the stomach before and after nitrate administration.	127
Figure 10.9: Total vitamin C in the stomach before and after nitrate administration.	128
Figure 10.10: Graph showing nitrosation potential in different regions of the stomach before and after nitrate administration.	129
<u>CHAPTER ELEVEN</u>	
Figure 11.1: Positions of the microdialysis probes.	145
Figure 11.2: Median serum nitrate concentrations before and after nitrate administration.	146

<u>LIST OF FIGURES</u>	<u>PAGE</u>
Figure 11.3: Median salivary nitrite concentrations before and after nitrate administration.	147
Figure 11.4: Concentrations of nitrate, nitrite and thiocyanate in the upper aerodigestive tract before and after nitrate administration.	148
 <u>CHAPTER TWELVE</u>	
Figure 12.1: Positions of the microdialysis and pH probes in subjects with Barrett's oesophagus.	164
Figure 12.2: Median serum nitrate concentrations before and after nitrate administration.	165
Figure 12.3: Median salivary nitrite before and after nitrate administration.	166
Figure 12.4: Concentrations of nitrite in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.	167
Figure 12.5: Concentrations of thiocyanate in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.	168
Figure 12.6: Concentrations of ascorbic acid and in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.	169
Figure 12.7: Concentrations of total vitamin C in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.	170
Figure 12.8: Median (IQR) ascorbic acid to total vitamin C ratio before and after nitrate.	172
Figure 12.9: Ascorbic acid to nitrite ratio before and after nitrate administration.	173
 <u>CHAPTER THIRTEEN</u>	
Figure 13.1: Nitric oxide probe and nitric oxide meter (ISO-NO Mark II).	186
Figure 13.2: Serum nitrate before and after nitrate.	187
Figure 13.3: Saliva nitrite concentrations before and after nitrate.	188

<u>LIST OF FIGURES</u>	<u>PAGE</u>
Figure 13.4a: Simultaneous recording of pH and nitric oxide <i>before</i> nitrate administration in patient one.	189
Figure 13.4b: Simultaneous measurement of pH and nitric oxide concentration <i>after</i> nitrate administration in patient one.	190
Figure 13.5a: Simultaneous recording of pH and nitric oxide <i>before</i> nitrate administration in patient two.	191
Figure 13.5b: Simultaneous measurement of pH and nitric oxide <i>after</i> nitrate administration in patient two.	192
Figure 13.6a: Simultaneous recording of pH and nitric oxide <i>before</i> nitrate administration in patient three.	193
Figure 13.6b: Simultaneous recording of pH and nitric oxide <i>after</i> nitrate administration in patient three.	194
Figure 13.7: Intracellular effects of nitric oxide generated in the lumen of the upper gastrointestinal tract.	200

<u>LIST OF TABLES</u>	<u>PAGE</u>
<u>CHAPTER TWO</u>	
Table 2.1: Incidence of cardia adenocarcinoma (per 100 000 of the Population).	9
Table 2.2: Incidence of adenocarcinoma of the oesophagus (per 100000 of the population).	10
<u>CHAPTER FOUR</u>	
Table 4.1: Incidence of oesophageal adenocarcinoma in Barrett's oesophagus.	30
Table 4.2: Prevalence of oesophageal adenocarcinoma in oesophagectomies performed for high grade dysplasia.	31
<u>CHAPTER TEN</u>	
Table 10.1: Median (IQR) pH, nitrite, ascorbic acid, total vitamin C and thiocyanate concentrations in the different regions of the upper gastrointestinal tract before nitrate administration.	130
Table 10.2: Median (IQR) pII, nitrite, ascorbic acid, total vitamin C and thiocyanate concentrations in the different regions of the upper gastrointestinal tract following nitrate administration.	130
<u>CHAPTER TWELVE</u>	
Table 12.1: Nitrite, thiocyanate, ascorbic acid, total vitamin C, ascorbic acid / nitrite ratio and pH in the different regions of the upper GI tract in subjects with Barrett's oesophagus pre and post nitrate meal.	171

SUMMARY OF THESIS

1. A novel technique utilising the microdialysis technique to measure the concentrations of chemicals of nitrosation, nitrite, thiocyanate, ascorbic acid and total vitamin C in the lumen of the human upper gastrointestinal tract was developed and validated *in vitro*.
2. The recoveries of the chemicals of interest using the microdialysis probes were inversely proportional to the rate of perfusion of the microdialysis probes except for nitrite which was also dependent on the pH of the solution being sampled. We determined the optimal perfusion rate of the microdialysis probes as 0.15mL hr^{-1} . This perfusion rate enabled adequate volumes of microdialysis product to be obtained for subsequent analysis without reducing the recoveries of the chemicals by the microdialysis probe.
3. The microdialysis probe was accurate in sampling the chemicals of interest under dynamic conditions which mimicked the reaction of intermittent delivery of nitrite in swallowed saliva with gastric juice ascorbic acid under both high and low nitrite load.
4. Salivary nitrite in man is derived from the bacterial reduction of dietary nitrate which has undergone entero-salivary recirculation. In the human subjects without gastro-oesophageal reflux, high concentration of nitrite was detected in the saliva and the distal oesophagus following an oral nitrate load.
5. In subjects without gastro-oesophageal reflux, there was an increasing concentration gradient for ascorbic acid and total vitamin C at increasingly distal sites of the upper gastrointestinal tract whereas there was a decreasing concentration gradient for nitrite with virtually no nitrite detectable in the distal stomach. In the acidic stomach, the cardia was the site of greatest ascorbic acid oxidation and also the site of the lowest ascorbic acid to nitrite ratio both before and after an oral nitrate load indicating that the condition for the formation of carcinogenic *N*-nitrosamines was maximal at this anatomical site in subjects without gastro-oesophageal reflux.
6. Twenty to thirty percent of the nitrate in the saliva is reduced to nitrite by the bacteria within the oral cavity. The nitrate reduction appears to continue within the oesophagus leading to a greater recovery of nitrite to that in the

saliva in some subjects. No nitrate reduction was seen within the nasal cavity of healthy subjects.

7. In subjects with Barrett's oesophagus, concentration gradients for ascorbic acid, total vitamin C and nitrite showed a similar pattern to that of the subjects without gastro-oesophageal reflux. However, the ascorbic acid to total vitamin C ratio and ascorbic acid to nitrite ratio in the acidic environment were both lowest within the Barrett's segment suggesting that the oxidative stress and the nitrosation potential were greatest within this anatomical site during periods of acid reflux.
8. Within the Barrett's segment the nitrite concentrations fell greatly compared to that of the squamous oesophagus which was attributed to the conversion of acidified nitrite by the gastric juice ascorbic acid to nitric oxide. Using a miniaturised nitric oxide sensor and measuring the nitric oxide generation in real-time we were able to demonstrate nitric oxide generation of up to $50\mu\text{M}$ when saliva came into contact with acidic gastric juice within the Barrett's segment during periods of gastro-oesophageal reflux.

CHAPTER ONE

ANATOMY

1.1 Anatomy of the oesophagus

The oesophagus is a hollow tubular organ extending from the pharynx to the cardiac region of the stomach measuring approximately 25cm in length. The distal 2cm of its length resides in the abdominal cavity. The oesophagus begins proximally where the skeletal muscles, inferior pharyngeal constrictor merges with the cricopharyngeus and forms the upper oesophageal sphincter. The body of the oesophagus occupies the posterior mediastinum and is composed of inner circular and outer longitudinal layers of smooth muscles. The oesophagus passes through the hiatus in the diaphragm formed by the right crus of the diaphragm and leaves the thoracic cavity. The distal 2 to 4 centimetres of thickened circular smooth muscle forms the lower oesophageal sphincter which sits within the diaphragmatic hiatus. The position of the lower oesophageal sphincter is maintained within the hiatus by the phrenico-oesophageal ligament, a continuation of the transversalis fascia of the diaphragm.

The oesophageal mucosa is formed by non-keratinized stratified squamous epithelium consisting of three functionally distinct layers: the stratum corneum, the outermost layer of flattened (anucleate) cells connected laterally by tight junctions with rich lipid matrix within the intercellular spaces and act as a permeability barrier. The middle layer, stratum spinosum contains metabolically active cells with numerous intercellular connections through desmosomes which give them the characteristic appearance and provides structural integrity of the tissue. The stratum geminativum or the basal layer consists of cuboidal cells occupying up to 15% of the epithelial thickness and is capable of replication and the overlying cellular layers are derived from the basal cells.

The junction between the oesophageal mucosa and that of the stomach is readily seen endoscopically as the pale pink mucosa of the oesophagus abruptly changes to the salmon pink mucosa of the stomach. The demarcation line is the ora serrata, also known as the Z-line and under normal circumstances coincides with the proximal extent of the normal gastric mucosa.

Below the epithelium lies the lamina propria which is made up of a loose network of connective tissue containing blood vessels. The lamina propria forms a digitating invagination into the epithelium to form rete pegs, otherwise known as the dermal papillae. The muscularis mucosa is a thin layer of smooth muscle which separates the lamina propria from the submucosa. The function of this layer

is unclear. The submucosa is comprised of a dense network of connective tissue. Contained within this layer are blood vessels, lymphatic channels, oesophageal glands and the Meissner's plexus.

1.2 Anatomy of the stomach

The stomach is a J-shaped organ that is in continuity with the oesophagus proximally and the duodenum distally. The function of the stomach is to act as a reservoir for ingested food and to initiate the digestive processes, both physical and enzymic, and release the partially digested chyme in aliquots into the duodenum.

The stomach is divided into four regions (Fig. 1.1). The cardia is the small, ill-defined area just adjacent to the junction with the oesophagus and the most fixed portion of the stomach. The cardiac incisure, which is formed by the acute angle between the left border of the oesophagus and the stomach, demarcates the upper margin. A line drawn horizontally across the stomach from the cardiac incisure separates the fundus above from the body below. The greater curvature commences at the cardiac incisure and continues along the left border of the stomach. The lesser curvature is continuous with the right margin of the oesophagus. The incisura angularis is located two-thirds along the lesser curvature and is demarcated by a sharp indentation. The incisura indicates the junction between the body and the gastric antrum. The antrum, however, is ill-defined anatomically along the greater curve. The gastric antrum extends to the pylorus which leads to the duodenum.

The outer longitudinal muscle layer is continuous with that of the oesophagus and duodenum. The middle layer consists of the circular layer which forms a muscular ring at the pylorus, the pyloric sphincter. An incomplete layer of oblique fibres form the innermost muscular layers of the stomach, which course over the gastric fundus and the anterior and the posterior walls of the stomach.

The surface of the mucosa has numerous small elevations created by shallow furrows which are further invaginated to form the gastric pits, or foveolae, and is continuous with the gastric glands at their base. The surface epithelium covering the stomach, including the foveole is columnar epithelium with basal nuclei and cytoplasmic granules containing mucin. There are regional differences in the cell populations comprising the gastric glands in the different regions of the

stomach. The mucus secreted by these cells protects the epithelial cells from the noxious injury from the acid and enzymes in the gastric secretions.

The cardia glands comprise less than 5% of the total gastric gland. The majority of the glands in this region consist mainly of mucous cells with small numbers of undifferentiated cells and endocrine cells. The main secretory products from the cardia glands are mucin and group II pepsinogen. Oxyntic glands comprise 75% of the gastric glands and are located in the fundus and the corpus of the stomach. Parietal, chief, endocrine, mucous neck and undifferentiated cells make up the oxyntic gland. The parietal cells secrete hydrochloric acid and intrinsic factor which is necessary for the absorption of vitamin B₁₂. In close association with the parietal cell are the mucous neck cells which contain acidic and sulphated mucin which are different from the surface mucous cells which secrete neutral mucin. It is thought that the mucous neck cell functions as stem cells for surface mucous, chief, parietal and endocrine cells. The chief cells, also known as the zymogen cells, secrete pepsinogen. The endocrine, or enteroendocrine cells, are scattered among the oxyntic glands and include the D cells, which secrete somatostatin and enterochromaffin-like cells which secrete histamine and serotonin depending on their silver staining characteristics. The pyloric glands comprise the remaining 20% of the gastric glands. The pyloric glands are composed of mucous, endocrine and small numbers of parietal cells which secrete pepsinogen II. The enteroendocrine cells, G cells which secrete gastrin and D cells which secrete somatostatin are also found in these glands.

1.3 Physiological functions of gastric acid.

Gastric acid has several important functions. It is important for activating pepsinogen to start the digestion of protein which is also important in releasing vitamin B₁₂ complexed to food protein. Gastric acid facilitates absorption of inorganic iron absorption in the duodenum. It stimulates pancreatic bicarbonate release through the action of secretin but also inhibits antral gastrin secretion by negative feedback. Gastric acid also has an antimicrobial effect and achlorhydria leads to bacterial overgrowth in the gastric lumen.

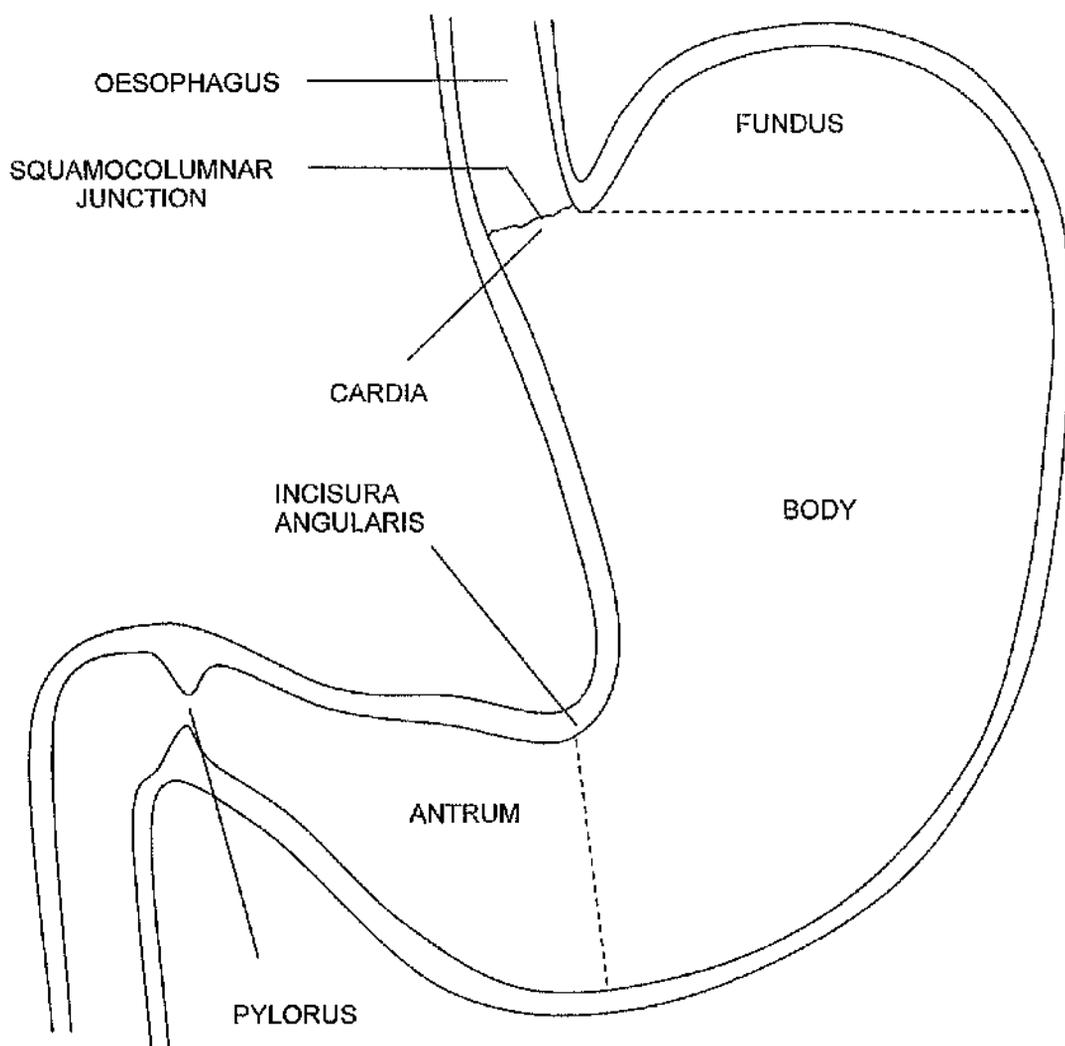


Figure 1.1: Anatomy of the stomach.
The stomach is divided into four anatomical regions: the cardia, fundus, body and the antrum.

CHAPTER TWO

EPIDEMIOLOGY OF CARDIA AND OESOPHAGEAL ADENOCARCINOMA

2.1 Introduction

In contrast to the falling incidence in the more distal gastric cancers, the incidences of adenocarcinoma of the oesophagus and cardia have increased dramatically in many developed countries in the last two decades.

Cardia cancer forms almost 50% of the gastric cancers diagnosed in white American men. The causes for the increases in these cancers are unclear.

In this section an overview of the epidemiology of the cardia and oesophageal adenocarcinoma will be presented.

2.2 Incidence of adenocarcinoma of the cardia

Since the late 1970s, the incidence of adenocarcinoma of the gastric cardia increased dramatically in the U.S.A. ^(1, 2, 3). This is in striking contrast to the falling incidence of non-cardia gastric cancer in U.S.A. and other countries ^(3, 4, 5, 6, 7, 8, 9). In the most recent study of white males in USA, the age-adjusted annual incidence rate of cardia adenocarcinoma increased from 2.1 per 100 000 during the period 1974 ~ 1976 to 3.3 per 100 000 during the period 1992 ~ 1994. The same trend is also seen for the black males with the annual incidence rising from 1.0 per 100 000 to 1.9 per 100 000 over the same period of time ⁽³⁾. The annual incidence rates for non-cardia gastric cancer has fallen from 5.1 per 100 000 for the period 1974 ~ 1976 to 3.7 per 100 000 during 1992 ~ 1994 in American white males. In the American blacks there was an initial increase in the incidence of non-cardia gastric cancer during the period 1974 ~ 1981 from 10.9 per 100 000 to 13.4 per 100 000 and declined again to 10.9 per 100 000 during the period 1992 ~ 1994 ⁽³⁾.

The incidence of cardia cancer in women is lower than that of males but the increasing trend seen in the females has been of a similar magnitude. In the study reported by Devesa, the incidence of cardia cancer in white women in USA increased from 0.3 per 100 000 during 1974 ~ 1976 to 0.6 per 100 000 during 1992 ~ 1994 ⁽³⁾ (table 2.1). Increasing trends for cardia cancer have been reported worldwide including Netherlands ⁽¹⁰⁾, Sweden ⁽⁴⁾, Denmark ⁽¹¹⁾, England ^(7, 12), Scotland ⁽⁸⁾ and Japan ⁽⁹⁾. All the above studies, except Powell et al studying the period 1962 ~ 1981, reported a falling incidence in the non-cardia gastric cancer.

Not all countries have noted an increase in cardia cancer. Finland ⁽¹³⁾ and Vaud ^(14, 15) in Switzerland have yet to demonstrate a significant increase in cardia cancer. Calvados in France ⁽¹⁶⁾ have seen a declining trend in the incidence. There is a suggestion more recently that the incidence of cardia cancer may have peaked in the USA ⁽³⁾.

As a result of the falling incidence in the non-cardia gastric cancer and the increasing incidence of cardia cancer, the proportion of cardia cancer contributing to the diagnosis of gastric cancer as a whole has increased. Cardia cancer currently forms approximately 13 to almost 50% ^(3, 7, 10, 14, 17) of the gastric cancer diagnosed in the western nations.

2.3 Incidence of adenocarcinoma of the oesophagus.

The incidence of the adenocarcinoma of the oesophagus has increased more rapidly than that of the cardia (see table 2.2). Among the white males in USA, the annual incidence of adenocarcinoma of the oesophagus rose from 0.7 per 100 000 population during 1974 - 1976 to 3.2 per 100 000 during 1992 - 1994, an increase of more than 350 % overtaking squamous carcinoma as the most common oesophageal cancer since 1990 ⁽³⁾. The incidence of adenocarcinoma of the oesophagus in the white female population also increased from 0.1 per 100 000 to 0.4 per 100 000 in the same study. Increasing incidences of oesophageal adenocarcinoma have also been reported in England and Wales ⁽¹⁸⁾, Scotland ⁽⁸⁾, Netherlands ⁽¹⁰⁾, Switzerland ⁽¹⁹⁾, Norway ⁽⁵⁾, Denmark ⁽¹¹⁾ and other European countries ⁽²⁰⁾ with annual increases in incidence exceeding 20 % in some regions. In some countries, oesophageal adenocarcinoma has overtaken squamous carcinoma of the oesophagus as the most common cancer affecting the male oesophagus ^(3, 8, 18).

STUDY (Country)	PERIOD	SEX	RACE	INCIDENCE per 100 000		
				START	END	% CHANGE
Powell ⁽¹²⁾ (W. Midlands, UK)	1962-1981	*	*	0.75	2.0	+168
Hansson ⁽⁴⁾ (Sweden)	1970-1985	M	*	1.9	3.0	+58
		F	*	0.5	0.8	+60
Blot ⁽²⁾ (USA)	1976-1987	M	White	2.4	3.2	+33
			Black	1.2	1.7	+42
		F	White	0.42	0.56	+33
			Black	0.45	0.6	+33
Bytzer ⁽¹¹⁾ † (Denmark)	1970-1991	*	*	1.6	2.9	+81
Dodaran ⁽¹⁸⁾ (England & Wales)	1971-1993	M	*	2.4	6.0	+147
		F	*	0.6	1.6	+151
Devesa ⁽³⁾ (USA)	1974-1994	M	White	2.1	3.3	+57
			Black	1.0	1.9	+90
		F	White	0.3	0.6	+100
			Black	NA	NA	NA
Kocher ⁽⁷⁾ † (England)	1960-1996	M	*	1.3	2.4	+85
		F	*	0.3	0.5	+75
Silvo ⁽¹³⁾ (Finland)	1976-1995	M	*	2.1	2.1	0
		F	*	0.6	0.47	-22
Brewster ⁽⁸⁾ (Scotland)	1977-1996	M	*	3.1	5.2	+68
		F	*	1.0	1.4	+40
Levi ⁽¹⁵⁾ (Swiss)	1976-1997	M	*	3.1	3.2	+3
		F	*	0.5	0.1	-80

Table 2.1: Incidence of cardia adenocarcinoma (per 100 000 of population).

Key: Start: beginning of study period. End: end of study period.

includes fundus

† age-standardised incidence rate per 100 000 world population

STUDY (country)	PERIOD	SEX	RACE	INCIDENCE per 100 000		
				START	END	% CHANGE
Powell ⁽¹²⁾ (W. Midlands, UK)	1962-1981	*	*	0.14	0.63	+350
Bytzer ⁽¹¹⁾ # (Denmark)	1970-1991	*	*	0.3	2.3	+666
Hansen ⁽⁵⁾ † (Norway)	1958-1992	M	*	0.1	0.8	+700
		F	*	0.0	0.1	>100
Dodaran ⁽¹⁸⁾ (England & Wales)	1971-1993	M	*	1.5	5.2	+252
		F	*	0.4	1.1	+167
Devesa ⁽³⁾ (USA)	1974-1994	M	White	0.7	3.2	+357
			Black	0.4	0.6	+50
		F	White	0.1	0.4	+300
			Black	NA	NA	NA
Brewster ⁽⁸⁾ (Scotland)	1977-1996	M	*	3.9	8.0	+105
		F	*	1.2	2.3	+92
Wijnhoven ⁽¹⁰⁾ (Netherlands)	1978-1996	M	*	0.5	2.2	+340
		F	*	0.1	0.6	+514
Levi ⁽¹⁹⁾ (Swiss)	1976-1998	M	*	0.5	1.9	+280
		F	*	0.1	0.2	+100

Table 2.2: Incidence of adenocarcinoma of the oesophagus (per 100000 of the population).

Key: Start: beginning of study period. End: end of study period.

† adjusted according to world standard population, not age-adjusted

age and sex adjusted

2.4 Sex distribution of non-cardia gastric cancer, adenocarcinoma of the cardia and oesophagus.

Gastric cancer has a typical male to female ratio of 2:1 ^(2, 5, 7, 8, 14). Cardia cancer has a male to female ratio of more than 3:1 ^(2, 4, 7, 8, 10, 14, 18). Some studies have reported even greater male preponderance, particularly in the younger population. For example, Hansson noted the male to female ratio of 7:1 in those who were aged less than 50 ⁽⁴⁾. Similar finding was noted by Levi where the male to female ratio was also higher in the younger age groups with subjects 50 – 59 years had a ratio of 11.9 compared to 7.2 for 60 – 69 years and 7 for 70 – 79 years and 3.4 for 80 + years ⁽²¹⁾

The sex ratio of adenocarcinoma of the oesophagus, like that of the cardia cancer, is male predominant with a typical ratio of 3:1 or more ^(2, 5, 7, 8, 11, 18, 19).

2.5 Race and adenocarcinoma of the cardia and oesophagus.

Cardia and oesophageal adenocarcinoma are diseases which afflict predominantly the white population. In the U.S.A., adenocarcinoma of the cardia is approximately twice as common in the white men as in the black men; oesophageal adenocarcinoma is five times more common in the whites than in the blacks ⁽³⁾. In contrast, squamous carcinoma of the oesophagus and non-cardia gastric cancer were more common in the blacks being approximately six times and three times more common in the blacks respectively ⁽³⁾.

In Singapore, the Chinese males have a higher cumulative rate of cardia and oesophageal adenocarcinoma than the Indian or the Malay males. Oesophageal adenocarcinoma was twice as common in Chinese males in Hong Kong compared to Chinese males in Singapore. Jews born in USA or Europe were more likely to be afflicted with cardia cancer than Jews born in Israel ⁽²²⁾. These studies suggested that the genetic predisposition for developing cardia and oesophageal adenocarcinoma differed between racial groups and that the risk of developing these cancers was modified by environmental factors.

2.6 Socioeconomic status and cancers of the upper gastrointestinal tract.

Socioeconomic status appears to have an influence upon the risk for the different cancers of the upper gastrointestinal tract. Both squamous oesophageal carcinoma and non-cardia gastric cancers are more common in those of the lower socioeconomic status. There is an inverse association between income, education, and social deprivation for both these tumors^(8, 23, 24, 25, 26, 27, 28, 29, 30). Part of the inverse association between social class and non-cardia gastric cancer can be explained by *Helicobacter pylori* (*H. pylori*) infection which is more prevalent in the lower social class^(31, 32). Adverse socioeconomic conditions⁽²⁸⁾ including past domestic overcrowding at home during childhood⁽³³⁾ have been associated with an increased risk of gastric cancer mortality rates and has been associated with a higher *H. pylori* infection rate in such population⁽³⁴⁾.

There is less socioeconomic divide in adenocarcinoma of the cardia. Some studies have suggested an increased risk for cardia cancer in people in higher social class. In a study in U.S.A., cardia adenocarcinoma was non-significantly greater in young men who had some college education by 30% compared to those who did not receive any high school education⁽³⁵⁾. A more recent study showed that 81 % of gastric cardia cases had at least high school education compared to 58 % in distal gastric cancer cases⁽³⁶⁾. A study in West Midlands suggested that in males there were 16% more cardia cancer cases and 27% less antral carcinoma than expected from the distributions of cancer affecting all sites in social class 1 and 2. Conversely, in social class 4 and 5 there were 10% less cardia and 8% more antral cancer than expected. Both differences were highly statistically significant ($p < 0.001$)⁽¹²⁾. A Scottish study also suggested that men who were most deprived also had lower incidence rates for cardia cancer but this was not seen for women⁽⁸⁾. Other studies have shown a non-significant reduction in risk in high income earners and those who had better education^(23, 37).

Oesophageal adenocarcinoma, like adenocarcinoma of the cardia, does not have a clear association between socioeconomic status. No association between level of education and oesophageal adenocarcinoma has been noted^(23, 30, 38). Studies between social class and oesophageal adenocarcinoma are also inconclusive. Powell noted a higher incidence of oesophageal adenocarcinoma in

men who were social class 1 or 2 ⁽¹²⁾. A study of women in Scotland showed an increasing trend in those who were in the lower social class but was not significant statistically ⁽³⁹⁾. A further study in Scotland found no association between deprivation category and oesophageal adenocarcinoma in both men and women ⁽⁸⁾. There were two studies in USA which suggested a reduced risk in those who were high income earners ^(23, 40).

A study conducted in Maryland, U.S.A., showed an increased mortality from gastric cardia and cardio-oesophageal cancer in administrative jobs (Women: OR (95% CI) 3.9 (1.5 – 9.8); Men: OR 1.4 (1.1 – 1.9)) and in health professionals (Women: OR 1.8 (0.6 – 5.3); Men 2.4 (1.3 – 4.6)) ⁽⁴¹⁾.

In summary, there is ample evidence to suggest that squamous carcinoma of the oesophagus and non-cardia gastric carcinoma occur more commonly in the less privileged whereas adenocarcinoma of the oesophagus and gastric cardia appear to show no such trend and there are some suggestions that the two cancers affect the upper middle class, white population.

CHAPTER THREE

AETIOLOGY OF ADENOCARCINOMA OF THE CARDIA AND OESOPHAGUS

3.1 Introduction

The causative factors for the development of adenocarcinoma of the cardia and oesophagus are unknown. There are a several factors which have been reported to be of aetiological importance in cardia and oesophageal adenocarcinoma. Socioeconomic status was discussed in the previous chapter. Others include smoking, body habitus, gastro-oesophageal reflux, *Helicobacter pylori*, Barrett's oesophagus and diet. The following sections discuss each of these factors in turn and their relevance in the aetiology of the cardia and oesophageal adenocarcinomas.

3.2 Smoking.

Smoking as a major risk factor for the development of squamous carcinoma of the oesophagus has been well established^(42, 43). The risk for developing squamous oesophageal carcinoma being elevated several fold compared to non-smokers and both dose and duration of smoking increases the risk for developing squamous oesophageal carcinoma.^(19, 23, 43, 44)

Smoking does not appear to be a strong risk factor in non-cardia gastric cancer. Some studies have shown a significant association between smoking^(45, 46, 47), others have shown only a weak association with less than a two-fold risk^(23, 36, 48, 49) or no association at all^(24, 50, 51). In contrast, the risk for both adenocarcinoma of the cardia and the oesophagus is at least two-fold in smokers compared to non-smokers and both duration and amount of cigarettes smoked increases the risk^(23, 36, 38, 44, 46, 49, 52). The increased risk of oesophageal adenocarcinoma in smokers may only be applicable to men as some authors have shown that smoking does not appear to be an important risk factor for developing adenocarcinoma of the oesophagus and cardia in women^(36, 39, 46).

Thus, the association with smoking is strongest in squamous carcinoma of the oesophagus with the risk being increased several-fold, followed by adenocarcinoma of the cardia and oesophagus, with at least a two-fold increase in risk and finally distal gastric cancer which appears to have only a weak association with smoking.

3.3 Body Size.

Body mass is a major risk factor for adenocarcinoma of the oesophagus with numerous studies indicating increased risk for adenocarcinoma of the oesophagus in individuals with higher body mass index (BMI) with up to several-fold increase in risk compared to those who are lean^(19, 36, 39, 53, 54, 55). Even when the body mass size was studied many years prior to the diagnosis of cancer the positive association was still present^(36, 39, 54). For example, in a well-conducted Swedish population-based case-control study, those classified as obese (BMI > 30kg/m²) 20 years before the diagnosis of cancer had a 16-fold increase in risk for developing oesophageal adenocarcinoma compared to those with a BMI of less than 22. A clear dose-response increase in risk was seen with increasing BMI⁽⁵⁴⁾. Obesity appears to be a greater risk factor for oesophageal adenocarcinoma in those diagnosed younger than 50 years as well as in non-smokers⁽⁵³⁾.

The association between body mass index and cardia adenocarcinoma is weaker than that for oesophageal adenocarcinoma. Increasing BMI increases the risk for developing cardia adenocarcinoma up to four-fold^(36, 53, 54, 56). If we take the example of the Lagergren study, the multivariate-adjusted odds ratio for obese patients (BMI >30 kg/m², 20 years prior to interview) the odds ratio was four-fold for cardia adenocarcinoma compared to the 16-fold increase for oesophageal adenocarcinoma. BMI also appears to be a greater risk factor for younger subjects than older subjects for cardia adenocarcinoma⁽⁵⁶⁾.

In contrast to both adenocarcinoma of the oesophagus and cardia, body size appears have no clear association for both squamous oesophageal carcinoma and distal gastric cancer with some studies showing inverse associations with BMI or no association at all^(19, 25 - 27, 36, 50, 53, 55, 56).

3.4 Gastro-oesophageal reflux.

Gastro-oesophageal reflux is a common symptom with almost 40 % of the American population suffering from reflux symptoms with more than 13 % of the population reporting heartburn at least once a week ⁽⁵⁷⁾.

A study from U.S.A. indicated that the risk of developing cardia and oesophageal adenocarcinoma was increased by two-fold in the presence of symptoms ⁽⁵⁸⁾. This study however did not differentiate between the two tumors. A Swedish study indicated that those who had reflux symptoms at least once a week, excluding symptoms which developed within five years of the diagnosis of cancer to exclude reverse causality, had almost an 8-fold increase in risk of developing oesophageal adenocarcinoma, a two-fold increase in risk for cardia adenocarcinoma and no association between reflux symptoms and squamous oesophageal carcinoma. For those individuals with more frequent, more severe and longer duration of symptoms the risk for oesophageal adenocarcinoma increased to 43-fold and only to just over four-fold for cardia adenocarcinoma which indicated that gastro-oesophageal reflux was an important risk factor for oesophageal adenocarcinoma but much less important for cardia adenocarcinoma ⁽⁵⁹⁾.

The presence of Barrett's oesophagus predisposes to the development of adenocarcinoma of the oesophagus. Barrett's metaplasia is a consequence of severe acid reflux into the oesophagus. This entity will be discussed in a separate chapter.

3.5 *Helicobacter pylori* and cancers of the upper gastrointestinal tract.

The association between *H. pylori* and gastric cancer is well known. It is classified as a class I carcinogen by the World Health Organisation. Individuals positive for *H. pylori* IgG more than 10 years prior to the diagnosis of cancer have approximately a six-fold increase for developing gastric cancer ⁽⁶⁰⁾. The risk increases to 21-fold when cytotoxin associated antigen A (CagA) antibodies are used to identify those infected with *H. pylori* by identifying those who are false negative on *H. pylori* IgG ⁽⁶¹⁾.

For many years, however, non-cardia gastric cancer incidence has been falling worldwide and this is true even in countries with high incidence of non-cardia gastric cancer⁽³⁻⁹⁾. This decline in incidence has been largely attributed to the fall in the prevalence of *H. pylori* infection^(62, 63, 64) and the resultant fall in chronic gastritis and atrophy⁽⁶⁵⁾.

In contrast to the increased risk for developing non-cardia gastric cancer, an inverse association of *H. pylori* infection, in particular those with the Cag A positive *H. pylori*, and oesophageal and cardia adenocarcinoma have been reported. A case-control study in Norway demonstrated a negative association between *H. pylori* infection and cardia cancer with an odds ratio of 0.4 for cardia cancer in subjects positive for *H. pylori* IgG⁽⁶⁶⁾. In contrast, a Swedish and an American study suggested that there was no association between *H. pylori* antibody status and cardia cancer with an odds ratio between cases and controls of 0.92 and 0.94 in the respective studies^(67, 68). However, in both studies odds ratio of *H. pylori* infection in patients with cardia cancer was significantly lower than in non-cardia gastric cancer consistent with other studies^(69, 70). In a recent study, a non-significant risk reduction was seen in both cardia and oesophageal adenocarcinoma in those were positive for *H. pylori* IgG (OR 0.7, 95 % CI 0.4-1.1) but when analysed by Cag A status, the risk for cardia or oesophageal adenocarcinoma was significantly lower than population-based controls (OR 0.4, 95 % CI 0.2-0.8)⁽⁷¹⁾.

In a prospective study, Weston demonstrated that the prevalence of *H. pylori* in patients with Barrett's oesophagus with high-grade dysplasia (14.3 %) and or adenocarcinoma (15 %) were significantly lower compared to those with Barrett's oesophagus with low-grade dysplasia (36.2 %), Barrett's oesophagus without dysplasia (35.1 %) or those with gastro-oesophageal reflux disease (44.2 %)⁽⁷²⁾. Another study observed that the *H. pylori* infection was just as common in control population undergoing gastroscopy for symptoms other than gastro-oesophageal reflux (42 %) compared to Barrett's patients without dysplasia (24 %), Barrett's patients with dysplasia or adenocarcinoma (37 %) and cardia adenocarcinoma (21 %) (p = non-significant for each compared to control). When analysed according to Cag A status, 53 % of the controls were infected with Cag A strains compared to 10% in patients with Barrett's oesophagus with or without dysplasia / adenocarcinoma (p = 0.005). Thirty three percent of those with cardia

adenocarcinoma had Cag A strains but because of the small number of cardia cancer cases infected with *H. pylori* (n = 3) there was no statistical significance between this group and controls ⁽⁷³⁾.

These studies suggest that *H. pylori* infection appears to be protective for gastro-oesophageal junction cancers in particular those with Cag A seropositivity. The mechanism for this protective effect is unclear although greater inflammation and gastric atrophy being more common in those with Cag A strain infection led to the speculation that perhaps reduced acid secretion may protect against cancers which are associated with gastro-oesophageal reflux ^(71, 74). However, a recent study using pepsinogen I as a marker for gastric atrophy suggested that there was no association between gastric atrophy and oesophageal adenocarcinoma but for adenocarcinoma of the cardia both atrophy and elevated pepsinogen I levels were associated with increased risks (OR of 4.5 and 2.9 respectively) ⁽⁷⁵⁾. Re-analysing the data by subdividing cardia tumors, an increased risk for tumors above or on the gastro-oesophageal junction was noted for those with elevated pepsinogen I level, which was associated with gastro-oesophageal reflux. Tumors below the junction demonstrated an increased risk in those who had a low pepsinogen I level consistent with atrophy ⁽⁷⁵⁾. The latter tumor is likely to be positively associated with *H. pylori* atrophic gastritis; the former tumor being similar to oesophageal adenocarcinoma, is negatively associated with *H. pylori* and likely to be positively associated with gastro-oesophageal reflux ⁽⁷⁶⁾.

3.6 Diet and adenocarcinoma of the upper gastrointestinal tract.

Diet has been long viewed as being important in the aetiology of many cancers including cancers of the upper alimentary tract as nutrients ingested first come into contact with this anatomical region and the process of digestion begins. The topic of diet and upper gastrointestinal tract cancer is beyond the scope of this thesis. An overview of the literature relevant to this thesis will be discussed.

One of the most consistent findings about diet and non-cardia gastric cancer is the negative association with intake of vegetables and fruits. Consumption of raw vegetables and fruit reduces the relative risk for non-cardia gastric cancer by up to 70% ^(48, 50, 77, 78, 79).

For cardia cancer, the protection offered by vegetables and fruit are less convincing than that for non-cardia gastric cancer. Some studies have demonstrated a protective effect of fresh vegetables for cardia cancer (OR 0.4 - 0.6) ^(46, 50, 80) whereas others did not find a protective effect of vegetables for cardia cancer ⁽⁸¹⁾. The protective effect of fruit for cardia cancer was demonstrated in some studies (OR 0.2 - 0.5) ^(50, 80) whereas others have shown no significant protective effect ^(35, 46, 81). Vegetables (OR 0.45 - 0.6) ^(30, 81, 82) and fruit (OR 0.2 - 0.5) ^(30, 39) both appear to protect from oesophageal adenocarcinoma. Consumption of dietary fibre has also been associated with a significantly reduced risk for non-cardia gastric cancer, oesophageal and cardia adenocarcinoma ^(82, 83, 84, 85).

Protein and fat intake have been positively associated with non-cardia cancer ^(84, 86, 87), cardia and oesophageal adenocarcinoma ^(50, 84). The risk of cancer varies depending on the source of the protein and fat; animal protein or fat have been shown to increase risk whereas that of plant origin appear to have little effect or even a protective effect ^(84, 86).

Consumption of ascorbic acid (vitamin C) has been consistently shown to reduce the risk for non-cardia gastric cancer by up to 67% ^(78, 80, 83, 84, 86, 87, 88). Ascorbic acid reduced the risk from cardia and oesophageal adenocarcinoma by up to 70% and 60% respectively ^(50, 80, 82, 84); others have shown no such protective effect for both these tumors ⁽⁸⁹⁾.

Beta-carotene reduces the risk for non-cardia gastric cancer by up to 67% ^(29, 80, 83, 84, 87). However when adjusted for ascorbic acid, α -tocopherol and nitrates, the protective effect of β -carotene was lost ⁽⁸⁷⁾. Beta-carotene was protective from cardia cancer in only one of four studies ^(50, 80, 84, 89). Beta-carotene appears to be protective for oesophageal adenocarcinoma ⁽⁸⁴⁾, perhaps only in those with reflux symptoms ⁽⁸⁹⁾.

Alpha-tocopherol reduced the risk for non-cardia gastric cancer by up to 48% when unadjusted for other antioxidants ^(80, 84, 86, 87). When adjusted for ascorbic acid, β -carotene and nitrate, the protective effect of α -tocopherol was lost ⁽⁸⁷⁾. Interestingly when α -tocopherol intake was examined in relation to ascorbic acid intake, the protective effect α -tocopherol could only be seen in those with middle and the highest tertile of ascorbic acid intake ⁽⁸⁶⁾ suggesting that ascorbic acid is important as a co-factor for α -tocopherol to exert its antioxidant effect in

preventing non-cardia gastric cancer. Alpha-tocopherol does not appear to protect from either cardia or oesophageal adenocarcinoma^(50, 80, 84, 89).

Nitrate was a source of great interest in the 1970s and 1980s as a potential precarcinogen in gastric carcinogenesis. The major source of nitrate in man is derived from the ingestion of vegetables; cured meat and some water supplies are also known sources of nitrate but contain significantly less than vegetables⁽⁹⁰⁾.

A number of studies have suggested some association between high nitrate concentrations in the drinking water and gastric cancer^(91, 92, 93).

The study in Columbia demonstrated that intestinal metaplasia and chronic atrophic gastritis were more common in the inhabitants of areas with high gastric cancer risk than in low risk areas. However, in the high risk areas more subjects consuming surface water had intestinal metaplasia and chronic atrophic gastritis compared to well users (in which nitrate content was much higher) which suggested that higher nitrate consumption may be associated with gastric cancer but that nitrate itself was not responsible for these precursor lesions⁽⁹²⁾. Others have shown no association between nitrate in the drinking water and gastric cancer but the levels of nitrate found in the drinking water were low in both these studies^(94, 95).

As for dietary nitrate and gastric cancer, Forman and colleagues reported that the salivary nitrate and nitrite concentrations in areas of low gastric cancer incidence were significantly higher than those of areas with high gastric cancer incidence which reflected nitrate intake⁽⁹⁶⁾. A number of studies reported an inverse association between gastric cancer and dietary nitrate^(83, 97, 98). More recent studies showed that dietary nitrate ingestion 20 years prior to the diagnosis of gastric cancer was associated with a significantly reduced risk (OR 0.55) but multivariate analysis showed that only vitamin C intake showed a significant protective effect suggesting a confounding effect of other dietary constituents⁽⁸⁷⁾. Others have shown no association between dietary nitrate and gastric cancer^(25, 88, 95).

There appears to be little published evidence regarding nitrite ingestion and the risk of gastric cancer. Risch and Mayne in separate studies reported a significantly increased risk for gastric cancer with an adjusted OR of 2.6 and 1.6 respectively^(83, 84). Other studies have shown no statistically significant association between nitrite ingestion and gastric cancer^(25, 87, 95, 98, 99). An

increased risk of gastric cancer with increasing nitrite ingestion for a given level of protein intake (low, medium and high) has been reported ⁽⁸⁶⁾.

Nitrate and nitrite do not appear to be associated with an increased risk for cardia cancer in two published studies ^(50, 84) and a single study suggested that nitrite alone was not a risk factor for oesophageal adenocarcinoma ⁽⁸⁴⁾. However, the study by Mayne showed that low vitamin C intake in combination with a high nitrite intake had a 2-fold and 3-fold increase in risk for cardia and oesophageal adenocarcinoma respectively compared to those with high vitamin C and low nitrite intake ⁽⁸⁴⁾. Such finding may be in keeping with nitrosamine formation within the acidic environment of the stomach in those with low vitamin C and high nitrite intake.

3.6.1 Summary of diet and upper GI cancer.

There are important limitations to consider when interpreting dietary studies. Firstly, many studies have assessed recent dietary habits and used this data to calculate the association between nutrients and cancer. The accepted time interval between exposure and carcinogenesis is said to be around 20 years and therefore recent dietary habits may not necessarily reflect intake 20 years previously. Second, the dietary recall for many years previously is unlikely to be precise and may distort the result. Thirdly, preference of a certain food group is associated with a reduced intake of another food group e.g. high meat intake is associated with a low vegetable and fruit intake ⁽³⁰⁾. Therefore a positive association between meat and cancer may be a marker of poor vegetable and fruit intake rather than meat being positively associated with cancer 'per se'. Fourthly, many different nutrients are found in a single food item and it may be difficult to ascribe an association between cancer and the nutrient in question as other nutrients may confound the effect of a single nutrient which may mask or magnify its effect. For example we have seen that nitrate appears to be protective taken at face value but since majority of dietary nitrate is derived from vegetables, nutrients such as ascorbic acid is also found which is also protective from cancer and in fact when nitrate is adjusted for ascorbic acid the protective effect disappears. This suggests that the apparent protective effect of nitrate is due to the concomitant presence of ascorbic acid and other micronutrients. Many of the studies have not made

adjustments for the myriad of nutrients that may be present together which may have therefore incorrectly ascribed the nutrient in question being positively or negatively associated with cancer.

With the above limitations borne in mind some general conclusions can be made. Fibre, ascorbic acid, α -tocopherol and β -carotene appear to be consistently shown to protect from non-cardia gastric cancer and in turn these nutrients are found in abundance in fruits and vegetables and hence these food groups also consistently shown to be inversely associated with the risk for non-cardia gastric cancer. Data on cardia and oesophageal adenocarcinoma are still limited but ascorbic acid and vitamin E may lack the protective effect seen in distal gastric adenocarcinoma according to the available evidence but more data are required before firm conclusions can be made. Evidence for nitrates suggests that it is protective for non-cardia gastric adenocarcinoma. As mentioned earlier, this is likely to be due to the confounding effects of other micronutrients found in vegetables and nitrate on its own is unlikely to be protective and may prove to be harmful as detailed later in the thesis. As for nitrites, its major source is from the reduction of dietary nitrate which has undergone enterosalivary recirculation. A study suggested that increased nitrite consumption for a fixed protein intake was associated with a significant risk for distal gastric cancer⁽⁸⁶⁾. Another study suggested a low vitamin C intake coupled to a high nitrite intake was associated with an increased risk for oesophageal, cardia and non-cardia adenocarcinoma⁽⁸⁴⁾. These studies hint at the potential role of *N*-nitrosamines in the aetiopathogenesis of adenocarcinomas of the upper gastrointestinal tract.

CHAPTER FOUR

BARRETT'S OESOPHAGUS

4.1 Definition.

Barrett's oesophagus is defined as the presence of columnar metaplasia in the distal oesophagus. Description of Barrett's metaplasia includes distal oesophageal mucosa containing fundic mucosa, junctional mucosa and mucosa with intestinal metaplasia. Only the latter entity predisposes to the development of adenocarcinoma of the oesophagus.

4.2 Epidemiology.

Barrett's oesophagus is predominantly a disease of middle-aged men with a male to female ratio of 2 to 1⁽¹⁰⁰⁾. Approximately 6 – 12% of the patients undergoing endoscopy for gastro-oesophageal reflux symptoms are noted to have Barrett's oesophagus and is dependent on the criteria used to define Barrett's oesophagus. A population-based study in Olmstead demonstrated that Barrett's oesophagus is found in approximately 1% of unselected autopsies (7 of 733) of whom only two patients were diagnosed before death⁽¹⁰¹⁾. Those using the older definition of columnar mucosa \geq 3cm report lower prevalence compared to those who include those with $<$ 3cm of intestinal metaplasia⁽¹⁰²⁾. The prevalence of Barrett's oesophagus in asymptomatic, predominantly male veterans over the age of 50 was noted to be 25%⁽¹⁰³⁾. Up to a third of the patients with Barrett's metaplasia either have no or minimal reflux symptoms^(104, 105). These findings explain why the majority of the patients with Barrett's oesophagus remain undiagnosed.

In some families Barrett's oesophagus and oesophageal adenocarcinoma appears to be inherited as an autosomal dominant characteristic^(106, 107, 108). This may be related to the fact that there may be a genetic predisposition to the development of reflux in families of patients with Barrett's oesophagus and oesophageal adenocarcinoma⁽¹⁰⁹⁾.

4.3 Histological Features.

Barrett's mucosa can be recognised endoscopically from the normal pale pink squamous mucosa by its velvety, salmon- pink appearance extending above the gastro-oesophageal junction. Microscopically, Barrett's oesophagus is diagnosed by identifying 'specialised' columnar epithelium which replaces the native squamous epithelium of the oesophagus.

Ultra-structurally the Barrett's metaplastic epithelium is made up of a mixture of cell types. These include cells resembling gastric cells, small intestinal cells and cells with variable microvilli appearance. Goblet cells which secrete acid mucins are found in association with the latter two cell types and not with the gastric-like cells⁽¹¹⁰⁾. Intestinal metaplasia has been subdivided into complete and incomplete depending on the maturity of the enterocytes. Complete intestinal metaplasia is characterised by the presence of mature absorptive cells and do not secrete mucin and is termed type I. Incomplete intestinal metaplasia consist of few or absent absorptive cells, goblet cells and columnar cells that secrete mucin and depending on the type of mucin secreted it is designated type IIA, which secrete neutral mucins, (now designated as type II) and IIB, which secrete sulphomucins (now designated type III)⁽¹¹¹⁾. Intestinal metaplasia in Barrett's oesophagus is associated with all types but type II predominate and type I is rare or absent^(110, 111). Previous study suggested an association between gastric cancer and intestinal metaplasia that was associated with sulphomucins⁽¹¹²⁾. This finding has been extrapolated to Barrett's oesophagus and it has been suggested that the presence of type IIB/III intestinal metaplasia predisposed to the development of oesophageal adenocarcinoma^(111, 113).

Barrett's oesophagus was previously only diagnosed when at least 3cm of columnar mucosa was visible above the proximal gastric fold. This arbitrary distance was adopted as it can be difficult for an endoscopist to determine the end of the tubular oesophagus and the beginning of the hiatus hernia, which is almost always found in Barrett's oesophagus. To add to the difficulty, it was suggested that even the "normal oesophagus" could be lined by 2cm of columnar epithelium and therefore biopsy of this region will provide a false positive diagnosis of Barrett's oesophagus⁽¹¹⁴⁾.

More recently, it has been recognised that Barrett's oesophagus can be shorter than 3cm and can be detected by the proximal extension of the columnar-lined mucosa from the gastro-oesophageal junction, confirmed histologically by the presence of intestinal metaplasia and Barrett's oesophagus is now defined as the presence of intestinal metaplasia anywhere within the oesophagus ⁽¹¹⁵⁾. Another related entity is intestinal metaplasia of the cardia which is defined as the presence of intestinal metaplasia at a normal-appearing gastro-oesophageal junction ⁽¹¹⁶⁾. The metaplastic mucosa of varying lengths termed long-segment Barrett's, short-segment Barrett's and intestinal metaplasia of the cardia depending on the extent of the metaplasia likely represent a continuum of a single disease process with a common aetiological agent, although intestinal metaplasia at the cardia appears to have another aetiology as we shall discuss later.

4.4 Malignant transformation in Barrett's oesophagus.

Malignant transformation of the Barrett's mucosa appears to be restricted to the intestinal-type mucosa ^(117, 118). The majority of oesophageal adenocarcinomas are surrounded by or adjacent to the metaplastic mucosa ^(119, 120) although in some resection specimens of oesophageal adenocarcinoma, intestinal metaplasia is absent and the likely explanation is that the tumor has overgrown the area of metaplastic mucosa obliterating the evidence ⁽¹²⁰⁾.

The incidence of adenocarcinoma developing in Barrett's oesophagus quoted in the literature is between one case in 441 patient-years to one case in 52 person-years (table 4.1). The reason for the wide discrepancy between the quoted figures may be attributable to a number of potential factors. Some institutions which specialise in managing Barrett's oesophagus will undoubtedly be referred patients with dysplasia who are more likely to develop adenocarcinoma i.e. there is a referral bias. Some studies have included subjects with high-grade dysplasia at the initial endoscopy which will bias towards higher incidence of adenocarcinoma ^(118, 121). Some studies looking at screening have included patients without intestinal metaplasia in the Barrett's segment which inevitably will include those who are not destined to develop adenocarcinoma reducing the estimation for developing malignant lesion ⁽¹²²⁾. Some studies will be biased due to the patient characteristics, for example studies conducted in the Veterans

Administration in the U.S.A. will be over-represented by the male population and this will increase the chance for identifying oesophageal adenocarcinoma since these malignancies are much more common in the male population (see chapter 2). Studies that have incorporated short segment Barrett's oesophagus, defined as Barrett's mucosa of less than 3cm, have lower incidence of detecting adenocarcinoma^(123, 124). Overall, the individual studies are small in number and even one or two additional cases of oesophageal adenocarcinoma will greatly affect the estimate. It can be seen from the table 4.1 that the higher estimates are in those studies with patient number <100.

4.5 Dysplasia in Barrett's oesophagus.

Pathological specimens of oesophageal adenocarcinoma frequently have areas of dysplasia in Barrett's epithelium which are remote from the tumor itself^(119, 125). Dysplasia is defined as an unequivocal neoplastic alteration of the epithelium and categorised as negative, indefinite, low grade and high grade dysplasia. Dysplastic cells are distinguished by histological changes exceeding that of reparative process and cytologic abnormalities: cellular and nuclear pleomorphism, nuclear hyperchromatism, loss of nuclear polarity and marked stratification of nuclei⁽¹²⁶⁾. It is believed that the metaplastic Barrett's epithelium progresses from non-dysplastic through low and high-grade dysplasia and eventually carcinoma^(118, 125, 127, 128) although it is well documented that some patients progress from no dysplasia to frank adenocarcinoma during surveillance endoscopies which are aimed at the early detection of adenocarcinoma^(124, 129). Miros's study showed that only those with dysplasia progressed to adenocarcinoma⁽¹²¹⁾ and therefore the absence of dysplasia prior to the diagnosis of adenocarcinoma most likely reflects sampling interval and or sampling error.

The natural history of low-grade dysplasia is becoming clearer. During a three-year follow up of 102 patients with Barrett's oesophagus 8% of those free of dysplasia developed low or high-grade dysplasia. Those with low grade dysplasia at any time during surveillance endoscopy 12% developed high-grade dysplasia or adenocarcinoma and two patients out of five who had high grade dysplasia at any time developed adenocarcinoma⁽¹³⁰⁾.

Reports have suggested that adenocarcinoma is found in up to 73 % of oesophagi resected for high-grade dysplasia (see table 4.2). Such high proportions are likely to reflect the small number of patients studied, the lack of intensive biopsy protocols to detect the tumor present (sampling error) and inclusion of patients with mucosal abnormalities suggestive of neoplasia and those with proven adenocarcinoma on pre-operative biopsy specimens (selection bias). Malignant degeneration of high-grade dysplasia is not inevitable. Intensive biopsy sampling can accurately classify high-grade dysplasia and adenocarcinoma and may obviate the need for oesophagectomy in those with high-grade dysplasia without evidence of carcinoma ⁽¹³¹⁾. A study by Levine using intensive biopsy protocols in 70 patients with high-grade dysplasia suggested 17% had prevalent adenocarcinoma, 26% developed adenocarcinoma during a mean follow up of 27 months with surveillance endoscopy ⁽¹³²⁾. More recent data by the same group demonstrated that out of 76 patients with high-grade dysplasia 59% developed adenocarcinoma during a mean follow up over a 5-year period. In those subjects who had aneuploidy or 4N (tetraploid) fractions on flow cytometry 61% developed adenocarcinoma compared to 42% in those without flow cytometric abnormalities over a three-year period ⁽¹³³⁾. Schnell followed 79 patients with high-grade dysplasia and found that 11% developed adenocarcinoma in the first year of diagnosis of high-grade dysplasia and 20% during an average of 7 years' follow up ⁽¹³⁴⁾. The risk of malignant degeneration also depends on the extent of high-grade dysplasia. Buttar followed 100 patients and found cancer in 38% at 1 year and 56% at 3 years in those patients with diffuse-high grade dysplasia and 7% and 14% at 1 and 3 years in those with focal high-grade dysplasia ⁽¹³⁵⁾. In reality, difficulty in differentiating between intramucosal carcinoma and high-grade dysplasia ⁽¹³⁶⁾ means that many clinicians will continue to advocate oesophagectomy in those who are found to have high-grade dysplasia and are fit enough for surgery ⁽¹³⁷⁾.

Author	Patient no.	Mean follow up: years (range)	Patient-years of follow up	No of cancers	Incidence per patient-year
Spechler 1984 ⁽¹²⁸⁾	107	3.3 (0.1 – 20)	350	2	1 in 175
Cameron 1985 ⁽¹³⁸⁾	104	8.5	884	2	1 in 441
Robertson 1988 ⁺ (129)	56	2.9 (0.5 – 8)	168	3	1 in 56
Hameetman 1989 ⁽¹¹⁸⁾	50	5.2 (1.5 – 14)	260	5	1 in 52
Miros 1991 ⁽¹²¹⁾	81	3.6 (0.5 – 8)	289	3	1 in 96
van der Burgh 1996 [*] (139)	155	9.3	1440	8	1 in 180
Wright 1996 [†] (140)	108 M 58 F	2.7 2.9	294 167	5 1	1 in 59 1 in 167
Drewitz 1997 [†] (125)	123 LS 47 SS	4.8 (0.5 – 13)	590 226	3 1	1 in 195 1 in 226
Streitz 1998 ⁽¹⁴¹⁾	136	3.7	510	7	1 in 73
Katz 1998 ⁽¹³⁰⁾	102	4.8 (3.0 – 6.4) / median (IQR)	563	3	1 in 188
O'Connor 1999 [†] (124)	106 LS 30 SS	4.2 (1 – 14.1)	445 126	2 0	1 in 223 0 in 126
Conio 2001 USA [†] (142)	117 LS 37 SS	5.0 2.3	585 85	4 0	1 in 146 0 in 85
Conio 2003 Italy [†] (143)	107 LS 59 SS	6.6 (2 – 13.9)	706 389	4 1	1 in 177 1 in 398

Table 4.1: Incidence of oesophageal adenocarcinoma in Barrett's oesophagus.

Key: + Barrett's \geq 5cm only studied, * No endoscopic surveillance, [†] includes short segment Barrett's (<3cm),

SS = short segment Barrett's (<3cm), M = male, F = female.

Author	Patient Number	Adenocarcinoma (%)
Lee 1985 [†] (144)	2	1 (50)
Hamilton 1987 [#] (125)	3	1 (30)
Altorki 1991 [*] (145)	7	2 (29)
Pera 1992 (146)	18	9 (50)
Rice 1993 (147)	16	6 (38)
Peters 1994 (148)	9	5 (56)
Edwards 1996 (149)	11	8 (73)
Heitmiller 1996 (150)	30	13 (43)
Cameron 1997 (151)	19	2 (10)
O'Connor 1999 (124)	2	0 (0)

Table 4.2: Prevalence of oesophageal adenocarcinoma in oesophagotomies performed for high grade dysplasia.

† The author presented four patients with high-grade dysplasia (HGD) but two patients had cancer diagnosed before operation and are excluded in this analysis.

The author presented 5 patients with HGD. One had exophytic tumor with only dysplasia diagnosed as invasive carcinoma after oesophagectomy; another case had invasive cancer on repeated biopsy for HGD 3 weeks later which were not included in the above analysis.

* The author presented 9 cases of HGD. Two out of 4 patients who were found to have carcinoma had malignant cells on prior cytology brushings therefore are not included in this analysis.

4.6 Pathogenesis of Barrett's oesophagus.

The precise pathological process leading to the development of Barrett's oesophagus is unknown. The most important factor for the development of Barrett's metaplasia is gastro-oesophageal reflux. It is unclear which constituents of refluxed material is the most important. Acid and pepsin does not appear to be the only damaging constituents since Barrett's oesophagus has developed after total gastrectomy with oesophagojejunostomy ⁽¹⁵²⁾. This would suggest that contents of the small intestine which include pancreatic and biliary secretions may be important in the development of this condition.

4.6.1 Gastro-oesophageal reflux and Barrett's oesophagus.

Gastro-oesophageal reflux is instrumental in the development of Barrett's oesophagus. However, it is unclear why some people with gastro-oesophageal reflux develop Barrett's whilst others do not. Compared to those with gastro-oesophageal reflux disease without Barrett's oesophagus, subjects with Barrett's oesophagus typically have a lower resting lower oesophageal sphincter pressure - in particular those with longer Barrett's segment ^(105, 153); a greater degree of oesophageal acid exposure and impaired acid clearance, particularly in the supine position - as measured by 24-hour pH monitoring, which would suggest that any acid reflux that occurred in the Barrett's oesophagus will be present in the oesophagus for a longer duration than those with reflux oesophagitis ^(105, 154, 155). One study, however, noted that the manometric findings and the 24-hour pH measurement in subjects with severe circumferential oesophagitis were similar to subjects with Barrett's oesophagus ⁽¹⁵⁶⁾. The degree of oesophageal acid exposure as measured in both the upright and supine position in patients with Barrett's oesophagus is related to the length of Barrett's metaplastic mucosa ^(153, 157). In other words the longer the Barrett's segment, the greater is the degree of gastro-oesophageal reflux.

4.6.2 Hiatus hernia.

Sliding hiatus hernia appears to play an important role in the pathogenesis of gastro-oesophageal reflux disease. Patients with reflux oesophagitis are more likely to have hiatus hernia than those without oesophagitis ⁽¹⁵⁸⁾ and gastro-oesophageal reflux occurs to a greater degree than those without hiatus hernia ^(159, 160). Hiatus hernia is demonstrated in 60 - 94 % of patients with oesophagitis ^(158, 161, 162, 163) and the severity of oesophagitis correlates with the presence and the size of the hernia ^(164, 165). In patients with Barrett's oesophagus, a subgroup of refluxers with the greatest oesophageal acid exposure, greater than 90% of the patients have demonstrable hiatus hernia ⁽¹⁶⁶⁾.

The mechanisms by which hiatus hernia predispose to gastro-oesophageal reflux appears to be through its effect on the lower oesophageal sphincter pressure and impairing oesophageal acid clearance. Patients with hiatus hernia demonstrated reduced lower oesophageal sphincter pressures compared to those without a hernia ^(165, 167) shorter sphincter length ^(165, 167) and reduced /absent intra-abdominal length of sphincter ^(159, 168). Maneuvers which abruptly raise the intraabdominal pressure are more likely to result in reflux in subjects with hiatal hernias ⁽¹⁶⁷⁾. The presence of a non-reducing hiatal hernia is associated with an impaired diaphragmatic pressure augmentation during inspiration ⁽¹⁶⁸⁾. The spatial separation of the lower oesophageal sphincter and the diaphragmatic high pressure zone reduces the effective pressure exerted at the level of the lower oesophageal sphincter ⁽¹⁶⁹⁾. Clearance of the refluxed gastric content is impaired in the presence of a hiatus hernia and as a result there is increased oesophageal acid exposure ^(164, 165, 168, 170) and reflux of the contents of the hernia back into the oesophagus has been demonstrated using barium and radio-isotope scanning ^(168, 170).

In summary, it can be seen that hiatus hernia predisposes to gastro-oesophageal reflux by impairing the sphincter mechanisms of the lower oesophagus by a number of mechanisms and prolongs the exposure of the oesophagus to the noxious effect of the refluxed gastric contents.

4.6.3 Acid, pepsin and bile reflux.

Animal studies have established the importance of acid and pepsin in producing peptic oesophagitis. Redo demonstrated that hydrochloric acid alone at $\text{pH} < 2$ did not cause erosions or ulcerations of the oesophagus whereas 2% solutions of pepsin at $\text{pH} < 2$ caused ulcerations. Addition of bile to gastric juice attenuated the mucosal injury caused by gastric juice at acid pH ⁽¹⁷¹⁾. In a feline model high acidity was required ($\text{pH} < 1.3$) for producing oesophagitis when only hydrochloric acid was employed whereas oesophagitis was produced when pepsin was present with hydrochloric acid even under conditions of $\text{pH} > 1.3$ and the damage was prevented by the presence of a pepsin inhibitor ⁽¹⁷²⁾. Furthermore, hydrochloric acid alone at $\text{pH} 2$ did not cause oesophagitis or increase mucosal permeability in the rabbit oesophagus whereas addition of pepsin at $\text{pH} 2$ caused both macroscopic damage and increased mucosal permeability to various ions including H^+ ions ⁽¹⁷³⁾. Taurodeoxycholate, a conjugated bile acid at $\text{pH} 2$ increased mucosal permeability but did not cause macroscopic change and trypsin did not alter gross appearance or the permeability ⁽¹⁷³⁾. This would indicate that pepsin and acid are the key components leading to oesophagitis in patients with peptic oesophagitis.

Acid reflux is clearly important in the pathogenesis of Barrett's oesophagus as noted previously. However, animal and human studies of Barrett's oesophagus suggest an important role of refluxing duodenal contents, which include pancreatic juice, in the pathogenesis of intestinal metaplasia of the distal oesophagus. A three-month study on dogs suggested that surgically damaged oesophageal mucosa exposed to gastric contents heals by columnar metaplasia but without the goblet cells ⁽¹⁷⁴⁾. This suggested that in the presence of epithelial disruption, continued reflux of acidic gastric contents leads to regeneration with columnar epithelium. Either gastric juice was inadequate stimulus to produce columnar metaplasia with goblet cells or the duration of the study was too short for its development. Rats surgically treated to produce reflux of duodenal contents with or without gastric acid and fed standard rat chow, developed intestinal metaplasia and adenocarcinoma of the oesophagus. Only 2 out of the 16 animals subjected to only reflux of gastric contents developed columnar-lined oesophagus and none developed any malignant lesions 50 weeks after the surgical procedure

(175). This suggests that bile reflux is an important component in the pathogenesis of Barrett's oesophagus and adenocarcinoma in this animal model.

In man, bile reflux also appears to be important in the pathogenesis of Barrett's oesophagus. In patients with Barrett's oesophagus bile reflux, as measured by bilirubin absorbance, is higher than in those with uncomplicated gastro-oesophageal reflux^(176, 177, 178) and bile salt concentrations in gastric and oesophageal aspirates of patients with Barrett's oesophagus are higher in individuals with Barrett's compared to those with uncomplicated reflux^(178, 179). Bile salts aspirated from the fasted stomach were found to be greater in those with increasing severity of gastro-oesophageal reflux and in particular those with complicated Barrett's oesophagus⁽¹⁷⁸⁾. Vaezi showed that the combination of acid and bile reflux in humans who have previously undergone partial gastrectomy led to the development of oesophagitis whereas patients with bile reflux alone did not demonstrate oesophagitis⁽¹⁸⁰⁾. The majority of patients with intact stomach and gastro-oesophageal reflux, especially those with Barrett's oesophagus, have simultaneous reflux of both acid and bile^(178, 181) although Barrett's metaplasia developing following gastrectomy has also been described⁽¹⁵²⁾.

The mechanism by which bile acids induce oesophageal injury appears to be related to the dissociation constant (pKa) of the bile acids and the pH of the prevailing environment. Conjugated bile acids at concentrations found in gastric juice produced ultrastructural damage to oesophageal epithelium at pH 1 but not at pH 7 whereas unconjugated bile acids produced damage at pH 7⁽¹⁸²⁾. An *in vivo* study of bile salt injury to the gastric mucosa in mice demonstrated that taurocholate with a pKa of 1.8 only produced damage at pH 1 whereas glychochenodeoxycholate with a pKa of 4.2 produced damage at pH 1 and pH 3⁽¹⁸³⁾.

The cellular damage produced by the bile salts in acid appears to be related to the increased epithelial permeability to the luminal H⁺ ions. Conjugated bile salt in hydrochloric acid cause back diffusion of H⁺ ions into human stomach⁽¹⁸⁴⁾ and the oesophagus in animal experiments^(173, 185). Taurine-conjugated bile acids was shown to cause back diffusion of H⁺ ions into the oesophageal epithelium from the lumen in rabbits at pH 2 whereas unconjugated bile acids caused back diffusion of H⁺ ions at pH 7 but not at pH 2⁽¹⁸⁶⁾. The pH dependent effect of the

different bile acid is due to the ionisation status of the particular bile acid at different pH. Below the pKa the bile acid is predominantly non-ionized and is permeable to the epithelial membrane whereas ionized species are less permeable. Additionally, unconjugated bile salts precipitate out in acid rendering them harmless. Epithelial barrier disruption by bile salts appears to be due to the accumulation of bile salts in the epithelium rather than the solubilization of mucosal lipid layer by bile acids ⁽¹⁸⁷⁾. Bile acids also appear to be mitochondrial toxins leading to the failure of oxidative phosphorylation and subsequently cell rupture ⁽¹⁸⁸⁾. However, the epithelial disruption and cytotoxicity of the bile salts does not explain how they may contribute to the carcinogenic process in Barrett's oesophagus.

More recent studies have focussed on the effect of acid and bile acids on cellular proliferation which may have an important role in the development of neoplasia in Barrett's oesophagus. In an ex-vivo organ culture model of Barrett's oesophagus, Fitzgerald showed that cellular proliferation was increased and differentiation decreased in Barrett's mucosa exposed to pulses of acid at pH 3.5 compared to those tissues which had either continuous exposure to acid or maintained at physiological pH ⁽¹⁸⁹⁾. Subsequent study by the same group in patients with Barrett's oesophagus demonstrated that patients who had complete suppression of acid reflux compared to those with incomplete suppression on proton pump inhibitor therapy, cellular proliferation was reduced and villin expression, a marker of epithelial maturation for this type of mucosa, was increased six months later whereas those with inadequate suppression showed no change from baseline ⁽¹⁹⁰⁾. These studies suggest intermittent exposure of Barrett's mucosa to acid increases cellular proliferation and reduced differentiation. Souza demonstrated an increase in cellular survival and proliferation in oesophageal adenocarcinoma cell line by reducing apoptosis and increase in cells entering G2 phase of the cell cycle after exposure to a brief pulse of acid mediated through the effects of mitogen activated protein kinases ⁽¹⁹¹⁾.

Induction of cyclooxygenase-2 (COX-2) expression in the Barrett's epithelium by acid and bile has been noted. Bile acids have been shown to stimulate COX-2 expression via the epidermal growth factor receptor in cholangiocarcinoma cell line ⁽¹⁹²⁾. Increasing COX-2 protein expression is seen progressing from normal oesophageal mucosa to Barrett's mucosa, Barrett's

mucosa with dysplasia and adenocarcinoma of the oesophagus. Exposure of the Barrett's oesophagus to acid and bile salts increased COX-2 expression, particularly by acid or bile acid individually ⁽¹⁹³⁾ and appears to be mediated by protein kinase C ⁽¹⁹⁴⁾. COX-2 may contribute to carcinogenesis by promoting cellular proliferation, inhibiting apoptosis and supporting angiogenesis ⁽¹⁹⁵⁾. Buttar demonstrated in an animal model of oesophageal adenocarcinoma that COX-2 protein was expressed, without a significant increase in COX-1 protein, and PGE₂ levels were increased in the oesophageal tissue. Tumor incidence was reduced in animals treated with Sulindac and a COX-2 specific inhibitor, both of which reduced the level of PGE₂ in the oesophageal tissue compared to controls ⁽¹⁹⁶⁾. It remains to be seen if COX-2 inhibitors may be a useful chemopreventative agent in patients with Barrett's oesophagus in man.

Although acid and duodenal contents including bile acids appear to be important in the pathogenesis of the Barrett's metaplasia and malignant degeneration, they may not be the mutagen leading ultimately to adenocarcinoma. The implication of the above studies is that acid and duodenal contents lead to epithelial disruption in the normal squamous oesophageal mucosa and lead to the development of Barrett's metaplasia. The same factors increase the cellular proliferation of Barrett's epithelium, for example by up-regulating COX-2 expression, and increase the susceptibility of the epithelium to mutagenic compounds. The mutagen(s) for the malignant degeneration in Barrett's mucosa is currently unknown. The present thesis will later discuss the potential mechanisms which lead to the mutagenic process in Barrett's epithelium.

4.7 Intestinal metaplasia of the cardia and carditis.

Barrett's oesophagus was previously defined arbitrarily as metaplastic epithelium ≥ 3 cm above the gastro-oesophageal junction. Recent studies have subdivided the Barrett's oesophagus in to those ≥ 3 cm as classical Barrett's or long-segment Barrett's and < 3 cm as short-segment Barrett's oesophagus (see above). More recently, it was noted that intestinal metaplasia can be frequently detected at the cardia in patients with gastro-oesophageal junction with apparently normal appearances with prevalence ranging between 6 % to 25 % ^(116, 197, 198, 199, 200). The precise pathogenesis of intestinal metaplasia at the cardia is unclear.

Intestinal metaplasia is thought to arise as a result of chronic inflammation⁽²⁰¹⁾ and can be induced by carcinogenic nitrosocompounds⁽²⁰²⁾.

There has been a debate as to the relative importance of *H. pylori* infection and gastroesophageal reflux in causing inflammation and intestinal metaplasia of the cardia. *Helicobacter pylori* has been implicated in having a causative role in carditis and intestinal metaplasia of the cardia^(203, 204). Chen demonstrated a higher prevalence of *H. pylori* infection at the cardia and other gastric sites in individuals with increasing severity of cardia inflammation whereas reflux symptoms showed a statistically non-significant inverse trend in relation to the severity of carditis. In addition those without carditis did not have *H. pylori* infection. However, overall only 27% of patients were positive for *H. pylori* on biopsy in the presence of carditis in that study⁽²⁰⁵⁾. Eradication of *H. pylori* has been shown to dramatically improve cardia inflammation whereas in those with persistent infection no improvement was seen⁽²⁰⁶⁾. Others have implicated gastro-oesophageal reflux as the cause of carditis and intestinal metaplasia at this site⁽²⁰⁷⁾. Öberg and colleagues suggested that the so-called cardiac mucosa does not normally exist and is due to metaplasia secondary to some noxious insult of the native mucosa at this site. They showed that individuals with cardiac-type mucosa had more objective evidence of gastro-oesophageal reflux. Virtually all patients who demonstrated cardiac mucosa had "carditis" which was associated with less antral *H. pylori* infection than those without cardiac mucosa and when the organism was found in cardia biopsy specimens it had no association with the presence or absence of carditis. In subjects with intestinal metaplasia of the cardia more subjects had oesophagitis and hiatal hernia compared to those with cardiac mucosa without intestinal metaplasia. Only 17% of the subjects with intestinal metaplasia of the cardia had antral *H. pylori* infection⁽²⁰⁷⁾.

The real answer appears to lie somewhere in between and that some cases of intestinal metaplasia are due to *H. pylori* and the others gastric acid related^(208, 209, 210). Carditis secondary to *H. pylori* infection has evidence of gastritis elsewhere in the stomach; those with a normal stomach, carditis is associated with gastro-oesophageal reflux^(208, 210). Using cytokeratin 7 and 20 staining pattern, Balaji showed that patients with intestinal metaplasia of the cardia and objective evidence of gastro-oesophageal reflux, negative for *H. pylori*, had incomplete type of metaplasia and in 90% of these patients cytokeratin staining pattern was the

same as that of Barrett's oesophagus. In contrast those who had cardia intestinal metaplasia who were positive for *H. pylori* with or without reflux, 50% and 41% respectively stained positive for Barrett's cytokeratin pattern⁽²⁰⁹⁾. Interestingly Balaji's study also highlighted patients who had cardia intestinal metaplasia with cytokeratin staining pattern of Barrett's metaplasia but had no objective evidence of gastro-oesophageal reflux or of *H. pylori* infection. The latter finding can be explained by the fact that at the cardia the pH is already acidic and individuals do not have to have reflux for the cardia to be subjected to the effects of gastric acid⁽²¹¹⁾. *Helicobacter pylori* infection, particularly the Cag A strain, is associated with a reduced risk for cardia and oesophageal adenocarcinoma^(66, 71 - 73) and the above studies suggest that the risk for developing cardia adenocarcinoma in patients with cardia intestinal metaplasia will be dependent on the aetiology of the metaplasia. A recent study suggested *H. pylori* may be both protective and predispose to cardia adenocarcinoma. Cardia cancer was negatively associated with *H. pylori* infection but was over-represented by *H. pylori* associated gastric atrophy compared to controls suggesting a dual aetiology for this type of cancer⁽⁷⁶⁾.

The above studies suggest that carditis and intestinal metaplasia at the cardia have dual aetiologies - *H. pylori* and gastro-oesophageal reflux. It is likely that intestinal metaplasia of the cardia unrelated to *H. pylori*, short-segment Barrett's and long-segment Barrett's form a continuum and the so-called Barrett's oesophagus encompasses a variable length of intestinal metaplasia extending proximally from the gastro-oesophageal junction and is better termed intestinal metaplasia of the distal oesophagus. The risk for developing cardia adenocarcinoma from cardia intestinal metaplasia related solely to reflux/acid exposure is unknown whereas for long-segment Barrett's and short-segment Barrett's oesophagus are 1 in 150^(118, 121 - 124, 128 - 139, 141 - 142) and perhaps in excess of 1 in 200^(123, 124, 142, 143, 212, 213) patient -- years respectively.

CHAPTER FIVE

NITROSAMINES IN CANCER

5.1 Introduction.

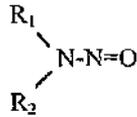
In the late 1960's through to the early 1980's there was a considerable interest in *N*-nitroso compounds as a potential carcinogen in human gastric cancer, many of which were potent carcinogens in animals. *N*-nitroso compounds could be synthesized *in vitro* from amines and nitrite under acidic conditions and there was considerable interest regarding the formation of *N*-nitroso compounds in the acidic environment of the stomach. However, this did not reflect the clinical practice where non-cardia gastric cancer developed in association with gastric atrophy and hypo-/achlorhydric stomach. Correa proposed a hypothesis where the stomach underwent a series of changes from gastritis to atrophic mucosa eventually developing hypo-/achlorhydria which then became colonised by bacteria capable of nitrosating amines in the presence of nitrite to generate carcinogenic *N*-nitrosamines which could potentially lead to mutagenesis and carcinogenesis in the gastric epithelium^(214, 215).

The subsequent sections will deal with *N*-nitroso compounds, describe the nitrosation chemistry, including bacterial nitrosation in more detail, and their potential relevance in cardia and oesophageal adenocarcinoma which form the basis of this thesis.

5.2 Nitroso compounds. What are they?

N-nitroso compounds are a diverse group of chemicals encompassing *N*-nitroso derivatives of primary, secondary and aromatic amines, amides guanidines and ureas. They have been extensively studied for their carcinogenic effects. *N*-nitroso compounds are found in the environment and are known to be widely distributed for example tobacco smoke, food, by-products of industrial processes to name but a few. This has been reviewed extensively⁽²¹⁶⁾ and will not be the subject of further discussion in this thesis.

N-nitroso compounds have a general structure



They can be divided into two classes with different chemical properties:

- (1) Nitrosamines where R_1 and R_2 are alkyl or aryl groups.
- (2) Nitrosamides where R_1 is an alkyl or aryl group, and R_2 is an acyl group.

Nitrosamines are generally stable compounds and require metabolic activation by mammalian enzymes prior to DNA alkylation (fig 5.1).

In contrast, nitrosamides (and related nitrosoureas and nitrosoguanidines) are unstable in neutral or alkaline aqueous solutions and do not require metabolic activation before they react and alkylate DNA.

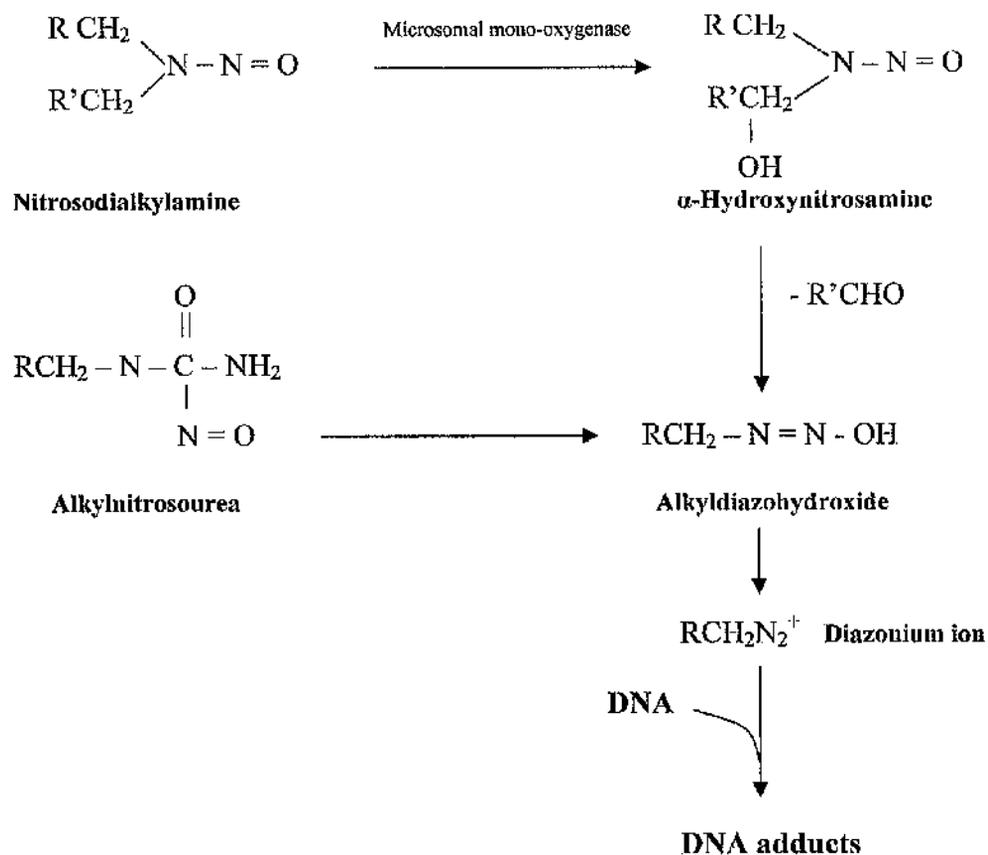


Fig 5.1: Bioactivation of *N*-nitroso compounds.

N-nitrosoureas and related compounds such as nitrosamides, nitrosoguanidines decompose at physiological pH to form electrophilic alkylating agents. *N*-nitrosamines are stable at physiological pH and require activation by cytochrome P450-dependent hydroxylation at the carbon atom adjacent to the *N*-nitroso group which is the critical step in the biotransformation.

5.3 Nitrosamines in animal models of oesophageal adenocarcinoma.

Several studies have used *N*-nitroso compounds to produce adenocarcinoma of the oesophagus in conjunction with surgical procedures to produce reflux of duodenal contents into the oesophagus^(217, 218, 219, 220, 221). Rats operated to produce gastroduodeno-oesophageal reflux developed adenocarcinoma (some were adenosquamous) of the oesophagus when treated with nitrosamines whereas those treated with nitrosamines alone developed only squamous oesophageal carcinoma suggesting that bile reflux modifies the carcinogenic property of the nitrosamines^(218, 219). In another similar study, rats operated to produce gastroduodeno-oesophageal reflux and administered nitrosamine developed columnar-lined mucosa in addition to oesophageal adenocarcinoma. A high fat diet increased the number of animals developing oesophageal adenocarcinoma and squamous oesophageal carcinoma⁽²²⁰⁾. In some studies surgical procedure which produced gastro-duodeno-oesophageal reflux alone was sufficient to produce both columnar mucosa resembling Barrett's oesophagus and oesophageal adenocarcinoma^(175, 221). The study by Miwa showed that normal duodenal secretions were important in the generation of columnar-lined mucosa and oesophageal adenocarcinoma⁽¹⁷⁵⁾. The development of oesophageal adenocarcinoma by different methods in these animal models appears to be related to the different rat strains used in different studies: Sprague-Dawley rats did not develop adenocarcinoma in the absence of nitrosamine administration (although glandular metaplasia with mucus-secreting cells developed)⁽²¹⁹⁾. Wistar rats on the other hand developed Barrett's oesophagus and oesophageal adenocarcinoma without any carcinogen^(175, 221). These studies suggest that duodenal secretions, which include bile and pancreatic secretions, can produce Barrett's oesophagus and oesophageal adenocarcinoma but such susceptibility is determined by the strain of the rat. The studies also suggest that the duodenal secretions modify the cellular differentiation of the carcinoma that subsequently develops in the rats exposed to nitrosamine.

Although the animal models provide an insight into the importance of the reflux of duodenal contents in producing Barrett's oesophagus and oesophageal adenocarcinoma, it must be borne in mind that in all animal models of gastroduodeno-oesophageal reflux, the normal anatomical relationship of the

oesophagus, stomach and duodenum have been altered and as such it does not precisely mirror the sequence of events which leads to duodeno-gastro-oesophageal reflux where the duodenal secretions enter the stomach and then reflux into the distal oesophagus. Developing an animal model which reflects the normal physiological process of duodenogastro-oesophageal reflux should be the logical next step.

5.4 *N*-Nitroso compounds and human cancer.

Direct evidence linking *N*-nitroso compounds to carcinogenesis in human is lacking and is based on substantial circumstantial evidence. *N*-nitroso compounds are carcinogenic in many animal species and produce cancer in numerous organs ⁽²²²⁾. Over 80% of nitrosamines and over 90% of nitrosamides are carcinogenic in many animal species including primates and it is unlikely that humans will be immune to the carcinogenic effect of nitroso compounds ⁽²²³⁾. Nitrosoureas used in cancer chemotherapy, which are alkylating agents, are implicated in second neoplasms developing in treated individuals ^(224, 225). Human liver metabolises nitrosodimethylamine (NDMA) like rat liver ⁽²²⁶⁾ and liver fractions from humans can convert NDMA to chemicals mutagenic to bacteria ^(227, 228, 229). Human liver and oesophagus can metabolize *N*-nitroso compounds into chemicals which can bind to DNA ^(226, 230, 231). Acute toxicity of *N*-nitrosodimethylamine in man produce hepatic necrosis with lesions resembling those seen in rodents ⁽²³²⁾ and 7-methylguanine and *O*⁶-methylguanine which are DNA adducts generated by nitroso compounds were found in the liver of a victim of NDMA poisoning ⁽²³³⁾. In Linxian, China, where there is a high incidence of oesophageal carcinoma, DNA adducts are elevated in oesophageal tissues unaffected by tumor in those who underwent oesophagectomy compared to samples from European patients ⁽²³⁴⁾. In addition urinary excretion of *N*-nitroso compounds is elevated in such patients compared to subjects from a low-risk region ⁽²³⁵⁾. These reports provide evidences that *N*-nitroso compounds are likely to be carcinogenic in humans.

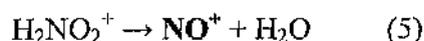
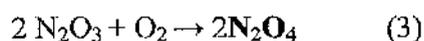
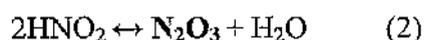
5.5 Nitrosation chemistry.

N-nitrosation involves the replacement of hydrogen which is attached to nitrogen by the nitroso group.



5.5.1 Acid-catalysed nitrosation.

Nitrous acid generated from the addition of nitrite to acid is the oldest method of preparing a nitrosating agent. Nitrous acid has a pK_a of 3.4 (at pH 3.4, 50% of the acid is dissociated into H^+ and NO_2^-) and therefore nitrosation by acidified nitrite is acid catalysed. Neither nitrous acid nor nitrite is able to nitrosate directly but nitrous acid generates a cocktail of chemicals including a number of nitrosating agents as shown below (indicated in bold print).

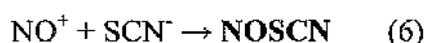
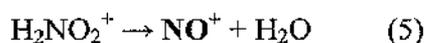
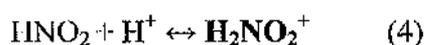


The nature of the nitrosating species depends on the prevailing pH. For example, at $\text{pH} < 2$, nitrous acid becomes protonated and generates nitrosating species in equations (4) and (5) whereas in weaker acids, $\text{pH} \approx 3$, N_2O_3 appears to be the major nitrosating species⁽²³⁶⁾.

Nitrosamine formation from secondary amines, the most widely studied reaction, follows the rate equation:

$$\text{Rate} = k [\text{R}_2\text{NH}][\text{nitrite}]^2$$

The optimal pH for nitrosation of secondary amines which have a $pK_a > 5$ is $pH \approx 3.4$, the pK_a of nitrous acid ⁽²³⁷⁾. The optimal pH for nitrosation is due to the balance between the formation of nitrosating species at lower pH, which increases the rate of nitrosamine formation, and the reduction in rate due to the formation of protonated amines at the lower pH which do not react with the nitrosating agent. The reaction is catalysed by the presence of halide anions and thiocyanate anions ^(237, 238). In the presence of thiocyanate, the optimum pH for nitrosation is $pH \approx 2.5$ ⁽²³⁷⁾. In this situation, the nitrosation species is formed as follows:



The rate equation for nitrosation of secondary amines follows:

$$\text{Rate} = k [\text{amine}][HNO_2][SCN^-]$$

Since the concentration of SCN^- is far greater concentration of nitrite/ HNO_2 at gastric pH (< 2), rate of nitrosation is greater than in the absence of SCN^- where the rate is proportional to $[\text{nitrite}]^2$.

For the nitrosation of amides and related compounds, the rate equation follows:

$$\text{Rate} = k [\text{amide}][HNO_2][H^+]$$

5.5.2 Bacterially catalysed nitrosation.

Bacteria can carry out *N*-nitrosation at neutral pH. This may be relevant in the achlorhydric stomach where bacterial overgrowth has been documented ⁽²³⁹⁾ and in other sites of the body where conditions favour both bacterial catalysation and nitrate reductase activity ⁽²⁴⁰⁾. The nitrosating ability of the different bacterial species depends on whether the organism is capable of denitrification.

Denitrifying bacteria appear to catalyse the formation of *N*-nitrosamine from nitrate and amines more effectively than non-denitrifying bacteria ⁽²⁴¹⁾. The proposed mechanisms for bacterial nitrosation include:

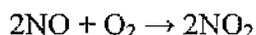
1. The bacteria reducing nitrate to nitrite concurrently lowering the pH of the culture medium promoting acid-catalysed nitrosation ⁽²⁴²⁾.
2. The bacteria providing a physical medium in which nitrosation is promoted for example the provision of a lipid phase by the bacterial membranes ⁽²⁴³⁾
3. The favoured theory is that bacterial enzymes catalyse the nitrosation reaction ^(244, 245).

The ability of bacteria to catalyse the nitrosation is dependent on the generation of nitric oxide or NO^+ -like species ^(246, 247). In the case of *E. coli*, a non-denitrifying bacteria, the nitrosation reaction is catalysed by the respiratory nitrate reductase ⁽²⁴⁸⁾ which is induced under anaerobic conditions ⁽²⁴⁹⁾. In the case of denitrifying bacteria such as *Pseudomonas aeruginosa* and *Neisseria mucosae*, *cd₁* nitrite reductase induced during anaerobic growth condition appears to be responsible for the nitrosation reaction ⁽²⁴⁷⁾.

5.5.3 Nitrosation by macrophages and neutrophils: the role of nitric oxide.

Macrophages stimulated by bacterial lipopolysaccharides and/or interferon γ nitrosates secondary amines at physiological pH ^(250, 251). L-arginine is the source of the nitrosyl group of the nitrosamine generated by activated macrophages ⁽²⁵²⁾ and involves the generation of nitric oxide as inhibitors of nitric oxide synthase and oxyhaemoglobin, a nitric oxide trapping agent inhibited nitrosation ⁽²⁵³⁾. Extravasated neutrophils also nitrosate amines by the same mechanism ⁽²⁵⁴⁾. The fact that the nitrosation of amines by macrophages required the presence of oxygen suggests that the nitrosation process involved the generation of N_2O_3 or N_2O_4 ⁽²⁵³⁾; similarly, nitric oxide in aerobic solution nitrosates morpholine at physiological pH, most likely due to the formation of N_2O_3 ⁽²⁵⁵⁾. Interestingly, activated macrophages and neutrophils simultaneously generate superoxide radicals with nitric oxide, which it consumes, and reduces nitrosation of amines. Superoxide dismutase increases the nitrosating potential of activated macrophages and ⁽²⁵³⁾ neutrophils ^(254, 256) whereas superoxide generating system (xanthine oxidase and hypoxanthine) attenuated nitrosation ⁽²⁵⁶⁾. Catalase had no such effect ⁽²⁵⁶⁾.

between nitrite and ascorbic acid is reduced ⁽²⁵⁹⁾. This is due to the recycling of the nitric oxide in the presence of oxygen to N₂O₃ ^(260, 261, 262)



5.6.2 Ascorbic acid inhibits bacteria-catalysed nitrosation.

Ascorbic acid in human gastric juice is considerably lower where histological abnormality such as chronic gastritis or intestinal metaplasia is present compared to the normal stomach ^(263, 264). Little or no ascorbic acid is present in hypo/achlorhydric gastric juice ^(264, 265). Acid suppressing medications such as omeprazole produce the same effect ⁽²⁶⁶⁾. Bacterial overgrowth has been documented in the naturally or drug-induced achlorhydric stomach ^(267, 268) and potentially catalyse the formation of *N*-nitrosamines in such conditions. Ascorbic acid has been shown to inhibit bacteria-catalysed nitrosation at neutral pH *in vitro* ⁽²⁶⁹⁾ and is likely to be effective in inhibiting bacteria-catalysed nitrosation *in vivo*.

5.6.3 Ascorbic acid inhibits N-nitrosation by activated macrophages and neutrophils.

Ascorbic acid has been shown to inhibit nitrosation by activated macrophages *in vitro*. However, ascorbic acid concentrations less than 50μM paradoxically increased the yield of nitrate and nitrosamine ⁽²⁷⁰⁾. When ascorbic acid-deficient rats stimulated by lipopolysaccharide were fed ascorbic acid, excretion of *N*-nitrosothioproline was significantly reduced which suggests that ascorbic acid can inhibit nitrosation by activated macrophages *in vivo* ⁽²⁵¹⁾. Ascorbic acid also inhibits *N*-nitrosation of amines by extravasated polymorphs ⁽²⁵⁴⁾.

5.7 Acid-catalysed nitrosation in the human stomach.

5.7.1 Introduction

Acid-catalysed nitrosation is potentially relevant in the acid-secreting human stomach. All the substrates relevant to the nitrosation reaction are present in the upper gastrointestinal tract. Nitrite is present in the saliva and is derived from the bacterial reduction of diet-derived nitrate in the oral cavity (see 5.7.2). The nitrite is swallowed and forms nitrous acid with acidic gastric juice to form nitrous acid and the nitrosating species which can react with nitrosatable amines to produce *N*-nitrosamines. The process is catalysed by thiocyanate anions, which are secreted in the saliva and present in gastric juice^(271, 272), via the formation of the nitrosating species nitrosothiocyanate (NOSCN) (see 5.5.1). Thiocyanate in human is derived from foods, in particular those of the *Brassica* species (e.g. broccoli, cabbage) and also from inhaling cyanide in tobacco smoke as well as ingestion of cyanide in the diet which is subsequently converted to thiocyanate by the enzyme rhodanese⁽²⁷³⁾. Ascorbic acid is a potent inhibitor of the *N*-nitrosation reaction. The healthy human stomach actively secretes ascorbic acid into the gastric juice and the concentrations in the gastric juice is higher than that of plasma^(274, 275). Ascorbic acid rapidly reduces the nitrosating species to nitric oxide and is itself oxidised to dehydroascorbic acid^(257 - 259, 276, 277, 278). This removal of intragastric nitrite by the ascorbic acid in gastric juice is probably the major mechanism preventing the generation of *N*-nitroso compounds within the lumen of the stomach. Thus the major determinant of *N*-nitrosation within the lumen of acid secreting stomach is the relative availability of nitrite and ascorbic acid. When the supply of nitrite exceeds that of ascorbic acid then nitrosation will occur, when ascorbic acid supply exceeds that of nitrite then nitrosation will be prevented.

5.7.2 Enterosalivary circulation of nitrate and the formation of salivary nitrite.

The major source of nitrite ingested by man is derived from enterosalivary recirculation of dietary nitrate. The average intake of nitrate in UK in 1985 was 54 mg/day and tap water contributed 10-20mg/day assuming a daily intake of 1 litre; nitrite intake was calculated to be 4.2 mg/day⁽²⁷⁹⁾. Most of the nitrate ingested in our diet comes from vegetable sources which contributes 75 – 80% of the intake^(90, 279). Very little nitrite is found in vegetables with concentrations rarely exceeding 2 mg/kg⁽⁹⁰⁾. Both nitrate and nitrite salts are used as curing agents in meat to develop and act as antioxidants. Nitrite, through the formation of nitric oxide, combines with myoglobin to stabilize the pink colour in cured meats⁽²⁸⁰⁾. Sodium nitrite is an antimicrobial agent that inhibits growth of *Clostridium botulinum* by delaying outgrowth of their spores⁽²⁸¹⁾.

The majority of nitrate ingested is absorbed from the proximal small intestine. Sixty percent is excreted in the urine^(282, 283). Approximately 25% is taken up by the salivary glands where it is concentrated and secreted into the buccal cavity⁽²⁸³⁾ (Fig 5.2). Man synthesizes approximately 1mmol/day of nitrate^(282, 284) which is likely to be derived from nitric oxide⁽²⁸⁵⁾. Twenty to 30% of the nitrate secreted into the buccal cavity is reduced to nitrite^(286, 287) by bacterial enzymes on the dorsum of the tongue during anaerobic respiration⁽²⁸⁸⁾. Approximately 5% of the ingested nitrate is thus converted to nitrite. The nitrite in saliva is swallowed and becomes acidified on entering the acidic gastric juice to form nitrous acid. Nitrous acid subsequently forms various nitrosating species in solution which is reduced to nitric oxide by the ascorbic acid secreted by the healthy human stomach and in the process ascorbic acid is oxidized to dehydroascorbic acid^(258, 259, 276, 277, 278).

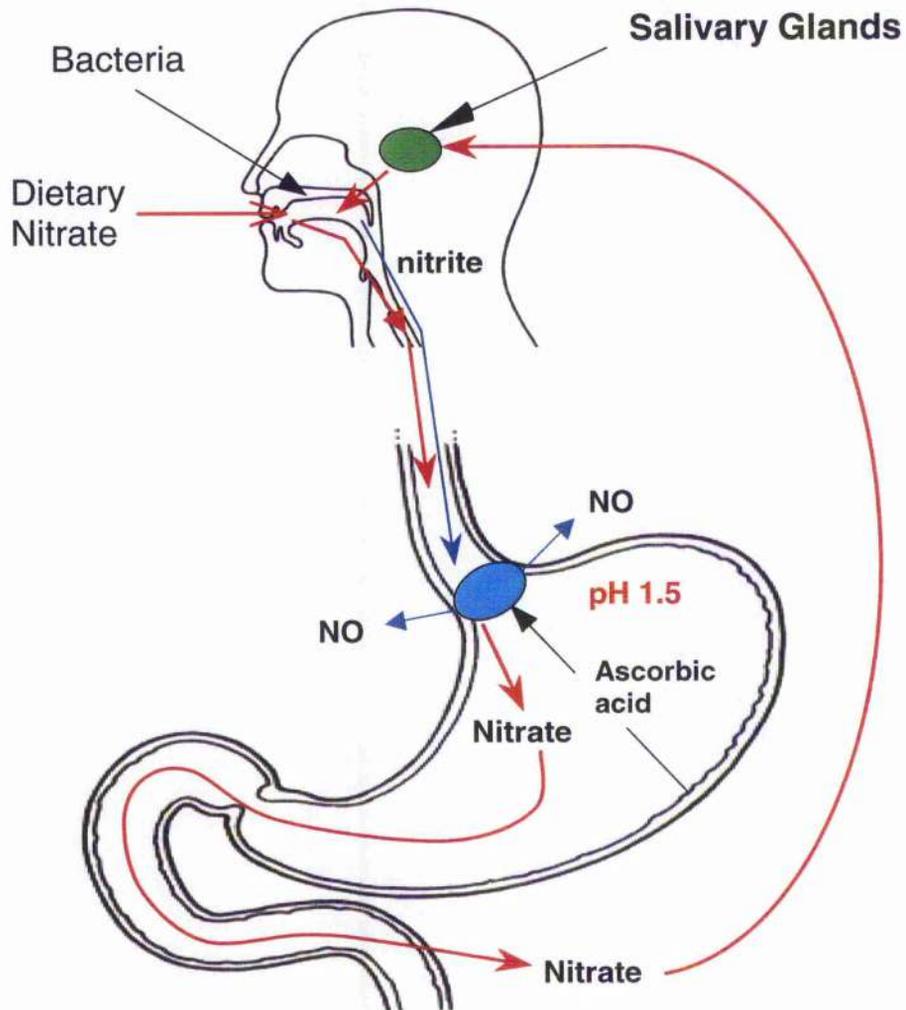


Fig 5.2: Enterosalivary recirculation of dietary nitrate in man.

Dietary nitrate is swallowed in the saliva and absorbed in the in the proximal small intestine. 25% is taken up and concentrated in the salivary glands and secreted into the oral cavity. Bacteria on the dorsum of the tongue reduce 20 - 30% of the nitrate to nitrite which is then swallowed in the saliva. The nitrite on contact with the gastric juice becomes acidified and rapidly reacts with ascorbic acid in gastric juice and generates nitric oxide in the process.

5.7.3 Ascorbic acid secretion in the human stomach.

Ascorbic acid is actively secreted into the gastric lumen as the concentration of ascorbic acid in gastric juice of histologically normal stomach is much higher than the concentrations of ascorbic acid found in the plasma^(274, 275). In histologically normal stomach, virtually all of the ascorbic acid is in the reduced form (range 83 – 100%)⁽²⁷⁴⁾. Ascorbic acid concentration in gastric juice is higher in histologically normal stomach than in stomachs with chronic *H. pylori* gastritis or atrophic gastritis^(263, 275, 289). Rokkas and colleagues have demonstrated that the Cag A(+) strain *H. pylori*, which produce greater gastric inflammation, have lower total vitamin C in the gastric juice compared to subjects infected by the Cag A(-) strain⁽²⁹⁰⁾. Gastric juice ascorbic acid concentration was shown to be inversely correlated with the degree of corpus inflammation^(263, 289, 291). Eradication of *H. pylori* restores the gastric juice ascorbic acid towards normal levels^(292, 293)

In addition to the *H. pylori* gastritis, the ascorbic acid concentration is reduced in subjects with gastric juice pH > 4, including subjects who become hypo/achlorhydric during treatment with a proton pump inhibitor, when the predominant form of ascorbic acid is in the oxidized form of dehydroascorbic acid^(264, 265, 266, 274). The reduced form of ascorbic acid is unstable in neutral pH and the presence of oxidants in neutral/achlorhydric gastric juice may explain the low ascorbic acid concentrations in achlorhydric gastric juice⁽²⁶⁴⁾.

5.8 Evidence for intragastric nitrosation in the acid-secreting human stomach.

In vitro experiments have demonstrated the formation of nitrosamines in gastric juice collected from humans when incubated with sodium nitrite and secondary amines^(294, 295, 296). Although large quantities of nitrite and amines were used, these experiments showed the potential for acid-catalysed nitrosation in human gastric juice. Subsequently it was shown that feeding nitrate to human subjects followed by proline led to increased urinary excretion of *N*-nitrosoproline indicating endogenous synthesis⁽²⁹⁷⁾. Using 3.5 mmol [¹⁵N] nitrate and 4.3 mmol L-proline Wagner demonstrated [¹⁵N] *N*-nitrosoproline excretion of up to 187 nmol/24hour again indicating endogenous nitrosation occurring in the stomach⁽²⁹⁸⁾.

Simultaneous ingestion of normal food high in nitrates (e.g. spinach) and amines (e.g. cod) results in increased urinary excretion of *N*-nitrosamines⁽²⁹⁹⁾. Such studies clearly indicate that human stomach is an important site for acid-catalysed nitrosation and that normal dietary constituents may lead to the intragastric synthesis of *N*-nitroso compounds. Furthermore, ascorbic acid has been shown to inhibit endogenous nitrosamine formation in man. Concomitant ingestion of ascorbic acid with proline in subjects living in a high-risk region for oesophageal cancer in China significantly reduced the urinary excretion of *N*-nitrosamino acids including nitrosoproline⁽²³⁵⁾. Using [¹⁵N] nitrate, it was shown that pre-loading subjects with ascorbic acid in addition to administering ascorbic acid with nitrate and proline reduced incorporation of [¹⁵N] nitrate into nitrosoproline by 81%⁽²⁹⁸⁾.

These studies clearly show that there is the potential for intragastric nitrosation to occur in human subjects ingesting nitrate in the presence of nitrosatable chemicals in the diet and ascorbic acid can inhibit intragastric nitrosation.

CHAPTER SIX

HYPOTHESIS

6.1 Nitrate utilisation.

Since the Second World War, nitrogenous fertilizer use has increased considerably as a result of intensive farming methods (Fig 6.1). Both the increase in the nitrate content of ground water ⁽⁹⁰⁾ and the increase in the nitrate content of some vegetables during 1960s to 1970s have been attributed to the increased use of nitrogenous fertilizers ⁽³⁰⁰⁾. In the United Kingdom the majority of public water supplies have less than the acceptable level of nitrate ($< 50 \text{ mg L}^{-1}$) as legislated by the European Community. However in 1985, just over 1 % of the tap water samples in the UK exceeded 50 mg L^{-1} of nitrate and $< 0.01 \%$ received water exceeding 80 mg L^{-1} ⁽⁹⁰⁾. A study in Oxford and SE Thames region demonstrated that drinking water contributed to approximately 20 % of nitrate intake ⁽⁹⁶⁾ suggesting that the water supply may be an important additional source of nitrate. Nitrates and nitrites are used curing agents for meat and both nitrate and nitrite concentrations in meat have declined since 1960 ⁽⁹⁰⁾. In the United Kingdom, dietary estimates from the Total Diet Study show that average dietary nitrate intake in 1979 ⁽²⁷⁹⁾ was approximately 61 mg per day and approximately 53 mg per day in 1997 with the vegetarians consuming 93 mg per day ⁽³⁰¹⁾.

6.2 Social Class and salivary nitrate/nitrite: a possible link between cardio-oesophageal adenocarcinoma.

Forman compared the salivary nitrate and nitrite concentrations in areas of low gastric cancer incidence (Oxford, Canterbury and Eastbourne) to areas of high gastric cancer incidence (Hartlepool, Sunderland, Bangor and Llandudno). Both the salivary nitrate and nitrite concentrations were higher in the more affluent regions (with lower incidence), compared to the less affluent regions (with high incidence) in both the male and female population. They also noted a significantly higher salivary nitrate and nitrite concentrations in the higher social class compared to the lower social classes within the region of low gastric cancer incidence ⁽⁹⁶⁾. Unlike distal gastric cancer which is a disease of the lower social class, adenocarcinoma of the cardia and oesophagus have no such distinctions and

some studies point to an increased risk of these cancers in higher social standings (see 2.6 above).

The rise in incidence of the oesophageal and cardia adenocarcinoma is apparent 20 to 30 years after the increased utilisation of nitrogenous fertilisers which is the accepted time scale between exposure to a carcinogen and development of cancer (Fig 6.1). The findings of non-cardia gastric cancer occurring more commonly in the lower social class whereas cardia and oesophageal adenocarcinoma may be occurring more often in the middle class subjects hint at the potential role of dietary nitrate in the pathogenesis of cardia and oesophageal adenocarcinomas.

6.3 The pathophysiological process leading to adenocarcinoma of the oesophagus and cardia occurs in the lumen of the distal oesophagus and the cardia.

The cardia is a unique site where the environment changes from the neutral pH of the oesophageal lumen to an acid pH of approximately 2.5⁽²¹¹⁾. This is also the site where salivary nitrite, derived from the enterosalivary recirculation of dietary nitrate, first meets acidic gastric juice. On contact with the acidic gastric juice, nitrite in saliva becomes protonated to nitrous acid which forms nitrosating species such as N_2O_3 and NO^+ which can react with nitrosatable amines to generate *N*-nitrosamines⁽²³⁶⁾ (Fig 6.2). This acid-dependent nitrosation is catalysed by the thiocyanate anion (SCN^-), present in the human saliva and gastric juice, by forming the nitrosating species, NO^+SCN^- . The major inhibitor of the nitrosation reaction in the human stomach is ascorbic acid which is actively secreted into gastric juice. The nitrosating species are rapidly reduced to nitric oxide by the ascorbic acid which itself is oxidised to dehydroascorbic acid in the process. When nitrite is present in excess of ascorbic acid, nitrosating agents can react with nitrosatable substances to form *N*-nitroso compounds. In subjects without gastro-oesophageal reflux, the cardia is presented with the greatest nitrite load and ascorbic acid will be the most depleted in this region, a condition likely to favour the formation of the carcinogenic *N*-nitroso compounds which may possibly lead to the development of adenocarcinoma of the cardia.

In subjects with Barrett's oesophagus, reflux of the gastric juice into the distal oesophagus will shift the nitrosative chemistry proximally into the Barrett's segment and the formation of *N*-nitroso compounds will potentially occur in the lumen of the distal oesophagus which may lead to the development of oesophageal adenocarcinoma.

In addition to the generation of intraluminal *N*-nitroso compounds, nitric oxide is generated at the cardia from the reaction between gastric juice ascorbic acid and salivary nitrite in subjects without reflux ⁽²⁷⁸⁾. In subjects with Barrett's oesophagus the process is likely to occur more proximally in the distal oesophagus during periods of acid reflux. Nitric oxide formed locally may then diffuse into the surrounding epithelium and shift the nitrosative stress intracellularly where in the presence of oxygen, it can form the nitrosating agent N_2O_3 and generate *N*-nitrosamines intracellularly ^(302, 303) and subsequent DNA alkylation. Furthermore nitric oxide inhibits key DNA repair enzymes ^(304, 305, 306) which may lead to mutagenesis and carcinogenesis at the cardia and the distal oesophagus.

6.4 Aims of the thesis.

The aims of this thesis were:

1. To study the nitrosation chemistry at the cardia in asymptomatic subjects without gastro-oesophageal reflux to test the hypothesis that in subjects without reflux the cardia is the site with optimal conditions for the formation of *N*-nitroso compounds.
2. To study the nitrosation chemistry in patients with Barrett's oesophagus and test the hypothesis that the optimal conditions for *N*-nitroso compound formation will be in the Barrett's segment during gastro-oesophageal reflux.
3. To study nitric oxide production in patients with Barrett's oesophagus during acid reflux.

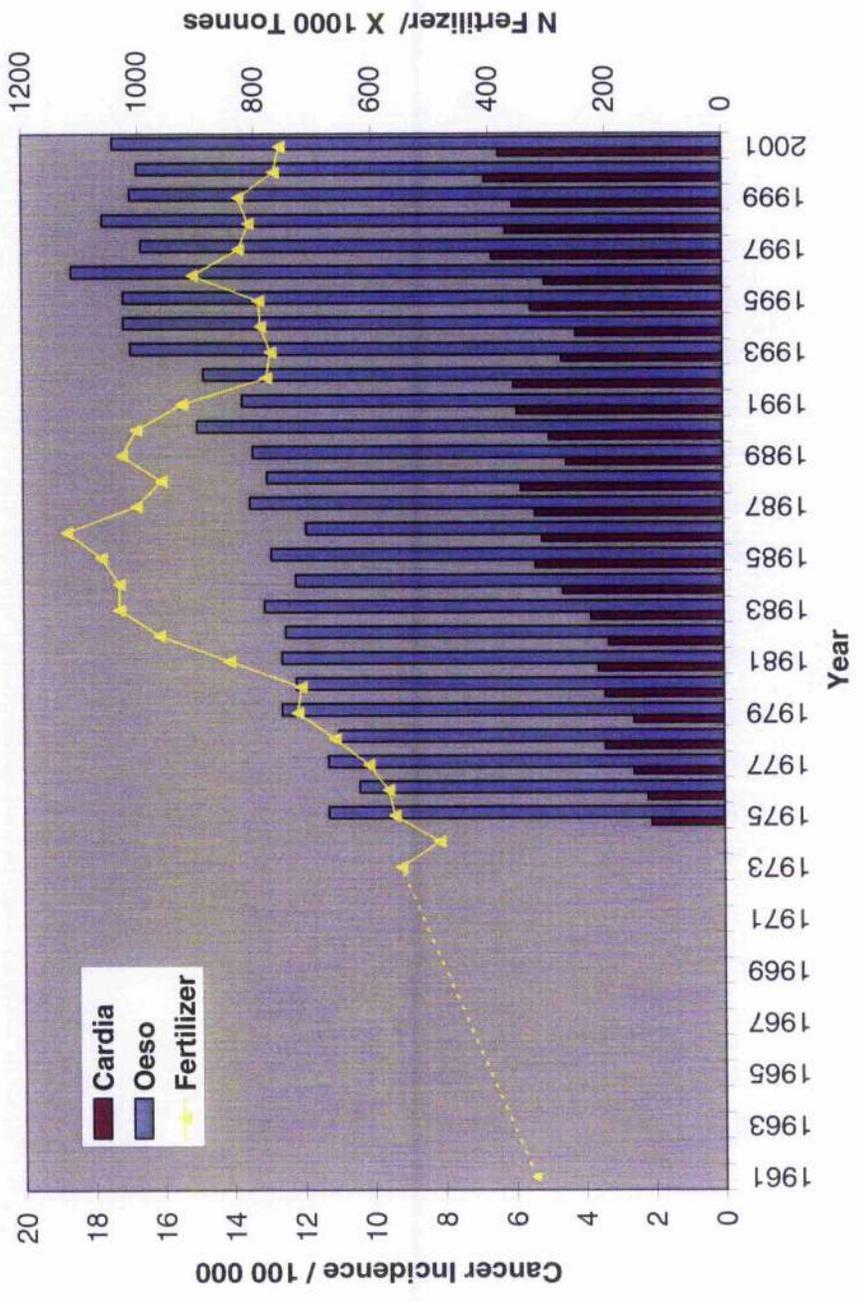


Fig 6.1: Nitrogenous fertilizer use in United Kingdom and the age-standardised incidence rate per 100 000 person-years at risk (European standard population) for adenocarcinoma of the cardia and oesophagus in Scottish male (307,308)

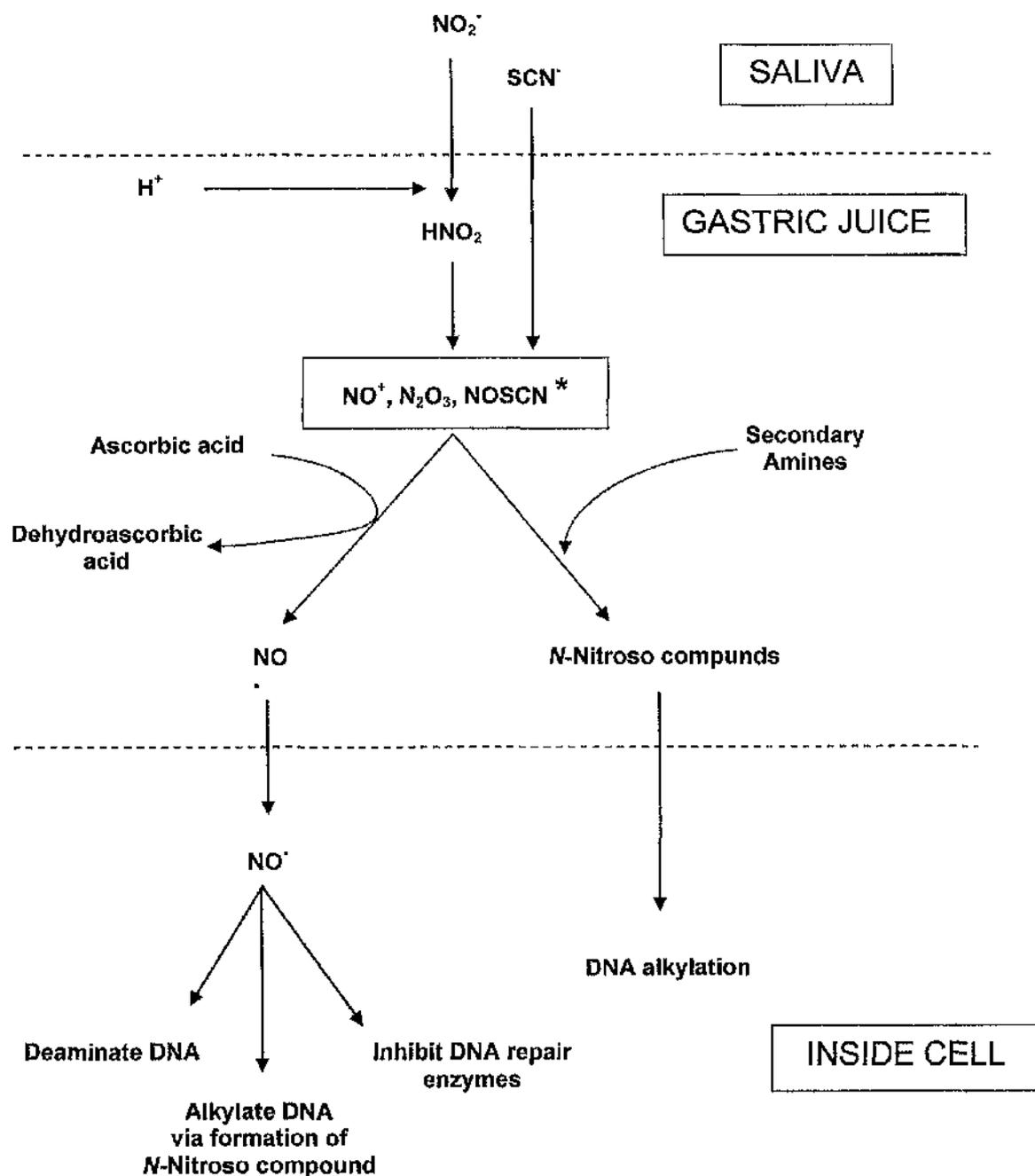


Fig 6.2: Nitrosation chemistry in the upper gastrointestinal tract.

Nitrite in saliva is swallowed and enters the gastric juice where it forms nitrous acid (HNO_2) and nitrosating species (* above). Thiocyanate (SCN^-), present in saliva and gastric juice, catalyses the acid nitrosation. Ascorbic acid (AA), in gastric juice competes with the secondary amines for the nitrosating species which it reduces to nitric oxide and the AA is oxidised to dehydroascorbic acid. When AA is depleted, this enables the nitrosating species to react with secondary amines to generate *N*-nitrosamines. The nitrosamines can diffuse into the cell and alkylate DNA. The nitric oxide formed intraluminally may diffuse into the surrounding epithelium and can cause DNA damage through DNA deamination, DNA alkylation and inhibiting DNA repair enzymes.

CHAPTER SEVEN

GENERAL METHODS

7.1 Introduction.

The experiments for this thesis required apparatus that had the capability of measuring chemicals in the lumen of the human upper gastrointestinal tract at several different levels simultaneously. The equipment needed to be small in order to provide reasonable comfort for the study subjects. The microdialysis probes provided all the prerequisite conditions for the experiments. The following sections outline the microdialysis probe, its principle and some of its applications and technical considerations when utilising the microdialysis probe for sampling chemicals of interest.

All chemicals used in the studies were obtained from Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK.

7.2 Microdialysis.

The principal use of the microdialysis probe is for sampling the extracellular chemicals. Analysis of the extracellular environment of the dog brain was first performed in the 1960s where a 'push-pull' technique in which fluid was infused and withdrawn simultaneously through two separate cannulae ⁽³⁰⁹⁾. Hollow fiber microdialysis probe was first developed by Ungerstedt and with further development it has become possible to implant the probe into tissues such as the brain, subcutaneous tissue and muscles amongst other applications ^(310, 311). Microdialysis probes have been used to measure luminal concentrations of chemicals in the rat uterus ⁽³¹²⁾, chemicals in the blood stream ^(313, 314), and the intestinal lumen ⁽³¹⁵⁾.

7.2.1 Microdialysis Probes.

There are a number of different types of microdialysis probes⁽³¹⁶⁾. All work on the principle of dialysing chemicals across a semipermeable membrane. Chemicals with low molecular mass diffuse into or out of the probe depending on the concentration gradient between the surrounding medium and the fluid used to perfuse the probes.

The probes we utilised for the experiments were the concentric type CMA flexible microdialysis probes (MAB 7.8.10, Biotech Instruments Ltd, Herts, UK) (figs 7.1 and 7.2). The overall length of the probe is 2.2 cm with a maximum diameter of 2 mm. The dialysing membrane is 10mm with a 15 kD molecular weight cut-off made of polyether-sulphone. The dead space of the inlet and outlet tubing is 25 μ L. The probe has a fine 1 metre-long inlet (afferent) tube leading to the microdialysis chamber and a similar outlet (efferent) tube returning from this chamber. The inlet tube is attached to a microdialysis pump (Univentor 864 microdialysis syringe pump, Biotech Instruments Ltd., UK) capable of driving 6 syringes simultaneously at a pre-selected rate (fig 7.3). The microdialysis probes are perfused with distilled water degassed with helium through the afferent tubing. Due to the equilibration across the semipermeable membrane, the concentration of solutes in the sample obtained from the efferent tubing provides a measure of their concentration in the test solution. There is a lag time which is depending on the rate of perfusion of the microdialysis probe for the perfusate to return from the microdialysis chamber to the end of the efferent tube.

7.2.2 Terminology.

The solution into which the microdialysis probe was immersed will be termed the test solution (or gastric juice). The fluid used to perfuse the microdialysis probes will be termed the perfusate. The samples collected from the efferent tube following microdialysis sampling will be termed the microdialysis product (also known as the dialysate). The concentrations of the chemicals recovered in the microdialysis product relative to the concentrations of the chemicals in the test solution will be termed the relative recovery (or recovery) and is given as a percentage of the concentrations in the test solution.

7.2.3 Sampling parameter considerations.

There are a number of technical factors that need to be considered when sampling with a microdialysis probe. The microdialysis sampling is rarely performed under conditions of equilibrium and the final sample represents a fraction of the actual concentration in the medium sampled. This is known as the relative recovery usually expressed as a percentage of the original sample concentration. For a given set of conditions the relative recovery of the chemical of interest will be constant and is determined by its concentration gradient. In turn the relative recovery is determined by a number of factors which include the following ^(316, 317):

1. The rate of perfusion: The flow rate utilised usually ranges between 0.5 – 5 $\mu\text{L min}^{-1}$ ⁽³¹⁰⁾. The slower the perfusion rate the greater the relative recovery of the analyte of interest as the process is diffusion dependent. A higher flow rate may increase the hydrostatic pressure sufficiently inside the probe, which is affected by the probe cross-sectional area, resulting in net extravasation of fluid out of the probe reducing the diffusion of analyte into the probe. However the limitation of the slower perfusion rate is the smaller volume of dialysate available for analysis which requires highly sensitive analytical methods. Provided that the temporal resolution is not critical then the collection interval can be increased to increase the yield.
2. The surface area of the semipermeable membrane: The greater the surface area the greater the relative recovery and this can compensate for selecting a higher perfusion rate.
3. The membrane and analyte properties: In addition to the physical size of the chemical of interest (analyte) and the pore size of the membrane, properties of both the membrane and the analyte may lead to physical or chemical interactions. The membrane is usually hydrophilic therefore the pores are filled with aqueous solution and lipophilic substances show lower relative recoveries than chemicals that are non-lipophilic. Electrostatic or adsorption interactions can also occur between the membrane and the analyte.
4. The composition and temperature of the perfusate may alter the recovery of the analyte: For example, using hypotonic and isotonic perfusates at both 24°C

and 38°C the recovery of paracetamol from rat cerebral cortex was lower in both the hypotonic perfusates particularly at 38°C compared to isotonic perfusates with similar recoveries at 24°C and 38°C⁽³¹⁷⁾. Generally, higher temperature increases recovery due to greater diffusion.

5. Physiological processes such as diffusion of the analyte in the medium, transport, uptake, release, binding and metabolism: Under certain experimental conditions, alterations in the extracellular environment by removal/addition of the chemicals of interest may alter the physiological process.

7.2.4 Setting up the microdialysis probe.

In order to perfuse the microdialysis probes with dialysing fluid, it is necessary to connect the inlet tubing to a syringe with an adapter. The adapter was made by sawing off the tip of a 21 gauge hypodermic needle (Becton Dickinson UK Ltd) to 1cm length, making sure that the bore was circular and not compressed as this will affect the perfusion pressure of the probes. The connection tubing on the inlet tube supplied with the microdialysis probes was attached to the adapter.

A 5 mL plastic syringe (Beckton Dickinson UK Ltd, Roborough, Plymouth, UK) was filled with double-distilled, deionised water degassed with helium and gently tapped until all gas bubbles were eliminated from the syringe. The syringe was then connected to the modified needle adapter. The syringes with the microdialysis probes attached were then mounted on the microdialysis pump and perfused at a preset rate. The probes are ready to use when droplets of fluid, the dialysate appears from the outlet tube.

The microdialysis membrane is fragile and must be handled very carefully. If the tip is bent as a result of handling mishap then the probe should be discarded.

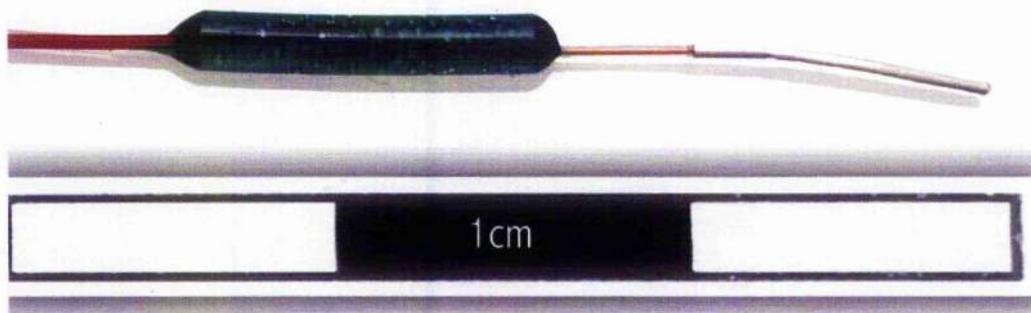


Fig 7.1: The flexible CMA microdialysis probe.
The microdialysis probe is of a concentric design (see fig 7.2).
The white semipermeable tip is made of polyethersulphone with a molecular cut-off of 15k Dalton.

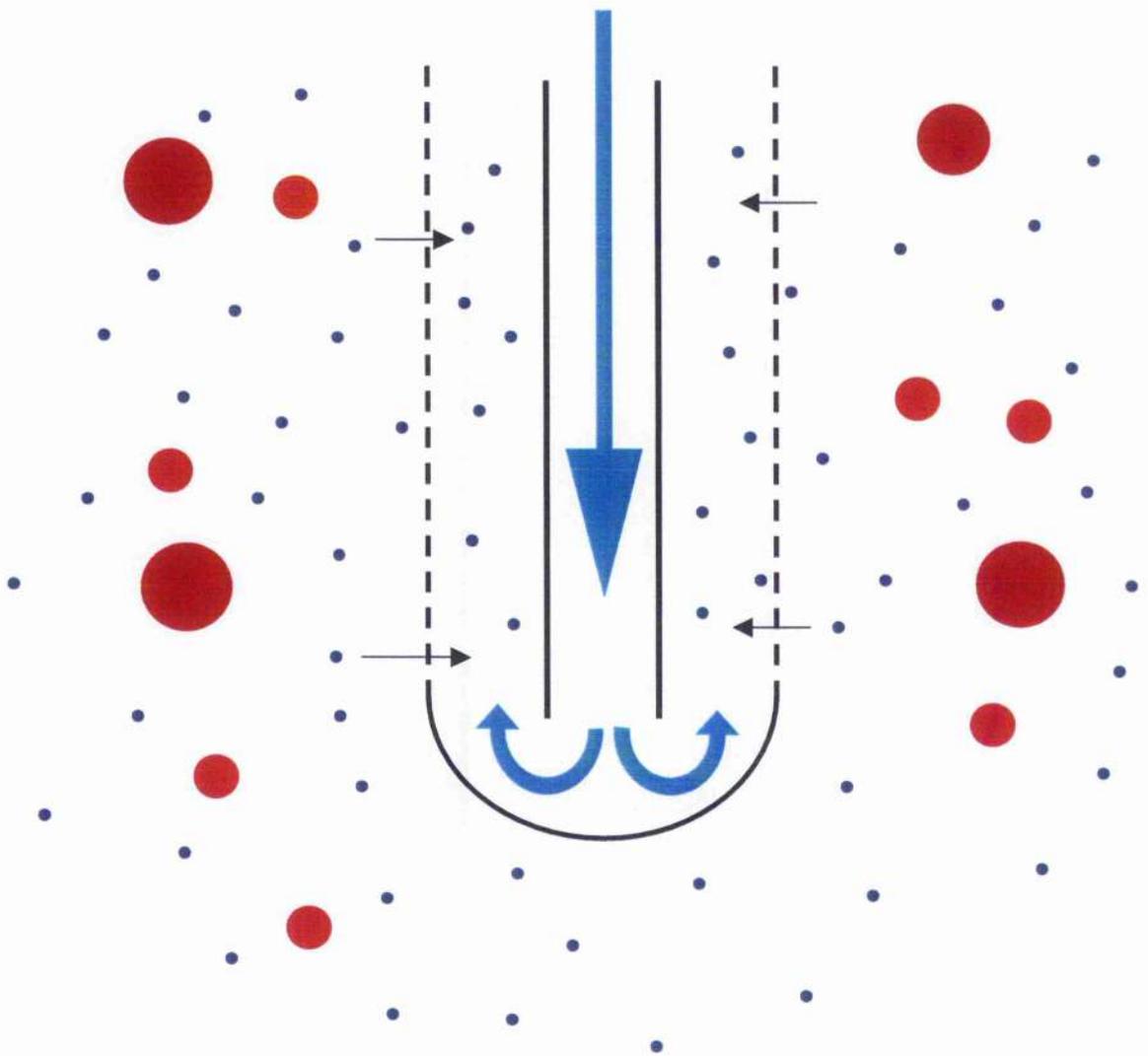


Fig 7.2: The concentric microdialysis probe tip.

The perfusate (blue arrow) enters the probe tip via the inner tube and washes out the chemicals small enough to diffuse through the semipermeable membrane via the outer tube thus maintaining the diffusion gradient.

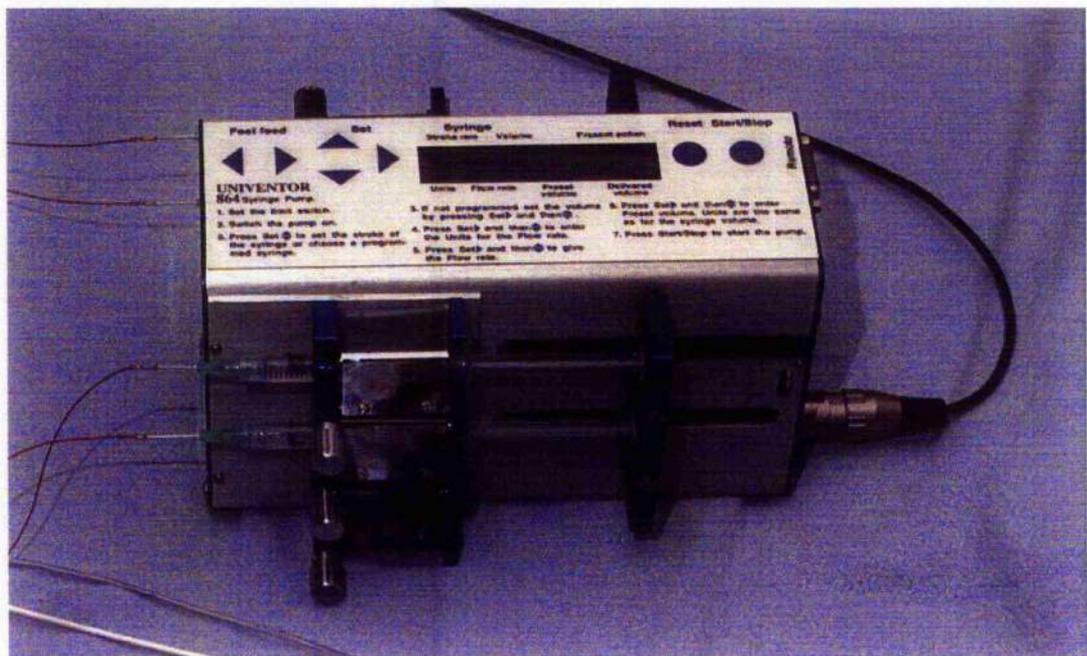


Fig 7.3: Univentor 864 microdialysis syringe pump (Biotech Instruments Ltd., UK)

The syringe drive is capable of driving 6 syringes simultaneously at a low flow rate. The figure also illustrates the 21G hypodermic needle adapted for attaching the microdialysis afferent tubing.

7.2.5 Collecting the microdialysis products.

The dialysate was collected in a 300 μL glass chromatography vial (03-CVG, Chromacol Ltd., Herts, UK). For nitrite and thiocyanate analysis, the microdialysis product was collected in a chromatography vial containing 20 μL of 1 M NaOH. This prevented the loss of nitrite which occurs at highly acidic pH leading to an underestimate of the nitrite concentration. For ascorbic acid and total vitamin C analysis, the microdialysis product was collected in 20 μL of equal volume (1:1) 2% m/v metaphosphoric acid / 0.5 % m/v sulphamic acid (M/S) which acted as a stabilising solution. One half of the product was then added to an equal volume of M/S further diluting the product by a factor of 2. This sample measured the ascorbic acid. The other half of the product was added to an equal volume of M/S plus 12 mg mL^{-1} dithiothreitol (DTT) (see 7.3.1). The DTT reduces oxidised ascorbic acid back to its reduced form and enables the estimation of the total vitamin C (which represents oxidised + reduced ascorbic acid).

7.3 Analysis of samples collected by microdialysis.

The microdialysis experiments are limited by the small volumes of dialysis products produced in a given experiment and thereby limited by the sensitivity of the assay method. Such methods must be able to handle small sample volumes often in the nano to microlitre ranges with concentrations as low as pico to nanomoles.

High performance liquid chromatography (HPLC) combined with various detectors is well-suited to the analysis of samples obtained from microdialysis experiments. We used the HPLC coupled to an electrochemical detector to measure the ascorbic acid and the total vitamin C concentrations.

Nitrite and thiocyanate concentrations were determined using colorimetric methods as the concentrations of these chemicals found in the human upper gastrointestinal tract are easily detectable by the methods described below.

7.3.1 Determination of ascorbic acid and total vitamin C by high performance liquid chromatography.

The ascorbic acid and total vitamin C levels were measured by high performance liquid chromatography employing an electrochemical detector as described by Schorah and colleagues⁽²⁷⁴⁾. The instruments comprised of an automated sample injector (Shimadzu SIL-10AD, Shimadzu Corporation, Japan) with a 50 μL loop, a pump (Shimadzu LC-10AT VP), an electrochemical detector (Shimadzu L-ECD-6A) set at 350 mV and 0.2 μA and an integrator (Shimadzu C-R3A Chromatopac). Ascorbic acid was separated using the reverse-phase, ion-pair chromatography on Phenomenex 5 μm C18 Luna 150 x 4.6 mm analytical column (Phenomenex, Macclesfield, Cheshire, UK) protected by a guard column 20 x 3 mm (Anachem, Luton, Bedfordshire, UK) hand-packed with Lichroprep RP-18 (25-40 μm , BDH-Merck Ltd., Poole, Dorset, UK). A precolumn (Phenomenex Security guard C18 ODS Octadecyl filters 4 x 3 mm (i.d.)) was also used. The mobile phase consisted of 0.1 M sodium acetate containing 0.1 M octylamine and 15 % acetonitrile with a final pH of 4.3, adjusted with glacial acetic acid. The flow rate was 0.8 mL min^{-1} and the retention time of ascorbic acid was approximately 3 minutes.

An aqueous stock standard solution (1 mg mL^{-1}) was prepared by adding 5ml of ascorbic acid stock solution to 5 mL of DTT (3.5 mg mL^{-1}). Working standards of 5 and 10 $\mu\text{g mL}^{-1}$ were prepared by diluting appropriate volumes of the stock solution on the day of analysis.

The autosampler was programmed to inject 25 μL of standards, quality control and samples. The first injection was a DTT solution (3.5 mg mL^{-1}) to remove oxidising sites on the column. Subsequent standards and samples were processed with standards and quality controls occurring every tenth sample. A blank sample of 1:1 metaphosphoric acid: sulfamic acid (M/S) diluted 1 in 2 using double-distilled water was also processed for each run and subtracted from the measured ascorbic acid of the samples as the M/S solution had a positive ascorbic acid reading when analysed. No more than 80 samples were analysed in a single run to ensure that ascorbic acid degradation was minimal as standard ascorbic acid samples were stable under the laboratory conditions over this time period.

The coefficient of variation for ascorbic acid concentrations between 3.5 μM to 57 μM using this method was < 2.2 %.

i) Metaphosphoric acid/sulphamic acid 1:1 solution (M/S)

Metaphosphoric acid 5.6 g was dissolved in distilled water to make up a total volume of 100 mL. Sulphamic acid 0.5 g was dissolved in distilled water to make up a total volume of 100 mL. Equal volumes of metaphosphoric acid and sulphamic acid were mixed to make up the 1:1 M/S solution.

ii) Metaphosphoric acid/sulphamic acid 1:1 solution plus dithiothreitol (M/S + DTT)

Dithiothreitol (DTT) is a reducing agent and allows the estimation of the total vitamin C in a given specimen by reducing dehydroascorbic acid (oxidised ascorbic acid) back to ascorbic acid. Total vitamin C measured by HPLC is the sum of reduced ascorbic acid + dehydroascorbic acid. Thirty milligram DTT was added to 2.5 mL M/S solution to make up the M/S + DTT solution (12mg mL⁻¹ DTT in M/S).

7.3.2 Determination of nitrite.

The nitrite (NO_2^-) concentration was determined colorimetrically using the modified Greiss reaction ⁽³¹⁸⁾.

i) Greiss Reagent

The modified Greiss reagent was synthesized from the following chemicals: 10 g sulphanilamide, 250 mg *N*-1-naphthylethylene diamine hydrochloride, 60 mL concentrated hydrochloric acid and 475 mL distilled water (0.019 g mL⁻¹ sulphanilamide and 0.47 g mL⁻¹ *N*-1-naphthylethylene diamine hydrochloride).

ii) Sodium nitrate standards

A stock solution of 100 mM sodium nitrite (NaNO_2) was made up by dissolving 3.45 g NaNO_2 in 500 mL of distilled water. The stock NaNO_2 solution

was diluted to 1 mM using phosphate buffered saline (PBS). The 1 mM NaNO₂ in PBS was further diluted using PBS to make the following concentrations of 'standards' 10, 25, 50, 75 and 100 μM.

iii) Colorimetric determination of nitrite

A 96-well plate was used to plate the samples for determination of the nitrite concentration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled H ₂ O		10μM NO ₂ ⁻		25μM NO ₂ ⁻		50μM NO ₂ ⁻		75μM NO ₂ ⁻		100μM NO ₂ ⁻	
B												
C												
D												
E												
F												
G												
H												

The first row of the plate was used for nitrite standards as indicated in the diagram, distilled water being equivalent to 0 μM NaNO₂. The remainder of the wells were used for plating samples for nitrite determination. Using a pipette 60 μL of standards and samples were plated into each well. Sixty microlitres of Greiss reagent was added to each well followed by 60 μL of distilled water to each well. The plate was left to develop at room temperature for 20 minutes. Colorimetric analysis was performed using a 540 nm filter. The concentration of nitrite (NO₂⁻) can be deduced from the NO₂⁻ standard curve (fig. 7.4).

The co-efficient of variation for the colorimetric determination of the nitrite concentrations up to 100 μM was < 3.5%.

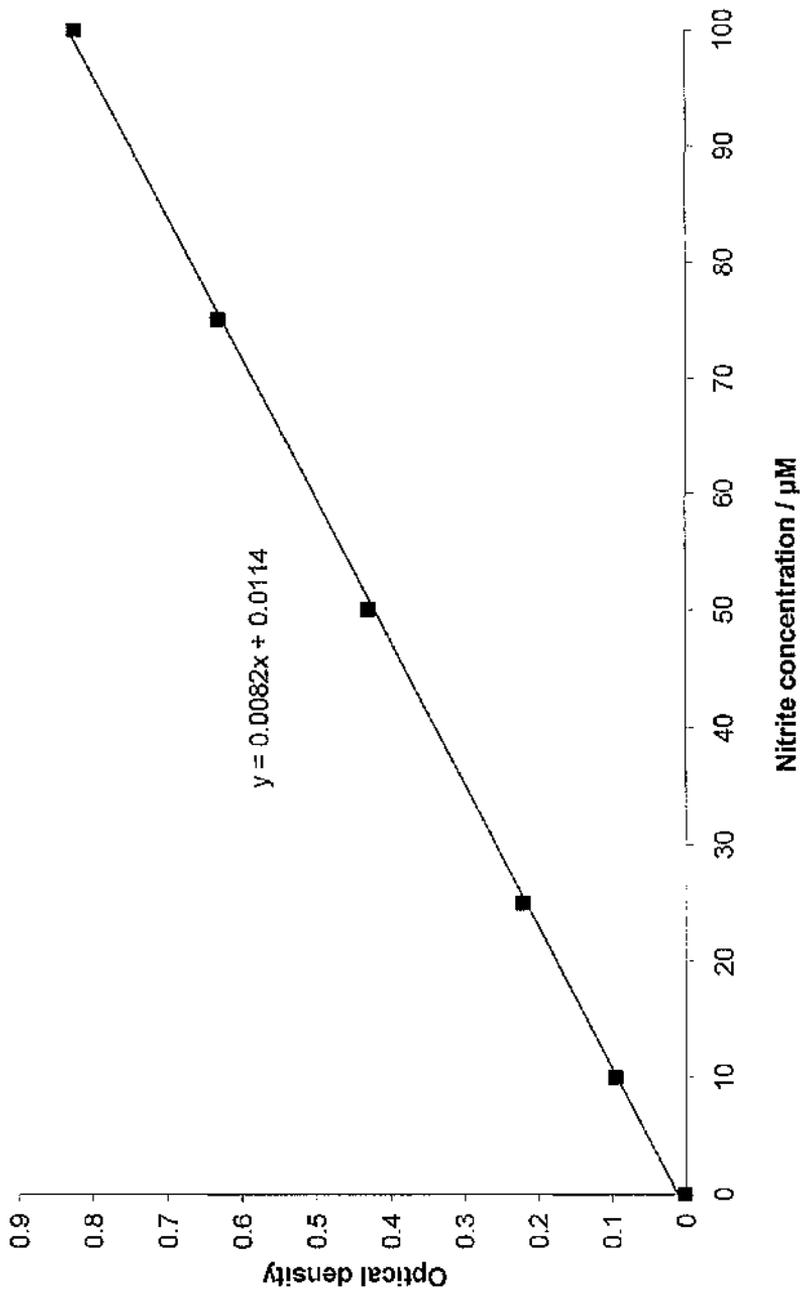


Fig 7.4 Standard curve for nitrite assay using the Greiss reagent.
The colorimetric assay was performed using a 540 nm filter.

7.3.3 Determination of thiocyanate.

The thiocyanate (SCN^-) concentration was determined colorimetrically using ferric nitrate known as the Bowler method ⁽³¹⁹⁾.

i) Ferric nitrate in nitric acid solution

Two-molar nitric acid was prepared in a fume cupboard by diluting 31.5 mL concentrated nitric acid with distilled water to make up a 250 mL solution. Eighty grams of ferric nitrate was dissolved in the 250 mL 2 M nitric acid solution. The solution was further diluted with distilled water to a total volume of 500 mL and then filtered using a filter paper. The reagent was kept in a brown bottle at 4°C until use when an aliquot was warmed to room temperature.

ii) Thiocyanate Standards

A stock solution of 1 M sodium thiocyanate (NaSCN) was made by dissolving 8.12 g NaSCN in 100mL of distilled water. Standard NaSCN solutions of 50, 100, 200, 500 and 1000 μM were prepared by diluting a stock solution of 1 M NaSCN using distilled water.

iii) Colorimetric determination of thiocyanate

A 96-well plate was used to plate the samples for determination of the thiocyanate (SCN^-) concentration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled H_2O		$50\mu\text{M SCN}^-$		$100\mu\text{M SCN}^-$		$200\mu\text{M SCN}^-$		$500\mu\text{M SCN}^-$		1mM SCN^-	
B												
C												
D												
E												
F												
G												
H												

The first row of the plate was used for SCN^- standards as indicated in the diagram, distilled water being equivalent to $0\mu\text{M SCN}^-$. Using a pipette, $100\mu\text{L}$ of standards and samples were plated into each well. One hundred μL of ferric nitrate in nitric acid solution prepared as described previously was added to the wells containing the standards and samples. Colorimetric analysis was performed immediately after adding the ferric nitrate reagent using a 450 nm filter. The concentration of SCN^- can be deduced from the SCN^- standard curve.

The coefficient of variation for the colorimetric determination of the thiocyanate concentration up to $1000\mu\text{M}$ was $\leq 4\%$.

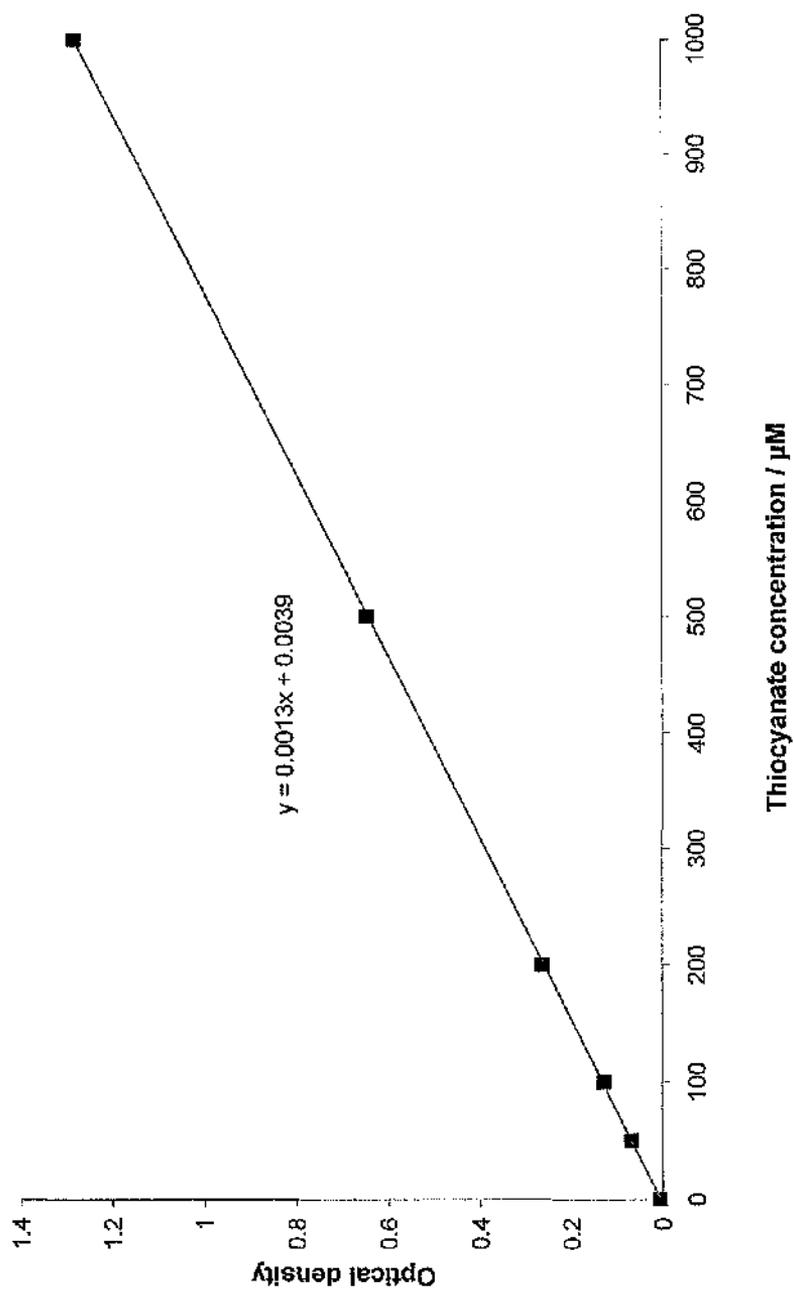


Fig 7.5 Standard curve for thiocyanate .

The Bowler method was used and thiocyanate concentrations were estimated colorimetrically using 450 nm filter.

7.3.4 Determination of nitrate.

Serum nitrate (NO_3^-) was determined using the *Aspergillus niger* nitrate reductase to reduce the nitrate to nitrite and analysed colorimetrically using the Greiss reagent as outlined above.

i) Nitrate standards

A stock solution of 100 mM sodium nitrate (NaNO_3) was made up by dissolving 4.25 g NaNO_3 in 500 mL of distilled water. This stock solution was kept in a stoppered brown glass bottle and kept at 4°C. The stock NaNO_3 solution was diluted to 1 mM using phosphate buffered saline (PBS). The 1 mM NaNO_3 in PBS was further diluted using PBS to make the following concentrations of 'standards' 10, 25, 50, 75 and 100 μM .

ii) Nitrate reductase reaction buffer

The reaction buffer was made up from the following chemicals:

a) β -Nicotinamide dinucleotide phosphate (NADPH)

Ten millilitres of distilled water was added to 50 mg NADPH. Eight hundred microlitre aliquots were pipetted into Eppendorf tubes to give 4 mg in 800 μL . Unused NADPH was stored at -20°C until subsequent use.

b) Flavin adenine dinucleotide (FAD)

Six millilitres of distilled water was added to 250 mg FAD. Eight hundred microlitre aliquots were pipetted into Eppendorf tubes to give 33.2 mg in 800 μL . Unused FAD was stored at -20°C until subsequent use.

c) Nitrate reductase

Four hundred and fifty microlitres of distilled water was added to 10 Units of *Aspergillus niger* nitrate reductase and mixed gently. Eighty microlitre aliquots were pipetted into Eppendorf tubes. Unused nitrate reductase was stored at -20°C until subsequent use.

d) PBS

e) *Distilled water*

The reaction buffer was made up after plating the samples and immediately before use. Eight hundred microlitres each of NADPH, FAD and PBS and 1520 μL of distilled water were mixed with 80 μL nitrate reductase at room temperature.

iii) *Colorimetric determination of nitrate*

The standards and samples were plated in two separate 96-well plates as shown below.

PLATE I: Total (Nitrate + Nitrite) Concentrations

	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled H_2O		$10\mu\text{M NO}_2^-$		$25\mu\text{M NO}_2^-$		$50\mu\text{M NO}_2^-$		$75\mu\text{M NO}_2^-$		$100\mu\text{M NO}_2^-$	
B	Distilled H_2O		$10\mu\text{M NO}_3^-$		$25\mu\text{M NO}_3^-$		$50\mu\text{M NO}_3^-$		$75\mu\text{M NO}_3^-$		$100\mu\text{M NO}_3^-$	
C												
D												
E												
F												
G												
H												

The first row of the plate was plated with nitrite (NO_2^-) standards and the second row with nitrate (NO_3^-) standards. Distilled water was equivalent to $0\mu\text{M NO}_2^-$ and NO_3^- .

PLATE II: Nitrite Concentration

	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled H ₂ O		10μM NO ₂ ⁻		25μM NO ₂ ⁻		50μM NO ₂ ⁻		75μM NO ₂ ⁻		100μM NO ₂ ⁻	
B												
C												
D												
E												
F												
G												
H												

The first row of the plate was used for nitrite standards as indicated in the diagram, distilled water being equivalent to 0 μM NaNO₂. The remainder of the wells were used for plating samples for nitrite determination.

The following steps were followed:

- 1) Add 60 μL of standards and samples as shown in the diagrams for plate I and II.
- 2) Add 30 μL of reaction buffer to each well of plate I.
- 3) Add 30 μL of distilled water to each well of plate II.
- 4) Both plates were incubated at 25°C in the dark for 2 hours.
- 5) Add 60 μL of Greiss reagent to each well of both plates.
- 6) Add 30 μL of distilled water to each well of both plates.
- 7) Leave at room temperature for 20 minutes.
- 8) Read plates I and II using a colorimeter with a 540 nm filter.
- 9) The nitrate concentration can be calculated from nitrite concentration in plate I (nitrite measured = nitrite + reduced nitrate) minus nitrite concentration in plate II (only nitrite). This calculated nitrate is then compared to the nitrate standard curve to obtain the actual concentration correcting for the efficiency of the nitrate to nitrite reduction by the nitrate reductase (reduction efficiency = NO₃⁻ standard / NO₂⁻ standard in plate I).

CHAPTER EIGHT

IN-VITRO STUDIES:

VALIDATION

OF

MICRODIALYSIS PROBES

8.1 Introduction

Before using the microdialysis probes in humans it was necessary to validate and construct the equipment for measuring the chemicals involved in nitrosation reaction in the lumen simultaneously at different levels of the human upper gastrointestinal tract. The initial experiments were performed using individual probes and later experiments were performed using multiple microdialysis probes assembled in a nasogastric tube which was used in the human subjects.

8.2 Effect of perfusion rate on recoveries of nitrite, thiocyanate, ascorbic acid and total vitamin C.

8.2.1 Methods.

The experiments were performed with the microdialysis probe suspended in a beaker, containing the chemical of interest, which was placed in a water bath set at 37°C. The beaker samples were stirred continuously with a magnetic stirrer.

The microdialysis probe was placed in individual solutions, pH adjusted at 37°C:

- 200 μM NaNO_2 adjusted to pH 9 with NaOH
- 500 μM NaSCN in pH 1.5 HCl
- 200 μM ascorbic acid in pH 2.5 HCl.

In the nitrite experiment, the microdialysis probe was perfused with distilled water adjusted to pH 9 with NaOH. In the thiocyanate experiment the microdialysis probe was perfused with distilled water. In the ascorbic acid experiments, the microdialysis probe was perfused with pH 2.5 HCl.

The concentration of the microdialysis product was compared to the average concentration of the chemicals in the samples taken from the beaker at 0 minutes and 40 minutes, corresponding to the beginning and the end of the experiment. The time of commencing and completing the collection of the microdialysis product was delayed according to the lag-time for the dialysate to travel the length of the outlet tubing. This was 15, 10, 7.5, 5, 3.75 and 2.5 minutes for 0.1, 0.15, 0.2, 0.3, 0.4 and 0.6 mL hr^{-1} . The dialysate was collected in a 300 μL glass

chromatography vial (03-CVG, Chromacol Ltd., Herts, UK) containing 20 μ L of 1M NaOH for nitrite and thiocyanate samples and 20 μ L of M/S for ascorbic acid and total vitamin C samples.

8.2.2 Data Analysis.

The results are given as percentage recovery – also known as the relative recovery compared to the original test solution. This was calculated as follows:

$$\left(\frac{[\text{X}] \text{ in microdialysis product} + \frac{[\text{X}]_0 \text{ beaker} + [\text{X}]_{40} \text{ beaker}}{2}}{2} \right) \times 100 \%$$

where [X] is the concentration of the chemical of interest, X; [X]₀ beaker is the concentration of X at 0 minutes; [X]₄₀ beaker is the concentration of X at 40 minutes.

8.2.3 Results.

The concentration of each compound in the test solution remained stable during the study. The recoveries for nitrite were 100, 92, 89, 74, 68, 50 %; for thiocyanate 97, 95, 95, 84, 88 and 79 %; for ascorbic acid 83, 74, 67, 53, 46 and 32 % and for total vitamin C 85, 78, 66, 56, 53 and 39 % at the respective perfusion rates of 0.1, 0.15, 0.2, 0.3, 0.4 and 0.6 mL hr⁻¹ (fig. 8.1)

8.2.4 Conclusion.

The experiment demonstrated that the relative recovery of a chemical was inversely proportional to the rate of perfusion of the microdialysis probe. Recovery of thiocyanate appears to be affected to a lesser degree than the recovery of nitrite, ascorbic acid and total vitamin C. The molecular weight of NaSCN is approximately 81 whereas NaNO₂ is 69 and that of ascorbic acid is 176. The possible explanations for the greater recovery of NaSCN, with a higher molecular weight than NaNO₂, include the differences in pH under which the experiments were conducted or the concentrations of the analyte used in the experiments, the former being the most likely explanation (see 8.6.3).

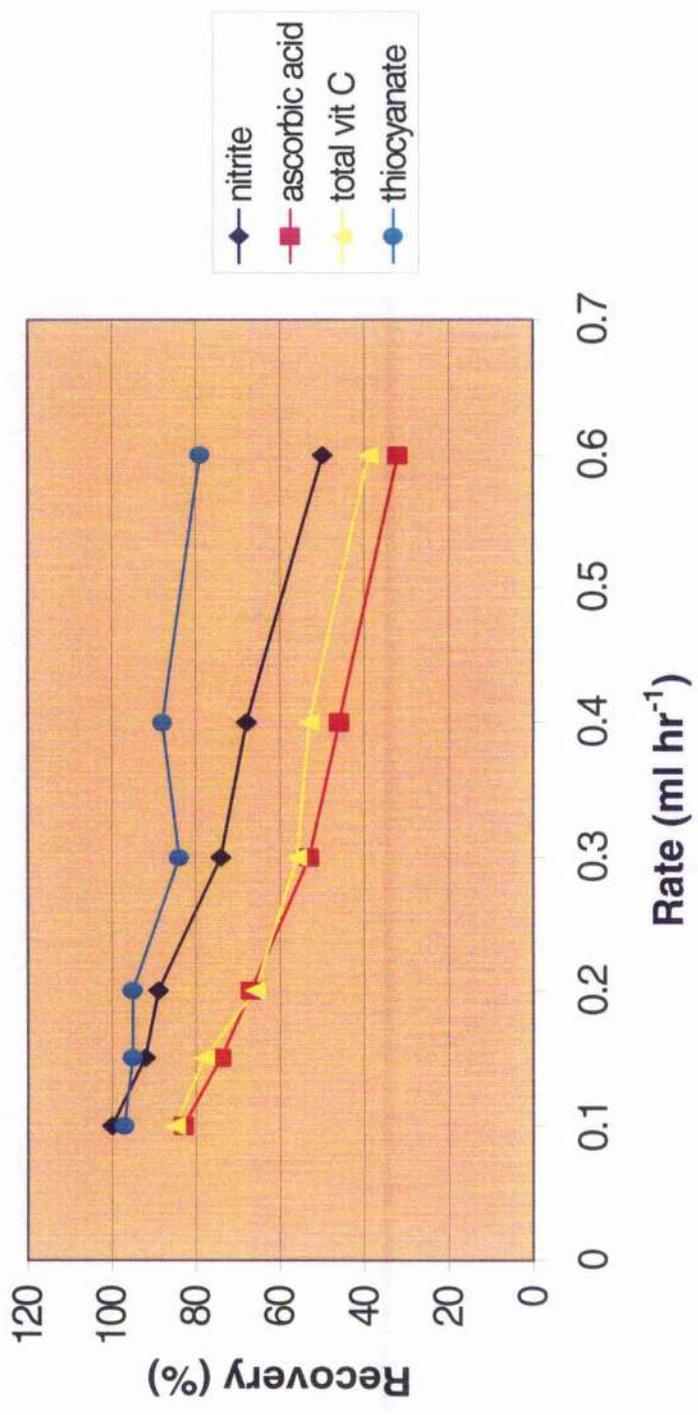


Fig 8.1: Recovery of nitrite, thiocyanate, ascorbic acid and total vitamin C at different rates of perfusion.

8.3 Effect of pH of the test solution being examined on the pH of the dialysate.

Gastric juice is highly acidic with its pH varying between 1 and 4. We therefore investigated the effect the pH of the test solution had on the pH of microdialysis product sample. The pH of the microdialysis product will depend upon the pH of the perfusate, the pH of the test solution and the recovery of hydrogen ions (H^+).

8.3.1 Methods.

In order to examine the effect of perfusing the microdialysis probe with a pH 7 solution while dialysing acidic solutions on the pH of the microdialysis product, a microdialysis probe was placed in a beaker of pH 1.0 HCl and another in a beaker of pH 2.0 HCl. The microdialysis probes were perfused at 0.1 mL hr^{-1} for 80 minutes with distilled water adjusted to pH 7.0 with NaOH. The H^+ concentration of the microdialysis product and of the acid in the beaker was measured by titration to pH 7 with NaOH.

A further experiment was performed to investigate the reverse situation where the microdialysis probe was dialysed with an acidic solution while dialysing a pH 7 solution. The microdialysis probe was placed in a beaker containing distilled water adjusted to pH 7 at room temperature and perfused with pH 2.5 HCl for 18 hours at 0.1 mL hr^{-1} to produce a sufficient volume of microdialysis product for accurate pH measurement.

8.3.2 Result.

In the former experiment where the microdialysis probe was perfused with pH 7 solution, the recovery of H^+ in the microdialysis product was 96 % and 92 % in the pH 1 and pH 2 experiments respectively giving a calculated pH of 1.03 and 2.07 respectively.

In the experiment studying the effect of perfusing the microdialysis probe with pH 2.5 HCl, the pH in the test solution fell from 7.0 to 6.09 over the time of

the experiment. The pH of the microdialysis product was 5.9. Conversion of pH to H^+ concentration for the dialysis fluid at pH 2.5 and the microdialysis product pH of 5.9 indicates 99.96 % equilibration of H^+ across the dialysis membrane.

8.3.3 Conclusions.

The above experiments indicated that throughout the range of stomach pH associated with acid nitrosation (pH 1- 4) the pH of the microdialysis product was virtually the same as the pH of the solution in which the probe was placed. This was the case whether the pH of the solution perfused was 2.5 or 7. This indicates very rapid equilibration of the H^+ ions across the dialysate membrane due to their small molecular size.

8.4 Effect of pH of the test solution on the recovery of various chemicals.

These experiments were performed to determine whether the effect of the pH of the test solution had any effect on the recovery of the chemicals of interest. The experiments were performed in both aqueous solutions and in human gastric juice obtained from subjects undergoing gastric acid secretion studies.

8.4.1 Methods.

i) Nitrite and Thiocyanate

The effect of pH on the recoveries of nitrite and thiocyanate was investigated using aqueous solutions of 200 μM $NaNO_2$ or 500 μM $NaSCN$ at pH 1.5, 2.5, 3.5, 5 and 7. Two experiments using four microdialysis probes were performed for each pH value. The pH of the test solutions were adjusted at 37°C using HCl or NaOH. The beaker containing the test solutions was immersed in a water-bath set at 37°C. The probes were perfused with distilled water at 0.15mL hr^{-1} .

The experiments were repeated with gastric juice instead of aqueous solutions. Three experiments were performed at pH 1.5 and 7 and single experiments at pH 2.5, 3.5 and 5 using four microdialysis probes per experiment. Nitrite and thiocyanate were analysed colorimetrically as described previously.

ii) Ascorbic acid and total vitamin C

Two experiments each, using four microdialysis probes, were performed using aqueous solutions of 200 μM ascorbic acid adjusted to pH 1.5, 2.5, 3.5, 5 and 7 at 37 °C using HCl or NaOH. The microdialysis probes were perfused with distilled water at 0.15 mL hr⁻¹. The experiments were repeated with human gastric juice, pH adjusted using NaOH.

8.4.2 Data analysis.

All data are presented as mean \pm S.D. Coefficient of variation is expressed as S.D./mean.

8.4.3 Results.

Aqueous solutions

i) Nitrite and thiocyanate

The mean nitrite recovery was slightly reduced at the lower pH values of pH 1.5 and 2.5 being 73 % (\pm 2.3) and 83 % (\pm 3.1) respectively compared with 88 % (\pm 2.4), 90 % (\pm 2.7) and 88 % (\pm 3.0) respectively for pH 3.5, 5 and 7 (Fig 8.2). The intra-probe coefficients of variation were < 6 %, < 5 %, < 5 %, < 6 % and < 4 % at pH 1.5, 2.5, 3.5, 5 and 7 respectively.

Thiocyanate recoveries were relatively unaffected by pH being 93 % (\pm 1.1), 91 % (\pm 2.6), 91 % (\pm 2.9), 88 % (\pm 3.5) and 89 % (\pm 1.5) at pH 1.5, 2.5, 3.5, 5 and 7 respectively (Fig 8.3). The intra-probe coefficients of variation were < 2 %, < 6 %, < 5 %, < 6 % and < 3 % at pH 1.5, 2.5, 3.5, 5 and 7 respectively.

ii) Ascorbic acid and total vitamin C

The ascorbic acid recoveries at pH 1.5, 2.5, 3.5, 5 and 7 were 71 % (± 5.2), 69 % (± 5.8), 69 % (± 4.2), 70 % (± 2.0) and 66 % (± 2.6) respectively (Fig. 8.4); for total vitamin C recoveries were 73 % (± 4.4), 74 % (± 4.8), 77 % (± 6.6), 78 % (± 4.6) and 69 % (± 2.5) respectively (Fig. 8.5).

The intra-probe coefficients of variation were < 10 %, < 13 %, < 7 %, < 6 %, < 4 % for ascorbic acid and < 12 %, < 11 %, < 11 %, < 8 %, < 5 % for total vitamin C at pH 1.5, 2.5, 3.5, 5 and 7 respectively.

Gastric Juice

i) Nitrite and thiocyanate

The nitrite recoveries were similar to those of the aqueous solution, showing slightly lower recoveries at pH 1.5 with 64 % (± 6.8) and pH 2.5 with 78 % (± 3.8) versus the higher pH 3.5, 5 and 7 with recoveries of 90 % (± 2.0), 86 % (± 2.0) and 85 % (± 4.1) respectively (Fig 8.2). The thiocyanate recoveries in gastric juice were lower than the recoveries in the aqueous solutions. The recoveries were 72 % (± 2.8), 79 % (± 2.4), 77 % (± 2.2), 75 % (± 1.8) and 70 % (± 2.8) at increasing pH 1.5, 2.5, 3.5, 5 and 7 respectively (Fig 8.3).

ii) Ascorbic acid and total vitamin C

The ascorbic acid recoveries from gastric juice at pH 1.5, 2.5, 3.5, 5 and 7 were 62 % (± 2.8), 67% (± 3.2), 63 % (± 2.4), 62 % (± 4.0) and 64 % (± 2.2) respectively; for total vitamin C recoveries were 66 % (± 3.8), 68 % (± 3.0), 72 % (± 4.6), 70 % (± 4.4), 70 % (± 3.6) respectively (Fig 8.4 and 8.5).

8.4.4 Conclusions.

Using a perfusion rate of 0.15 mL hr⁻¹ the recovery rate of each compound at 37°C was > 60 %. The recovery in aqueous solution was slightly higher than in gastric juice, particularly for the thiocyanate with a typical difference of >10 %; for nitrite, ascorbic acid and total vitamin C the differences were typically < 10 %.

The recovery of nitrite from both aqueous solutions and gastric juice at pH 1.5 and 2.5 was slightly reduced compared to that from pH 3.5, 5 and 7.

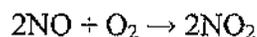
The microdialysis provided good reproducibility for the recovery of the chemicals of nitrosation in aqueous solutions with the intra-probe coefficient of variation being < 6 %, < 6 %, < 13 % and < 12 % for nitrite, thiocyanate, ascorbic acid and total vitamin C respectively. This indicated that the microdialysis probes are reliable tools for the measurement of the chemicals of nitrosation in the human upper gastrointestinal tract.

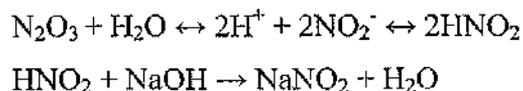
8.5 Elucidating the cause for reduced nitrite recovery at pH 1.5 and 2.5. Part I: permeability of the microdialysis tubing to nitric oxide.

The above experiments demonstrated that nitrite recovery in aqueous solution and gastric juice was reduced at pH 1.5 and to a lesser extent at pH 2.5 compared to higher pH values. When nitrite is acidified it produces nitrous acid and about 1 % molar equivalent of the nitrous acid exists in the form of nitric oxide. Nitric oxide is able to pass through various synthetic polymers. The fraction existing as nitric oxide may be able to escape through the collecting tube as the dialysate travels its length leading to the lower recovery. The following experiments were performed in order to investigate this hypothesis.

8.5.1 Method.

A 20 cm length of the microdialysis collecting tube was immersed in a solution of pH 1 HCl containing 5mM ascorbic acid and 100 μ M nitrite at 37°C. The nitric oxide concentration in this test solution was 91 μ M as measured with the nitric oxide sensor and meter (ISO-NO Mk.II, World Precision Instruments Inc, Sarasota, Florida U.S.A.). The collecting tube of the microdialysis probe was placed in this solution and perfused with 0.1 M NaOH at a rate of 0.1 mL hr⁻¹. The NaOH was used to perfuse the tube as this will entrap any nitric oxide, which diffuses into the tube, as nitrite:





The experiment was repeated with the beaker solution at pH 7.0, which generated no nitric oxide.

8.5.2 Results.

In the experiment performed at pH 1, the microdialysis product was found to have a nitrite concentration of 95 μM . In contrast, no nitrite was found in the microdialysis product in the second experiment where the beaker pH was 7 where no nitric oxide was generated.

8.5.3 Conclusions.

These studies confirmed that the collecting tube was permeable to any dissolved nitric oxide present in the dialysate. This would also imply that any unreacted H^+ ions and nitrite ions which have diffused into the microdialysis probe will be able to react to generate nitric oxide and lead to further losses during transit through the collecting tube measuring 1 metre in length.

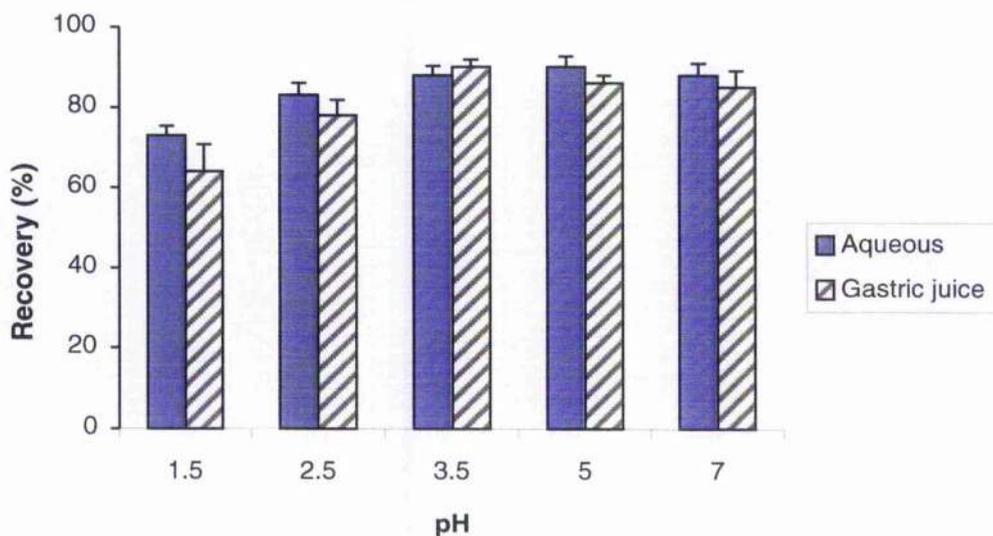


Fig 8.2: Mean nitrite recoveries in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C.

For aqueous solutions n = 8 for each pH.

For gastric juice n = 12 at pH 1.5 and 7, n = 4 at pH 2.5, 3.5 and 5.

Error bars represent + SD. Perfusion rate 0.15 mL hr⁻¹.

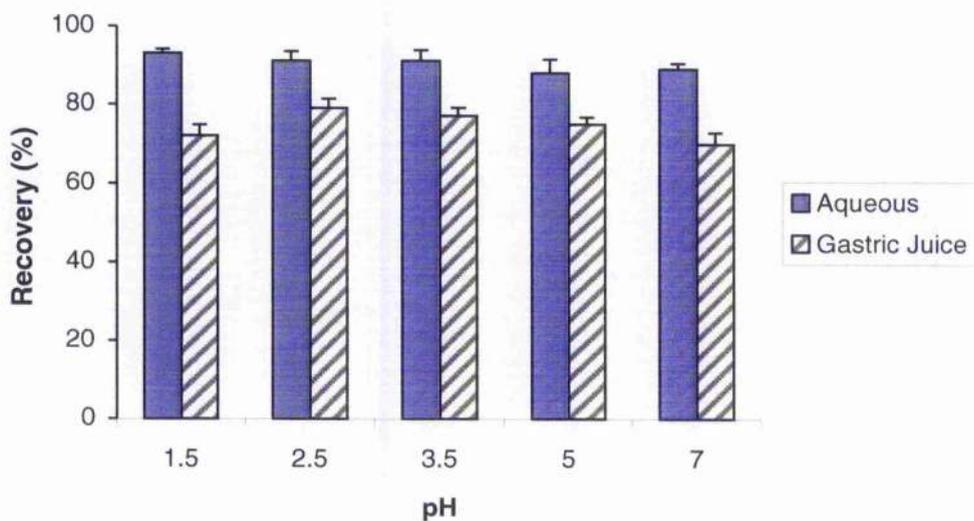


Fig 8.3: Mean thiocyanate recoveries in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C.

For aqueous solutions n = 8 for each pH.

For gastric juice n = 12 at pH 1.5 and 7, n = 4 at pH 2.5, 3.5 and 5.

Error bars represent + SD. Perfusion rate 0.15 mL hr⁻¹.

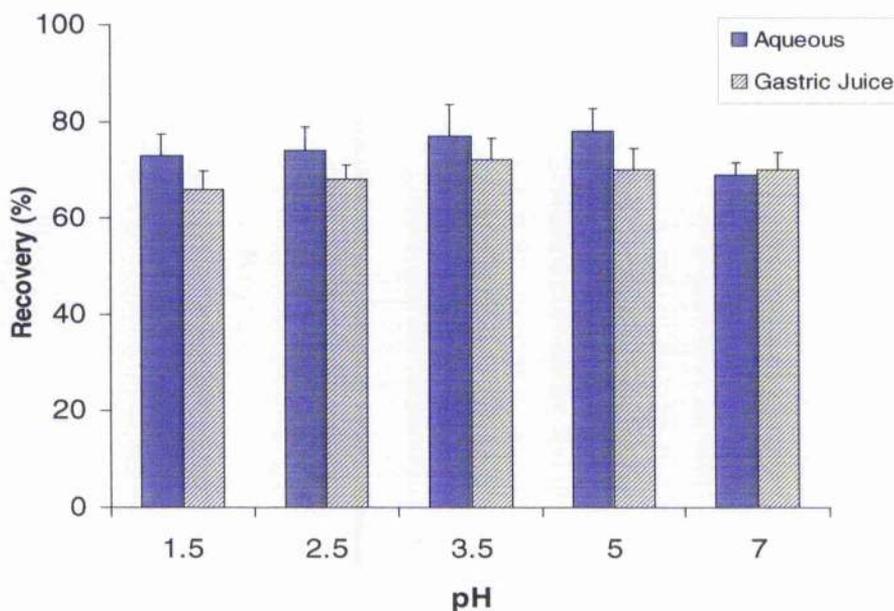


Fig 8.4: Mean ascorbic acid recovery in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C.

For aqueous solutions n = 8 for each pH.

For gastric juice n = 4 at each pH.

Error bars represent + SD. Perfusion rate 0.15 mL hr⁻¹.

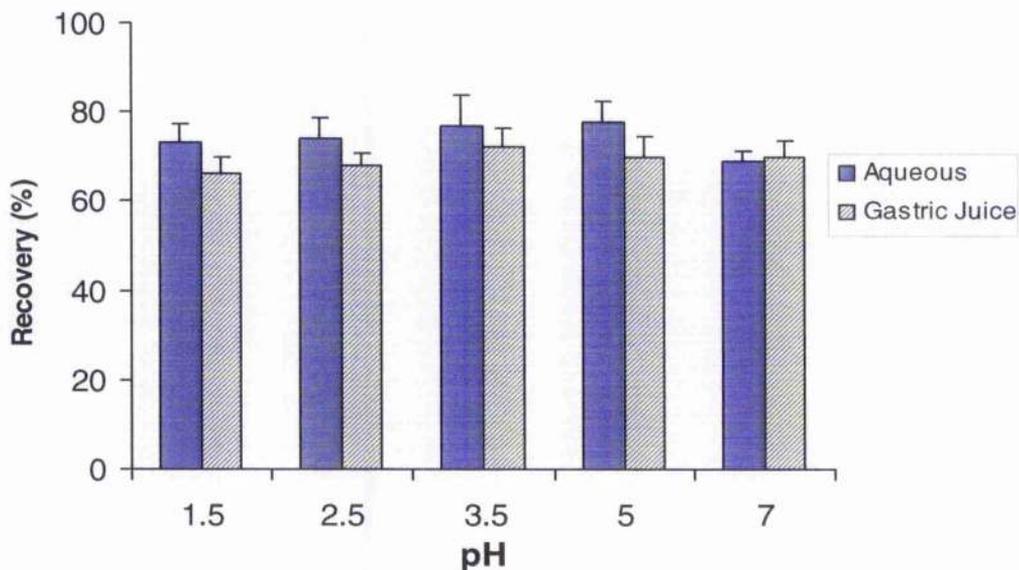


Fig 8.5: Mean total vitamin C recovery in aqueous solution and gastric juice at pH 1.5, 2.5, 3.5, 5.0 and 7.0 at 37°C.

For aqueous solutions n = 8 for each pH.

For gastric juice n = 4 at each pH.

Error bars represent + SD. Perfusion rate 0.15 mL hr⁻¹.

8.6 Elucidating the cause for reduced nitrite recovery at pH 1.5 and 2.5. Part II: formation of nitric oxide and loss of nitrite at pH 1.5 and 2.5.

The previous experiment suggested that nitrite is lost as nitric oxide during transit through the collecting tube by reacting with H^+ ions at pH 1.5 and 2.5. The amount lost in this way will depend on the time the dialysed fluid is present within the collecting tube, which is determined by the microdialysis perfusion rate. The hypothesis was tested in the following experiment.

8.6.1 Method.

The microdialysis probes were placed in aqueous solutions containing 200 μM $NaNO_2$ plus 500 μM $NaSCN$ at pH 1.5 and 37°C and perfused with distilled water at 0.1, 0.15, 0.2, and 0.3 $mL\ hr^{-1}$. By increasing the perfusion rate it was postulated that the recovery of nitrite at pH 1.5 will increase by reducing the transit time in the collecting tube thus allowing less time for nitrite and H^+ ions to react to generate nitric oxide. The improved recovery by reducing the transit and therefore the chemical reaction time will eventually be counter-balanced by the reduction in the recovery of nitrite consequent to the increased perfusion rate.

Four microdialysis probes were used simultaneously for each aqueous solution. The experiment was repeated with pH 7 aqueous solution where nitrite was stable. Further experiments were carried out in gastric juice pH 1.5 at 37°C to which 200 μM $NaNO_2$ was added but without adding $NaSCN$ (natural concentration of thiocyanate in the gastric juice was used). A single micro-dialysis probe was used for the gastric juice experiments.

8.6.2 Data Analysis.

The results are given as mean \pm S.D.

8.6.3 Results.

In contrast to the studies at higher pH, the nitrite recoveries at pH 1.5 actually *increased* as expected with increasing flow rate being 61 % (± 1.2), 72 % (± 2.4), 67 % (± 3.4), 71 % (± 3.7) at 0.1, 0.15, 0.2, and 0.3 mL hr⁻¹ respectively (Fig 8.6). The thiocyanate recoveries were 97 % (± 2.4), 92 % (± 0.6), 95 % (± 3.3), 83 % (± 4.1).

The nitrite recoveries in aqueous solution at pH 7 were 94 % (± 2.0), 87 % (± 2.4), 84 % (± 2.4), 66 % (± 3.2) and similar to that of thiocyanates which were, 96 % (± 1.1), 86 % (± 0.8), 88 % (± 1.4), 70 % (± 2.3) at 0.1, 0.15, 0.2 and 0.3 mL hr⁻¹ respectively.

The nitrite recoveries in gastric juice again *increased* with increasing microdialysis perfusion rate being 55 %, 75 %, 77 % and 68 % at 0.1, 0.15, 0.2 and 0.3 mL hr⁻¹ respectively; whereas for thiocyanate the recoveries decreased with increasing perfusion rates being 93 %, 84 %, 75 % and 67 % respectively.

8.6.4 Conclusions.

The above studies confirm the low recovery of nitrite at pH 1.5 (and 2.5) was due to the loss of nitrite as nitric oxide through the chemical reaction between H⁺ ion and nitrite to form nitrous acid and subsequently nitric oxide. Nitric oxide readily diffuses through polymers including that of the efferent tube of the microdialysis probe leading to the loss of nitrite as the dialysate travels the length of the tubing. The recovery of thiocyanate, ascorbic acid and total vitamin C (see previous experiments) all fell with increasing microdialysis perfusion rate irrespective of the pH of the solution, whereas the recovery of nitrite at pH 1.5 did not decrease and in fact slightly increased. At pH 7 where nitrite is stable, the recovery of nitrite showed the typical pattern of decreasing recovery with increasing microdialysis perfusion rate. The absence of decreasing recovery with increasing microdialysis perfusion rate for nitrite at pH ≤ 2.5 can be explained by the higher flow rate reducing the fraction lost as nitric oxide in the collecting tube and this offsetting the reduced initial recovery across the dialysis membrane.

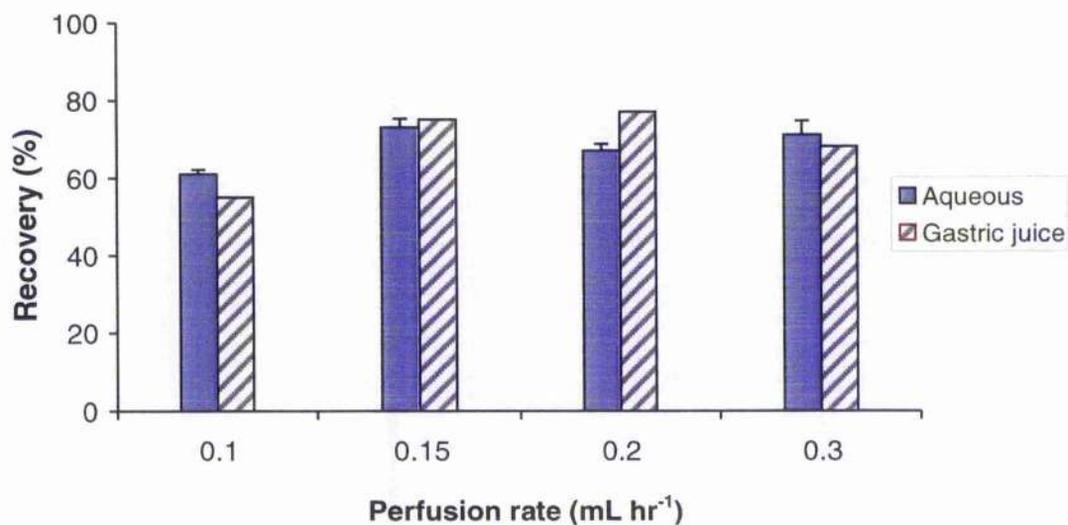


Fig 8.6: Mean nitrite recovery at pH 1.5 at different rates of perfusion in aqueous solutions and gastric juice.

For aqueous solutions, n = 4 for each perfusion rate

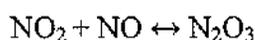
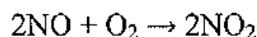
For gastric juice, n = 1 for each perfusion rate.

Error bars represent + SD.

8.7 Dynamic studies simulating the interaction between salivary nitrite and gastric juice ascorbic acid in acid stomach.

8.7.1 Introduction.

Nitrite and thiocyanate are delivered in the saliva which is swallowed intermittently and interact with the ascorbic acid in acidic gastric juice in the human stomach. Acidification of nitrite forms nitrous acid and nitrosating species such as N_2O_3 , nitrosonium ion (NO^+) or nitrosothiocyanate (NOSCN). These nitrosating species are reduced to nitric oxide by ascorbic acid, with the latter being oxidized to dehydroascorbic acid. The nitric oxide formed in this way can react with dissolved oxygen to reform nitrosating species which can in turn react with any remaining ascorbic acid. This recycling of nitrite will continue until the entire ascorbic acid is converted to dehydroascorbic acid or the oxygen is used up.



Studies were performed in order to ensure that the microdialysis probes were reliable under these dynamic conditions and that they were sufficiently sensitive to detect the presence of the chemicals under such conditions. Experiments were performed to simulate the conditions of both low and high gastric juice ascorbic acid concentrations. In the low dose ascorbic acid experiments we predicted that the ascorbic acid would not be detectable in the stomach sample and the microdialysis product. In the high dose ascorbic acid experiment, ascorbic acid should be detectable in both the stomach sample and the microdialysis product with no nitrite being detectable in the microdialysis product.

Further experiments were performed at pH 3.5 to test the reliability of the microdialysis probes under the condition where acidified nitrite reacts more slowly with ascorbic acid, and consequently the reaction between ascorbic acid and nitrite is much slower and potentially both these chemicals will co-exist.

8.7.2 Methods.

A specially constructed glass flask (artificial stomach, Fig 8.7) containing a 60 mL of 1 mM NaSCN in pH 2.5 HCl was used to simulate the stomach. Ascorbic acid was infused into the solution at a rate of either 18 $\mu\text{mol hr}^{-1}$ or 150 $\mu\text{mol hr}^{-1}$ simulating low and high ascorbic acid secretion rate into gastric juice respectively. Ten minutes after starting the ascorbic acid infusion, 1mL boluses of nitrite (1.2 mmol L⁻¹) were injected into the stomach every 3 minutes simulating the delivery of nitrite in saliva. A 2 mL sample was taken directly from the stomach at the time points corresponding to the start, middle and end of the microdialysis collection period and 0.5 mL aliquots were added to each of 0.5mL M/S 1:1 solution and 0.5 mL M/S + DTT for ascorbic acid and total vitamin C respectively. Another 0.5 mL aliquot was added to 50 μL 1 M NaOH for nitrite and thiocyanate analysis (latter only measured in 0.15 mL hr⁻¹ experiments). The microdialysis probes were perfused with distilled water at 0.1 mL hr⁻¹. The microdialysis products were collected in 20 μL of 1M NaOH for nitrite and 20 μL of M/S for ascorbic acid and total vitamin C. The samples were further diluted 1:1 with distilled water for nitrite (thiocyanate), 1:1 with M/S or 1:1 M/S + DTT for ascorbic acid and total vitamin C respectively. A nitric oxide sensor (World Precision Instruments Inc., Sarasota, Florida, U.S.A.) and meter (ISO-NO Mark II, World Precision Instruments, I.N.C., Sarasota, Florida, U.S.A.) were used to monitor the nitric oxide generated during the experiments.

Nitrite was analysed colorimetrically using the Greiss reagent, and ascorbic acid and total vitamin C analysed by HPLC as described above. Experiments were repeated at 0.15 mL hr⁻¹ perfusion rate but without monitoring nitric oxide production. Thiocyanate concentration was measured in the 0.15 mL hr⁻¹ but not in the 0.1 mL hr⁻¹ experiments.

Further experiments were performed at pH 3.5, infusing low and high-dose ascorbic acid into the artificial stomach in separate experiments. Microdialysis probes were perfused with distilled water at 0.15 mL hr⁻¹.

8.7.3 Data analysis.

The results are given as mean (\pm S.D).

8.7.4 Results.

Dynamic experiments at pH 2.5

i) Low-dose ascorbic acid infusion (Nitrite in excess of ascorbic acid)

Prior to commencing the nitrite delivery, infusing ascorbic acid at $18 \mu\text{mol hr}^{-1}$ into the simulated gastric juice resulted in a progressive rise in the ascorbic acid concentration in the samples taken directly from the stomach (performed in a separate experiment without the addition of nitrite). As soon as the nitrite administration commenced, ascorbic acid could not be detected but the concentration of the total vitamin C progressively increased indicating that the infused ascorbic acid was all oxidized to dehydroascorbic acid. The concentration of nitrite progressively increased as more nitrite was administered. There was only a small initial rise in nitric oxide reaching $8 \mu\text{M}$ on adding the first bolus of nitrite but no increase thereafter.

In the 3 studies performed at 0.1 mL hr^{-1} , the mean concentrations of nitrite, ascorbic acid and total vitamin C in the samples taken directly from the stomach during the 40min time period corresponding to the microdialysis collection period were $135 \mu\text{M} (\pm 21)$, $0 \mu\text{M}$ and $87 \mu\text{M} (\pm 57)$ respectively. The recoveries from the microdialysis probe compared to the stomach samples were $86 \% (\pm 13.8)$ for nitrite and $92 \% (\pm 15.4)$ for total vitamin C. No ascorbic acid was detectable in the microdialysis sample.

For the two 0.15 mL hr^{-1} experiments using two microdialysis probes each for nitrite and thiocyanate; two for ascorbic acid and total vitamin C. Ascorbic acid was again undetectable from both the stomach samples and four microdialysis samples. The mean nitrite, thiocyanate and total vitamin C concentrations in the stomach samples were $128 \mu\text{M}$, $727 \mu\text{M}$ and $59 \mu\text{M}$ respectively. The microdialysis recoveries for nitrite, thiocyanate and total vitamin C were $94 \% (\pm 6.4)$, $93 \% (\pm 4.2)$ and $94 \% (\pm 3.5)$ respectively.

ii) High-dose ascorbic acid infusion (ascorbic acid in excess of nitrite)

Ascorbic acid was infused into the stomach at 8-fold higher rate ($150 \mu\text{mol hr}^{-1}$). Ascorbic acid remained detectable from the samples taken directly from the stomach during nitrite administration with only a proportion being converted to

dehydro-ascorbic acid. The nitric oxide concentration progressively increased with each addition of nitrite producing a nitric oxide concentration equivalent to the concentration of nitrite added.

Five experiments were performed at 0.1 mL hr^{-1} . The mean nitrite, ascorbic acid and total vitamin C detected in the samples taken directly from the stomach were $27 \mu\text{M} (\pm 14)$, $352 \mu\text{M} (\pm 80)$ and $619 \mu\text{M} (\pm 213)$ respectively. The microdialysis recoveries for nitrite, ascorbic acid and total vitamin C were $0\% (\pm 0.8)$, $67\% (\pm 19.6)$ and $73\% (\pm 9.8)$ respectively.

Two experiments using four microdialysis probes (two each for nitrite and thiocyanate; two probes for ascorbic acid and total vitamin C) were performed at 0.15 mL h^{-1} . The mean concentrations of nitrite, thiocyanate, ascorbic acid and total vitamin C in the stomach samples were $20 \mu\text{M}$, $752 \mu\text{M}$, $619 \mu\text{M}$ and $828 \mu\text{M}$ respectively. The microdialysis recoveries for nitrite, thiocyanate, ascorbic acid and total vitamin C were 0% , $98\% (\pm 1.7)$, $54\% (\pm 2.9)$ and $82\% (\pm 1.0)$ respectively.

Dynamic experiments at pH 3.5

i) Low-dose ascorbic acid

For the three experiments, the mean concentrations of nitrite, thiocyanate, ascorbic acid and total vitamin C in the stomach samples were $137 \mu\text{M} (\pm 11)$, $774 \mu\text{M} (\pm 15)$, $10 \mu\text{M} (\pm 1.4)$ and $95 \mu\text{M} (\pm 15)$ respectively. The microdialysis recoveries for nitrite, thiocyanate, ascorbic acid and total vitamin C were $106 (\pm 6.8)$, $95 (\pm 2.5)$, $52 (\pm 4.9)$ and $78 (\pm 8.8)$ respectively.

ii) High-dose ascorbic acid

For the three experiments, the mean concentrations of nitrite, thiocyanate, ascorbic acid and total vitamin C in the stomach samples were $77 \mu\text{M} (\pm 6)$, $782 (\pm 44)$, $763 \mu\text{M} (\pm 59)$, $900 \mu\text{M} (\pm 118)$ respectively. The microdialysis recoveries for nitrite, thiocyanate, ascorbic acid and total vitamin C were $89 (\pm 11.7)$, $96 (\pm 4.2)$, $71 (\pm 2.5)$ and $85 (\pm 3.2)$ respectively.

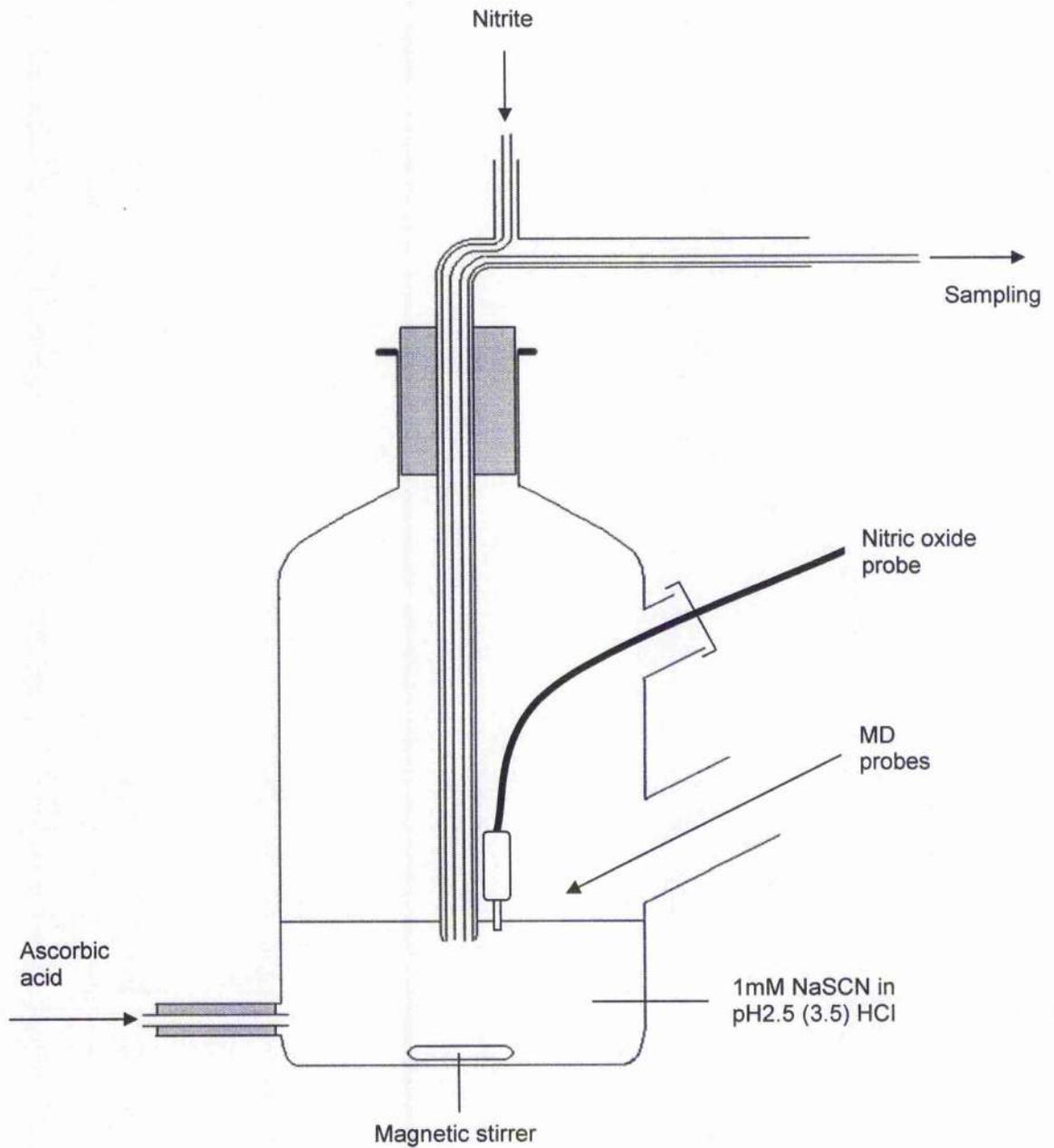
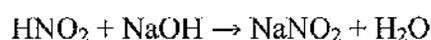
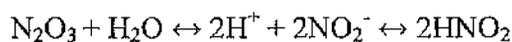
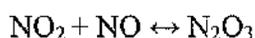
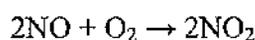


Fig 8.7: Artificial stomach used in the dynamic experiments.

The apparatus was placed in a water bath maintained at 37°C. The mixture was stirred gently using the magnetic stirrer. Ascorbic acid was delivered using a syringe drive and the nitrite bolus administered using a syringe.

8.7.5 Conclusions.

In the experiments conducted at pH 2.5 simulating the dynamic conditions of swallowed salivary nitrite interacting with low and high gastric juice ascorbic acid concentrations, the microdialysis probes proved to be reliable in measuring the chemicals relevant to nitrosation. In the low dose ascorbic acid experiments (nitrite in excess of ascorbic acid) the nitrite recovery was in excess of 86 % and greater than 90 % for both thiocyanate and total vitamin C. Ascorbic acid was non-existent in the stomach sample as expected and 0 % in the microdialysis samples. In the high dose ascorbic acid experiments (ascorbic acid in excess of nitrite) the recoveries were 0 %, > 90 %, > 50 % and > 70 % for nitrite, thiocyanate, ascorbic acid and total vitamin C respectively. The small amount of nitrite (20 μ M) detected in the stomach sample was derived from nitric oxide as follows:



The microdialysis probe reliably distinguishes this nitrite 'anomaly' as any nitric oxide entering the probe will be rapidly lost through the probe due to its highly permeable nature.

At pH 3.5 the reaction between ascorbic acid and nitrite is much slower and therefore both ascorbic acid and nitrite co-existed in solution. Both ascorbic acid and nitrite were recovered in the microdialysis product indicating that the microdialysis probes were reliable under such conditions. The nitrite recovery in the low-dose ascorbic acid experiment at pH 3.5 was higher than that in the stomach sample and is likely to reflect the loss of nitrite during sampling and processing of the stomach solution rather than an artefactual elevation of nitrite in the microdialysis sample.

CHAPTER NINE

PROBE FOR HUMAN USE

9.1 Assembly.

The probes were assembled for use in the human upper gastrointestinal tract and the recoveries of the assembled probes assessed. Four microdialysis probes were secured in exposed recesses of a nasogastric tube 3.3 mm in diameter (Rüsch U.K. Ltd., High Wycombe, U.K.) using waterproof taping (Sleek, Johnson & Johnson, Newark, N.J., U.S.A.). Recesses in the nasogastric tube were created by cutting four 4 cm X 3 mm oblong sections, spaced 1 cm apart, along the nasogastric tube. Each recess was isolated by injecting silicone into the lumen of the nasogastric tube to prevent fluid tracking between the individual microdialysis probes, which could interfere with measuring the local environment. Four perforations were made in the intact wall of the recesses of the nasogastric tube (it was not possible to fully expose the microdialysis probe in order to maintain the rigidity of the assembly which would otherwise compromise the fragile microdialysis probes). To protect the exposed dialysing section of the microdialysis tubes, a fine mesh was secured around the dialysing section of the microdialysis probe with 2/0 silk sutures (Ethikon Ltd, Edinburgh, U.K.). This configuration allowed free contact of the microdialysis probes to the external medium without trapping fluid within the lumen of the thin nasogastric tube surrounding the microdialysis probes and the probes consequently sampling stagnant fluid. The inlet (afferent) and outlet (efferent) tubings of the microdialysis probes were for the most part contained inside the lumen of the nasogastric tube and sealed with waterproof taping. The assembled probe was attached to a 5-channel pH probe (Synetics Medical Ltd., Enfield, Middlesex, U.K.) with each microdialysis probe lying adjacent to a pH sensor (Fig 9.1). The cardia probe had two pH sensors spaced 1 cm apart, the additional sensor located 1cm proximal to the cardia microdialysis probe in order to locate the pH step-up point which can accurately localise the gastro-oesophageal junction⁽²¹¹⁾. The outputs from the pH sensors were digitalised (DAS 1201 data acquisition board, Keithley Instruments Ltd., Reading, Berkshire, U.K.), monitored on a computer screen during the procedure and stored on computer for subsequent analysis.

9.2 Validation of the assembled probes for use in the human upper gastrointestinal tract.

Before using the assembled probes in humans, it was necessary to investigate whether mounting the microdialysis probes had any adverse effect on the recovery of the chemicals of interest.

9.2.1 Method.

Aqueous solution of NaSCN 500 μM at pH 1.5, adjusted with HCl, at 37°C was prepared in a large shallow glass beaker. It was important to have a shallow container as the temperature differential seen between the bottom and the upper part of fluid column in a tall thin glass cylinder alters the microdialysis recovery rates. The assembled microdialysis probes were immersed and positioned to avoid contact with the beaker sidewall which may reduce the recovery of the chemicals by reducing the surface area of the microdialysis probes available for diffusion. A magnetic stirrer was placed in the centre of the beaker to stir the solution gently. The experiment was conducted at 37°C. To the solution was added NaNO_2 to make a concentration of 200 μM . The microdialysis probes were perfused as previously using distilled water at 0.15 mL hr^{-1} . Samples were taken from the beaker at 0 and 40 minutes corresponding to the beginning and the end of the experiment. Collection of the microdialysis sample commenced at 10 minutes and ended at 50 minutes (i.e. a ten-minute delay) to account for the time taken for the dialysate to travel from the probe tip to end of the outlet tube. The microdialysis products were collected in 20 μL 1 M NaOH in the chromatography glass vial as previously mentioned. The experiment was repeated at pH 2.5 and then at pH 7 where pH adjustment was made using NaOH. Nitrite and thiocyanate concentrations were determined as mentioned previously.

The experiment was repeated for ascorbic acid and total vitamin C recovery at pH 1.5, 2.5 and 7 at 37°C. The ascorbic acid concentration in the beaker was 200 μM . The microdialysis product was collected in 20 μL 1:1 M/S, processed for ascorbic acid and total vitamin C as described previously and their concentrations were determined by HPLC.



Figure 9.1: Assembled microdialysis probes for human use.

Four microdialysis probes were set in a fine-bore nasogastric tube. A multi-channel pH sensor is attached adjacent to the microdialysis probes. A fine tenflon tube is also attached to allow intragastric instillation of potassium nitrate.

9.2.2 Data analysis.

Data are presented as mean (\pm S.D.). Coefficient of variation is expressed as S.D./mean.

9.2.3 Results.

The mean recoveries for nitrite, thiocyanate, ascorbic acid and total vitamin C for three experiments each at pH 1.5, 2.5, and 7 were 71 % (\pm 5.5), 81 % (\pm 4.1), 86 % (\pm 5.9) for nitrite; 93% (\pm 2.4), 92 % (\pm 3.8), 90 % (\pm 4.5) for thiocyanate; 61 % (\pm 5.5), 62 % (\pm 9.0), 57 % (\pm 3.8) for ascorbic acid and 65 % (\pm 5.7), 65 % (\pm 5.9), 61 % (\pm 6.5) for total vitamin C at the respective pH. The recoveries were thus slightly reduced in the assembly. The intra-probe coefficient of variation for the four microdialysis probes at pH 1.5, 2.5 and 7 respectively ranged from < 12 %, < 6 %, < 8 % respectively for nitrite recoveries; < 3 %, < 7 %, < 7 % respectively for thiocyanate recoveries; < 9 %, < 14 %, < 10 % respectively for ascorbic acid recoveries and < 11 %, < 12 %, < 19 % respectively for total vitamin C recoveries.

9.2.4 Conclusions.

We tested the function of the assembled probes suitable for the simultaneous measurement at four positions throughout the human upper gastrointestinal tract complete with pH electrodes alongside each microdialysis probes. The recovery was slightly reduced probably due to the surface of the microdialysis membrane being partially excluded by the surface of the nasogastric tube to which they were attached. The microdialysis probes assembled as above proved to be reliable in measuring the chemicals of nitrosation. The recovery of each probe in the assembled apparatus should be checked each time before it is used in order to detect any equipment damage.

For the human studies, we decided to use 0.15 mL hr⁻¹ as the perfusion rate for the microdialysis probes as this rate produced the best overall balance between satisfactory recoveries of the chemicals of interest and sufficient volume of microdialysis product for chemical analyses to be performed.

9.3 Disinfection of the microdialysis probes.

In order to use the microdialysis probes in humans it was necessary to disinfect the equipment with suitable disinfectants. The following experiments were performed in order to establish whether the disinfecting agents had any impact on the function of the probes. Initial experiments involved the use of glutaraldehyde (Cidex, Johnson and Johnson, Newark, N.J., USA) and the cleaning procedure was performed in an enclosed fume cupboard. Due to the regulations in the department, the cleaning agent was subsequently changed to Virkon S[®] (DAHS, Sudbury, Suffolk, UK).

9.3.1 Method and Results.

The used microdialysis probes were initially soaked for 20 minutes in Klerzyme (Shield Medicare Ltd., Farnham, UK) to remove proteinaceous material. The probes were then rinsed with water. The probes were soaked in Cidex or Virkon and perfused with distilled water at 0.6 mL hr^{-1} for 30 minutes. The probes were rinsed with water. Subsequently the probes were soaked in distilled water and perfused with 1 M ethylenediamine tetra acetic acid (EDTA) to remove any trace elements which will oxidize any ascorbic acid in subsequent experiments. The final cleaning stages involved soaking the probes in distilled water and perfusing them with distilled water at 0.6 mL hr^{-1} overnight.

The probes were checked for recovery using an aqueous solution at pH 7 containing $200\text{ }\mu\text{M NaNO}_2$ and $500\text{ }\mu\text{M NaSCN}$ and any substandard probes manifested by a significantly reduced recovery or significantly reduced product volume were discarded and replaced with new probes. Both the Cidex and Virkon did not have any detrimental impact on the recoveries of the microdialysis probes.

CHAPTER TEN

HUMAN VOLUNTEER STUDIES

10.1 Introduction.

Despite the cardia representing less than 5% of the surface area of the stomach, the majority of gastric cancers occur at the gastro-oesophageal junction in the absence of *H. pylori* infection⁽⁶⁶⁾. Intestinal metaplasia is a common finding at the cardia^(116, 197 - 200) and in a significant proportion of patients is associated with carditis and *H. pylori* infection^(203, 204). In the remainder of the patients intestinal metaplasia cannot be attributed to *H. pylori* and is likely to be related to the presence of acidic gastric juice / gastro-oesophageal reflux^(207 - 210). The mutagen responsible for the high incidence of metaplasia and neoplasia at the gastro-oesophageal junction remains unknown. Cancer of the cardia also differs from cancer of the more distal stomach with respect to associated gastric physiology. The former occurs in patients with normal gastric secretory function^(320, 321, 322); in contrast, cancer of the more distal stomach usually develops in subjects with *H. pylori*-induced atrophic gastritis and hypochlorhydria⁽³²²⁾.

Intestinal metaplasia and cancer at the gastro-oesophageal junction are being attributed to gastro-oesophageal reflux and the noxious effects of acid, pepsin and duodenal contents in the refluxate. However, this hypothesis cannot entirely explain cancer at the gastric cardia which has a columnar mucosa well able to withstand the physiological constituents of gastric juice. In addition, epidemiological studies indicate only a weak association between symptoms of reflux and cardia cancer⁽⁵⁹⁾.

For many years there has been interest in the potential role of endogenously produced *N*-nitroso compounds in the aetiology of upper gastrointestinal cancer⁽³²³⁾. Acidic conditions present in the stomach protonate nitrite to nitrous acid which subsequently gives rise to nitrosating species which are able to react with secondary amines and amides forming potentially carcinogenic *N*-nitroso compounds^(323, 324). The major source of nitrite entering the healthy acid-secreting stomach is saliva and arises from the enterosalivary re-circulation of dietary nitrate as discussed previously. Bacteria catalyse the reduction of nitrate to nitrite in the oral cavity and is inhibited by antibacterial agents^(325, 326). The salivary glands actively take up and secrete thiocyanate which is also found in gastric juice^(271, 272). Thiocyanate is a potent catalyst of the nitrosation of secondary amines by acidified nitrite^(237, 238). This delivery of nitrite and thiocyanate into the acidic

environment of the stomach has therefore been regarded as a potentially important source of endogenous formation of carcinogenic *N*-nitroso compounds^(323, 324). Ascorbic acid is an important inhibitor of acid-catalysed nitrosation. The healthy human stomach actively secretes ascorbic acid into the gastric juice^(274, 275). Ascorbic acid rapidly reduces the acidified nitrite / nitrosating species to nitric oxide, and itself oxidised to dehydroascorbic acid^(257 - 259, 276 - 278). The relative availability of nitrite compared to ascorbic acid determines whether *N*-nitrosation can proceed within the gastric lumen. When the supply of nitrite exceeds that of ascorbic acid nitrosation will occur; and conversely when ascorbic acid supply exceeds that of nitrite then nitrosation will be prevented. The removal of intragastric nitrite by the ascorbic acid in gastric juice thus prevents the generation of *N*-nitroso compounds within the gastric lumen.

Previous studies have regarded the gastric lumen as a homogeneous compartment with respect to acid-catalysed nitrosation. However, the gastric cardia is the most proximal region of the stomach where the greatest nitrite load will be delivered. Nitric oxide generated from the reaction of acidified nitrite and ascorbic acid has been shown to be the greatest at the cardia⁽²⁷⁸⁾. This suggests that the highest intragastric ratio of nitrite to ascorbic acid and thus the condition most suitable for generating *N*-nitroso compounds should occur in the region of the cardia. This chapter examines this hypothesis and provide an insight into the potential mechanism for mutagenesis and carcinogenesis at this anatomical site.

10.2 Method.

10.2.1 Subject selection and microdialysis procedure

Subjects who were negative for *H. pylori* as determined by the ¹⁴C-urea breath tests were enrolled in the study. All the subjects undergoing the study were endoscoped prior to the experiment to exclude any pathology and to record the positions of the gastro-oesophageal junction, the size of hiatal hernia, if any were present. A rapid urease test (CLO™, Delta West Pty Ltd., Bentley, Australia) was performed using two biopsy specimens: one from the antrum and another from the gastric body to confirm that the subjects were not re-infected by *H. pylori* since

some of the subjects had a negative urea breath test performed several years prior to the experiment. All CLO tests were confirmed to be negative.

On the day of the study the subjects presented having fasted overnight. They were instructed to avoid nitrate-rich foods for 24 hours prior to the procedure. The probe assembly was passed per orally and positioned so that one microdialysis probe was positioned in each of the following regions: the distal oesophagus 5 cm above the gastro-oesophageal junction, cardia, 5 cm distal to the gastro-oesophageal junction in the proximal stomach and 10 cm distal to the gastro-oesophageal junction in the distal stomach (Fig 10.1). The pH sensor sited 1 cm proximal to the cardia microdialysis probe recorded esophageal pH (> 4) and the pH sensor at the cardia microdialysis probe recorded gastric pH (≤ 2.5). This ensured that the cardia microdialysis probe was located just distal to the gastro-oesophageal junction as the pH step-up corresponds to the squamocolumnar junction under fasting conditions ⁽²¹¹⁾. The position of the assembly was checked by an abdominal X-ray. The subjects lay semi-recumbent throughout the study. The pH being recorded by each sensor was closely monitored during each study and the position of the tube adjusted if required to maintain its correct location relative to the gastro-oesophageal junction.

After the confirmation of satisfactory positioning, the microdialysis probes were perfused with degassed, distilled water at 0.15 mL hr^{-1} . The first 15 minutes' collections of the microdialysis samples were discarded and the following 40 minutes collected for analyses. Following this basal collection, 2 mmol of potassium nitrate in 25 mL of water was administered intragastrically via a fine Teflon feeding tube attached to the nasogastric tube. Forty minutes after administering the potassium nitrate further microdialysis collections were obtained for 40 minutes.

The microdialysis samples were collected in a 300 μL glass vials (Chromacol, Ltd., Herts, U.K.) containing preservatives. Ascorbic acid and total vitamin C were collected in 20 μL of 2 % metaphosphoric acid containing an equal volume of 0.5 % sulfamic acid (M/S). One half of the sample was diluted with an equal volume of M/S for ascorbic acid and the other half with an equal volume of 12 mg mL^{-1} dithiothreitol in M/S (M/S+DTT) for total vitamin C samples. The nitrite and thiocyanate samples were collected into 20 μL of 1 M NaOH. Separate collections were necessary as the preservative used for nitrite and

thiocyanate was incompatible with ascorbic acid / total vitamin C and vice versa. In order to collect separate specimens for nitrite / thiocyanate and ascorbic acid / total vitamin C the collection of microdialysis samples was alternated every 2 minutes into each collecting tube. The vitamin C samples were frozen at -70°C for analysis within four weeks and the nitrite and thiocyanate samples were stored at 4°C for analysis on the same day.

Venous blood was obtained from an indwelling intravenous catheter every 15 minutes from the start of the study. Serum nitrate was collected in a plain *Vacutainer*[®] tube, plasma ascorbic acid and total vitamin C determinations were collected in *Vacutainer*[®] tubes containing lithium heparin. Blood samples were allowed to stand for 20 minutes at room temperature and were centrifuged at 3000 r.p.m. at 4°C for 10 minutes and the supernatant for plasma ascorbic acid and total vitamin C were diluted with equal volumes of M/S solution or M/S+DTT respectively and snap frozen in liquid nitrogen and stored at -70°C until analysis. The supernatant for serum nitrate was transferred into a plain test tube and frozen at -20°C until analysis.

Samples of saliva were obtained every 15 minutes for nitrite and thiocyanate determination. This was done by asking the patient to spit into a sterile container and 500 μL was immediately added to 50 μL of 1 M NaOH in a 1.5 mL Eppendorf tube and stored at 4°C until analysis on the same day.

Prior to each study the recovery of each microdialysis probe fully assembled on the nasogastric tube was assessed for each individual chemical at 37°C at pH 1.5, 2.5 and 7. Any probe that was damaged was discarded and replaced with a new probe. Following each study, the individual probes were then removed from the nasogastric tube, cleaned and sterilized with Cidex (Johnson and Johnson, Newark, N.J.) and latterly Virkon[®] (DAHS, Sudbury, Suffolk, UK) before being remounted into a fresh nasogastric tube.

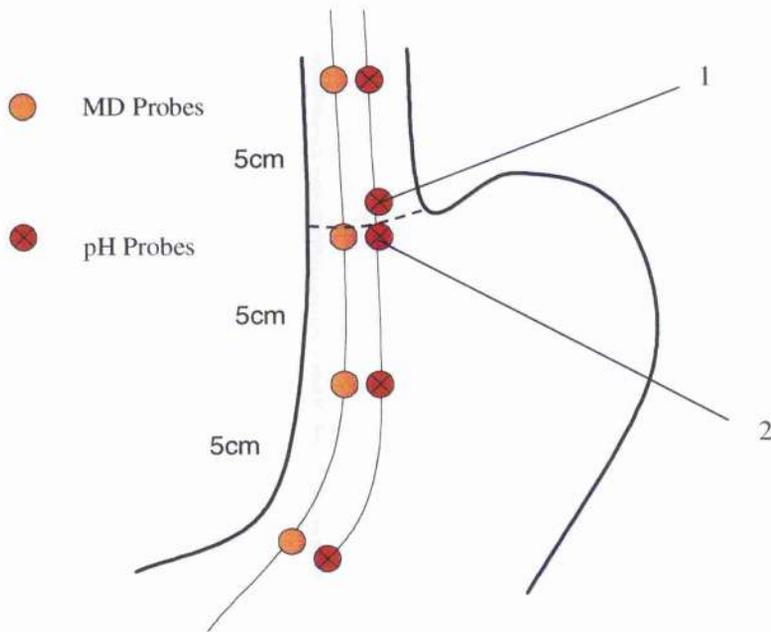


Fig 10.1: Schematic diagram of the positions of the microdialysis probes and the pH sensors.

The broken line indicates the gastro-oesophageal junction. The microdialysis probes are positioned from proximal to distally: distal oesophagus, cardia, proximal stomach and distal stomach. The pH sensor 1 lies just above the gastro-oesophageal junction and sensor 2 just below at the cardia. The pH of sensor 1 is > 4 and for sensor 2 is ≤ 2.5 . This enables accurate positioning of the microdialysis probe at the cardia.

10.2.2 Chemical analyses

The chemicals collected in this experiment were serum nitrate; salivary nitrite and thiocyanate; microdialysis nitrite, thiocyanate, ascorbic acid and total vitamin C. Nitrite, thiocyanate, ascorbic acid and total vitamin C were analysed as previously mentioned.

i) Saliva assay

Prior to analysis, the saliva samples were centrifuged at 13000 r.p.m for two minutes using a microcentrifuge and the supernatant was analysed. The supernatant was diluted 1:4 with distilled water. The samples were analysed for nitrite and thiocyanate colorimetrically on a 96-well microplate using the modified Greiss reagent for nitrite and the Bowler method for thiocyanate as described in 7.3.2 and 7.3.3 respectively.

ii) Microdialysis assay

The volumes obtained from the microdialysis experiments (approximately 55 μ l) only allowed for single measurement to be made for each sample therefore extreme care was taken to ensure that the samples were processed correctly. The microdialysis products for thiocyanate and nitrite were diluted 1:3 with distilled water and analysed colorimetrically in a 96-well microplate as above. For ascorbic acid and total vitamin C the samples were diluted 1:2 using M/S for ascorbic acid and M/S+DTT for total vitamin C and analysed by HPLC coupled to an electrochemical detector as described in 7.3.1.

iii) Serum nitrate assay

After thawing, the serum samples were filtered through a 10-kDalton microfilter (Microcon 10, Millipore UK Ltd, Watford, UK) centrifuged at 13000 r.p.m. for one hour to remove high-molecular-weight substances and an aliquot of filtrate was analysed as described in 7.3.4 by reduction of nitrate to nitrite and analysing the samples colorimetrically with the Greiss reagent. The original nitrite concentration in the serum was also determined. The difference between the total nitrite (reduced nitrate + original nitrite) and the original nitrite represented the actual serum nitrate concentration.

10.3 Statistical analyses.

All data are presented as median and interquartile range (IQR) unless otherwise stated. Paired data were analysed using 1-sample Wilcoxon test. For the comparison of pre- and post-nitrate data of the serum nitrate, plasma ascorbic acid, plasma total vitamin C, saliva nitrite and saliva thiocyanate, individual post-nitrate time points (60 to 120 minutes) were compared to the 45 minute sample which represented the concentrations of the chemicals of interest just before nitrite was administered.

In order to calculate the ascorbic acid to nitrite ratio, any samples with undetectable ascorbic acid or nitrite concentrations were allocated a concentration of 1 μM , the approximate lower limit of detection for both ascorbic acid and nitrite using our analytic methods. If both ascorbic acid and nitrite were undetectable, these samples were excluded from analysis. Statistical testing was performed using the 1-sample Wilcoxon test. Bonferroni correction was applied for multiple comparisons. A two-tailed $p < 0.05$ was considered statistically significant.

10.4 Ethics.

The study was approved by the North Glasgow University NHS Trust Ethics Committee and each subject gave written, informed consent.

10.5 Results.

Twenty one patients were recruited. Four patients were unable to tolerate the procedure for the duration of the experiment and were excluded leaving 17 patients available for analysis. The mean age was 32.1 years (range: 18-52 years) and 12 were males.

10.5.1 Blood.

i) Nitrate

Just prior to nitrate administration (45 min), the fasting serum nitrate concentration was 17.3 μM (14.0 – 22.0). Following the ingestion of 2 mmol potassium nitrate, the serum nitrate increased three-fold within 30 min, increasing to 59.4 μM (49.5 – 66.2) 40 minutes after nitrate administration at 90 minutes and remained at this level for at least the following 45 min ($p < 0.01$ for all post-nitrate time points (60 -135 mins.) vs pre-nitrate time point at 45 mins.) (Fig. 10.2).

ii) Ascorbic acid and total vitamin C

The plasma ascorbic acid concentration at the beginning of the experiment was 52.9 μM (32.6 – 64.0) and that of TVC was 56.2 μM (39.8 – 67.0). The serum concentration of ascorbic acid and TVC were unchanged following the nitrate administration.

10.5.2 Saliva.

i) Nitrite

Prior to insertion of the probe assembly (-15 min), the salivary nitrite concentration was 53.9 μM (45.7 – 146.1) and fell to 23.1 μM (10.1 – 42.2) at 15 minutes after the insertion of the microdialysis assembly ($p < 0.01$). Following the administration of potassium nitrate, the saliva nitrite concentration increased 8-fold to 201.2 μM (92.7 – 292.6) at 90 minutes ($p < 0.01$ vs 45 min (pre-nitrate)) and remained elevated for at least the following 45 minutes (Fig. 10.3).

ii) Thiocyanate

Prior to passing the probe assembly (-15 min), the salivary thiocyanate concentration was 2044 μM (948 - 4380) and this fell to 1238 μM (489 - 1739) 15 minutes after the placement of the assembly ($p < 0.01$) and remained at this level throughout the remainder of the study.

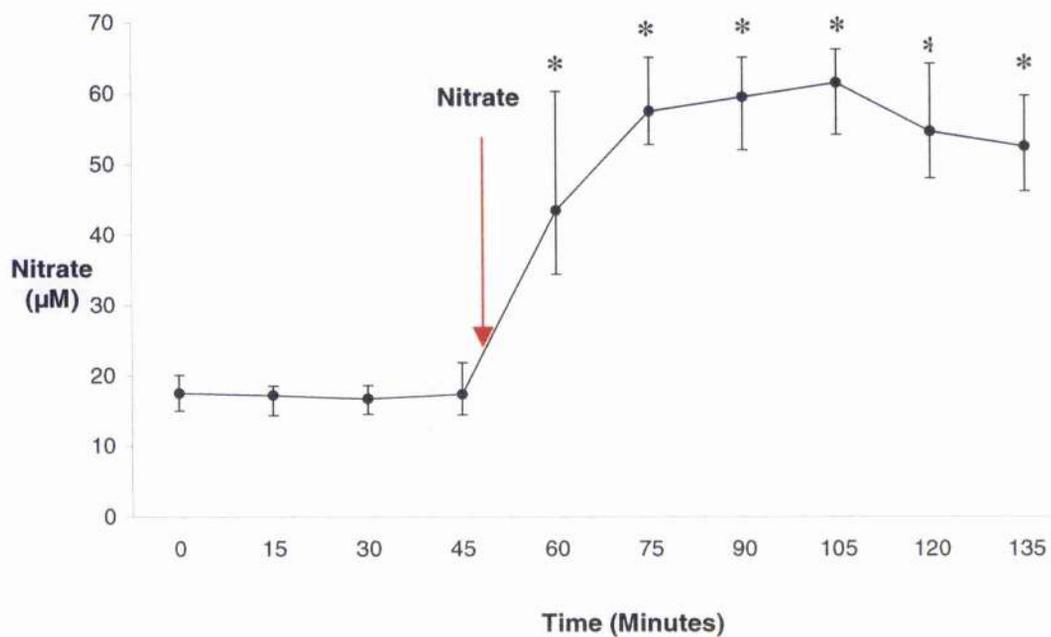


Fig 10.2: Median serum nitrate before and after nitrate administration.
 Vertical bars represent IQR. N = 17 for all time points.
 2 mmol potassium nitrate given at 50 minutes (indicated by red arrow).
 *p < 0.01 compared to 45minute sample.

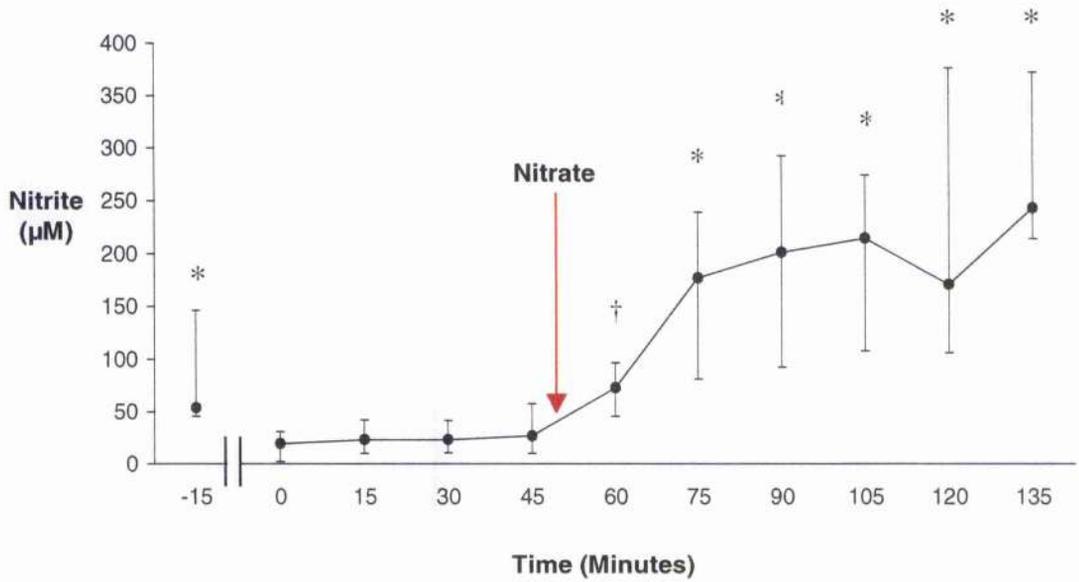


Figure 10.3: Median salivary nitrite before and after nitrate administration.

Vertical bars represent IQR. N = 17 at all time points.

- 15 minutes indicates sample before insertion of microdialysis assembly. 2 mmol potassium nitrate administered at 50 minutes (arrow).

† p < 0.05 compared to 45 minute sample.

* p < 0.01 compared to 45 minute sample.

10.5.3 Oesophagus.

i) pH

The median (range) pH of the distal esophagus was 7 (6.7 – 7.6) before and 7 (6.6 – 7.6) after nitrate was administered.

ii) Nitrite

The fasting nitrite concentrations in the distal esophagus was 17.6 μM (13.1 – 34.3) which was similar to the median fasting salivary nitrite concentration of 20.7 μM (10.3 – 41.1) during 0 – 45 minutes, prior to the nitrate administration (Fig. 10.4). Following the nitrate meal, the nitrite concentration in the distal esophagus reached 165.5 μM (103.4 – 237.6) ($p < 0.01$ vs fasting levels) which was non-significantly lower than that of the median salivary nitrite 215.8 μM (123.3 – 245.0) during 90 – 135 minutes, corresponding to the post-nitrate microdialysis collection period (Fig. 10.4). 7 of the 17 patients and 8 of 17 patients before and after nitrate administration had higher nitrite recovery from the distal oesophagus than in the saliva.

iii) Thiocyanate

The median thiocyanate concentration in the distal oesophagus before nitrate administration was 540 μM (398 - 1096) which was significantly lower than the thiocyanate concentration of 1123 μM (487 - 1698) in the saliva during 0 – 45 minutes ($p < 0.01$). Following nitrate administration, there was a non-significant increase in the thiocyanate concentration in the distal oesophagus to 629 μM (438 - 1237) and was again lower than the thiocyanate concentration of 1073 μM (577 - 1971) in saliva during 90 – 135 minutes ($p < 0.01$) (Fig. 10.5).

vi) Ascorbic acid and total vitamin C

Both ascorbic acid and total vitamin C concentrations were low in the oesophagus. The median ascorbic acid concentration was 11.6 μM (2.4 – 16.7) before the nitrate meal and was non-significantly lower at 6.9 μM (1.5 – 15.5)

after the nitrate meal. The total vitamin C concentration was 14.2 μM (3.7 – 17.1) before the nitrate meal and 7.8 μM (5.4 – 16.1) after the nitrate meal (N.S).

v) Ascorbic acid to total vitamin C ratio

This ratio indicates the proportion of ascorbic acid in the reduced form. The median ratio of ascorbic acid to total vitamin C in the squamous oesophagus before and after nitrate administration were 82% (40-100%) (N = 15) and 73% (52-100) (N = 15) respectively (N.S.)

10.5.4 Stomach.

i) pH

Prior to nitrate administration, the median pH of the cardia, proximal and distal stomach were 2.6 (2.5 – 3.6), 1.9 (1.6 – 2.9) and 1.7 (1.4 – 3.0) respectively (Table 10.1). Following the nitrate meal the pH of different regions of the stomach were 2.8 (2.5 – 3.7), 1.7 (1.5 – 3.0) and 1.5 (1.4 – 2.2) respectively (Table 10.2). The percentage of time the pH was above 4 in the cardia, proximal stomach and distal stomach were 18.5, 0 and 0%. Following the nitrate meal, the percentage of the time the pH was above 4 were 23, 0 and 0% at the respective sites.

ii) Nitrite

The median nitrite concentrations within the stomach were 0 μM (0 – 14.2) at the cardia and 0 μM for both proximal and distal stomach before nitrate was administered (Fig. 10.6; table 10.1). Following nitrate administration, the nitrite concentrations increased significantly at the cardia to 30.9 μM (12.4 – 57.9) ($p < 0.01$ vs cardia before nitrate) but remained at 0 μM (0 – 18.7) at the proximal stomach ($p < 0.01$ vs cardia after nitrate) and 0 μM at the distal stomach ($p < 0.01$ vs cardia after nitrate) (Fig. 10.6; table 10.2).

The nitrite concentration at the cardia, 0 μM (0 – 14.2) before the nitrate meal and 30.9 μM (12.4 – 57.9) after the nitrate meal were both significantly lower than that of the distal oesophagus which were 17.6 μM (13.1 – 34.3) before the nitrate meal and 165.5 μM (103.4 - 237.6) after the nitrate meal (both $p < 0.01$ vs after nitrate).

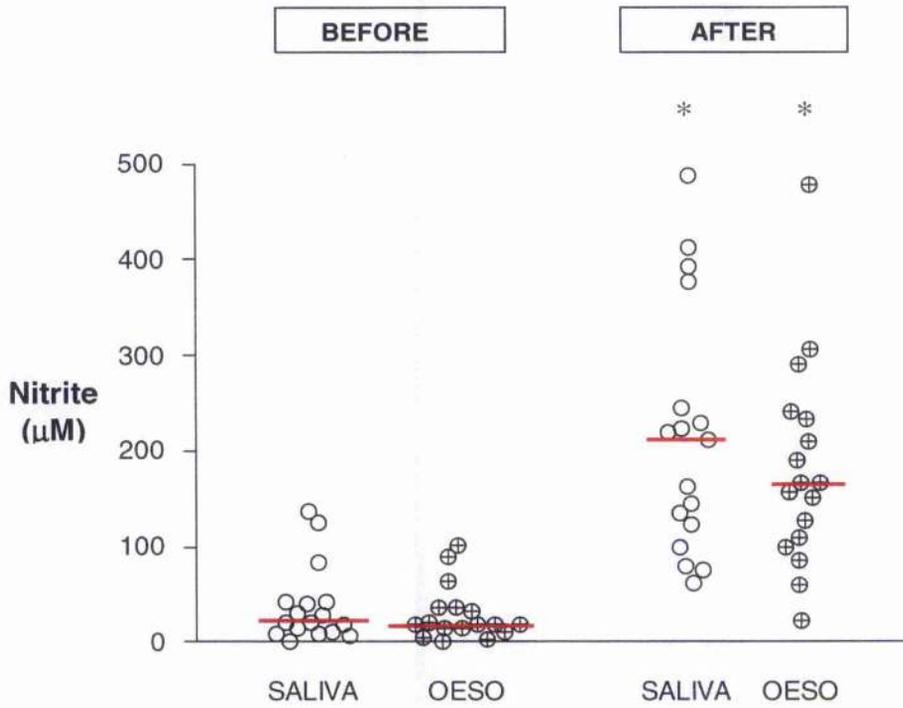


Figure 10.4: Individual salivary and oesophageal nitrite concentrations before and after nitrate administration.

Horizontal line represents median nitrite. Both saliva and oesophageal nitrite increased after the nitrate (* $p < 0.01$ vs before nitrate).

The salivary and oesophageal nitrite concentrations were not significantly different from one another both before and after the nitrate.

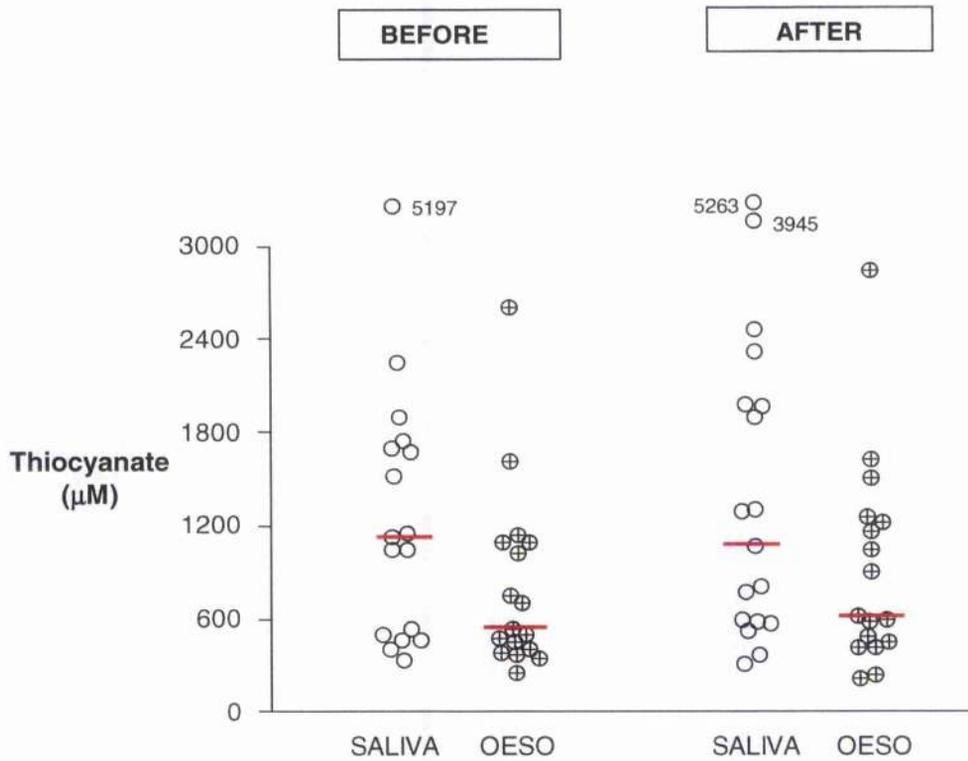


Figure 10.5: Individual salivary and oesophageal thiocyanate (SCN^-) concentrations before and after nitrate administration. Horizontal line represents the mean SCN^- . Outliers are indicated by the values next to the symbols. SCN^- in saliva was significantly higher than SCN^- in the distal oesophagus before and after nitrate (* $p < 0.01$ compared to oesophagus). SCN^- concentrations were similar both before and after nitrate for saliva and oesophagus.

iii) Thiocyanate

Prior to nitrate administration, the mean thiocyanate concentrations in the cardia, proximal stomach and distal stomach were 554 μM (267 - 833), 697 μM (286 - 914) and 486 μM (289 - 933) respectively (N.S.) (Fig. 10.7; table 10.1). Following nitrate administration, the respective thiocyanate concentrations were 469 μM (273 - 1057), 651 μM (381 - 1009) and 650 μM (325 - 1139) (table 10.2).

iv) Ascorbic acid

There was a proximal to distal gradient in intra-gastric ascorbic acid with the lowest level occurring at the cardia both before and after the nitrate meal (Fig. 10.8; tables 10.1 and 10.2). The median concentration was 9.3 μM (4.5 - 13.9) at the cardia, 20.8 μM (9.5 - 69.3) at the proximal stomach ($p < 0.01$ vs cardia) and 27.7 μM (10.3 - 104.2) at the distal stomach ($p < 0.01$ vs cardia, N.S. vs proximal stomach). Following the nitrate meal the median concentration of ascorbic acid at the cardia and the proximal stomach were unchanged respectively at 6.9 μM (1.3 - 12.0), 19.9 μM (5.5 - 50.6) ($p = 0.018$ vs cardia), and increased non-significantly to 40.4 μM (12.3 - 101.0) at the distal stomach ($p < 0.01$ vs cardia).

v) Total vitamin C

The intra-gastric concentration of total vitamin C also showed an increasing concentration gradient moving distally from the cardia both before and after the nitrate meal (Fig 10.9; tables 10.1 and 10.2). Before the nitrate meal, the total vitamin C concentration was lowest at the cardia 11.9 μM (5.7 - 18.7) higher at the proximal stomach, 29.8 μM (12.4 - 87.8) ($p < 0.01$ vs cardia) and slightly higher at the distal stomach, 35.0 μM (15.0 - 111.2) ($p < 0.01$ vs cardia, N.S. vs proximal stomach). Following the nitrate meal, the total vitamin C levels for the cardia, proximal stomach and distal stomach were 14.9 μM (7.1 - 30.1), 35.1 μM (13.5 - 79.9) ($p = 0.036$ vs cardia) and 68.4 μM (22.7 - 128.1) ($p < 0.01$ vs cardia; $p = 0.018$ vs proximal stomach) respectively.

vi) Ratio of ascorbic acid to total vitamin C

Before nitrate administration the ascorbic acid to total vitamin C ratios were 72% (43-94%) in the cardia, 79% (50-91%) in the proximal stomach (N = 16) and 83% (69-95%) in the distal stomach (N = 16). Following nitrate administration, the mean ratio fell to 49% (9-75%) (N = 15), 50% (22-92%) and 60% (50-78%) for the oesophagus, cardia, proximal and distal stomach respectively ($p < 0.01$ for cardia pre vs post-nitrate; $p < 0.05$ distal stomach pre vs post-nitrate).

vii) Ratio of ascorbic acid to nitrite

The ascorbic acid to nitrite ratio is an indicator of nitrosation potential. The higher the ratio, the greater the ascorbic acid relative to nitrite and the condition is unfavourable for the formation of nitroso compounds; at low ratios when the nitrite concentration exceeds that of the ascorbic acid, the condition favours the generation of nitroso compounds.

Prior to nitrate administration, there was a marked gradient in the ratio of ascorbic acid to nitrite throughout the stomach (Fig. 10.10). The ratio was lowest in the cardia, median (range) 1.5 (0.2 - 112), higher in the proximal stomach, 20.8 (3.7 - 193) ($p < 0.01$ vs cardia) and highest in the distal stomach at 27.7 (7 - 351) ($p < 0.01$ vs cardia; N.S. vs proximal stomach). Following the nitrate administration, the ratio at the cardia fell to 0.3 (0 - 108) ($p = 0.018$ vs cardia pre-nitrate), but the ratio in the proximal stomach, 7.8 (0 - 264) ($p < 0.01$ vs cardia post-nitrate) and in the distal stomach, 40 (0.7 - 428) ($p < 0.01$ vs cardia post-nitrate) were not significantly different from their values before nitrate.

The proportion of patients showing ascorbic acid to nitrite ratio of less than one prior to nitrate was 7/16 (44%), 0/17 (0%) and 0/17 (0%) for the cardia, proximal stomach and distal stomach respectively. Following the nitrate meal, the proportions were 14/17 (82%), 3/16 (19%) and 1/16 (0.1%) respectively.

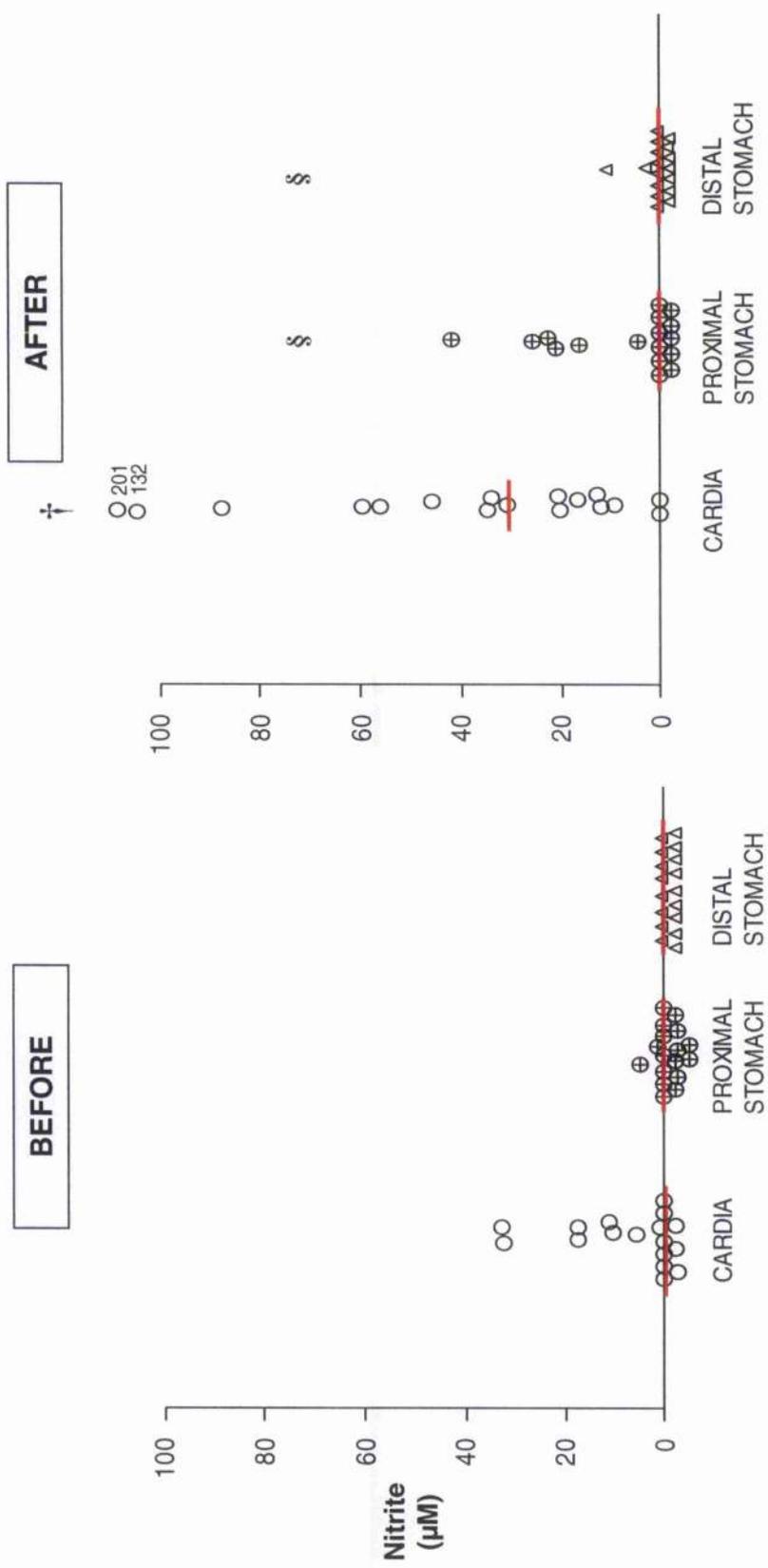


Fig 10.6: Concentrations of nitrite in the stomach before and after nitrate administration.

Horizontal line represents median concentrations. Outliers are indicated with values next to symbols.

† p < 0.01 vs before nitrate meal; § p < 0.01 vs cardia after nitrate meal.

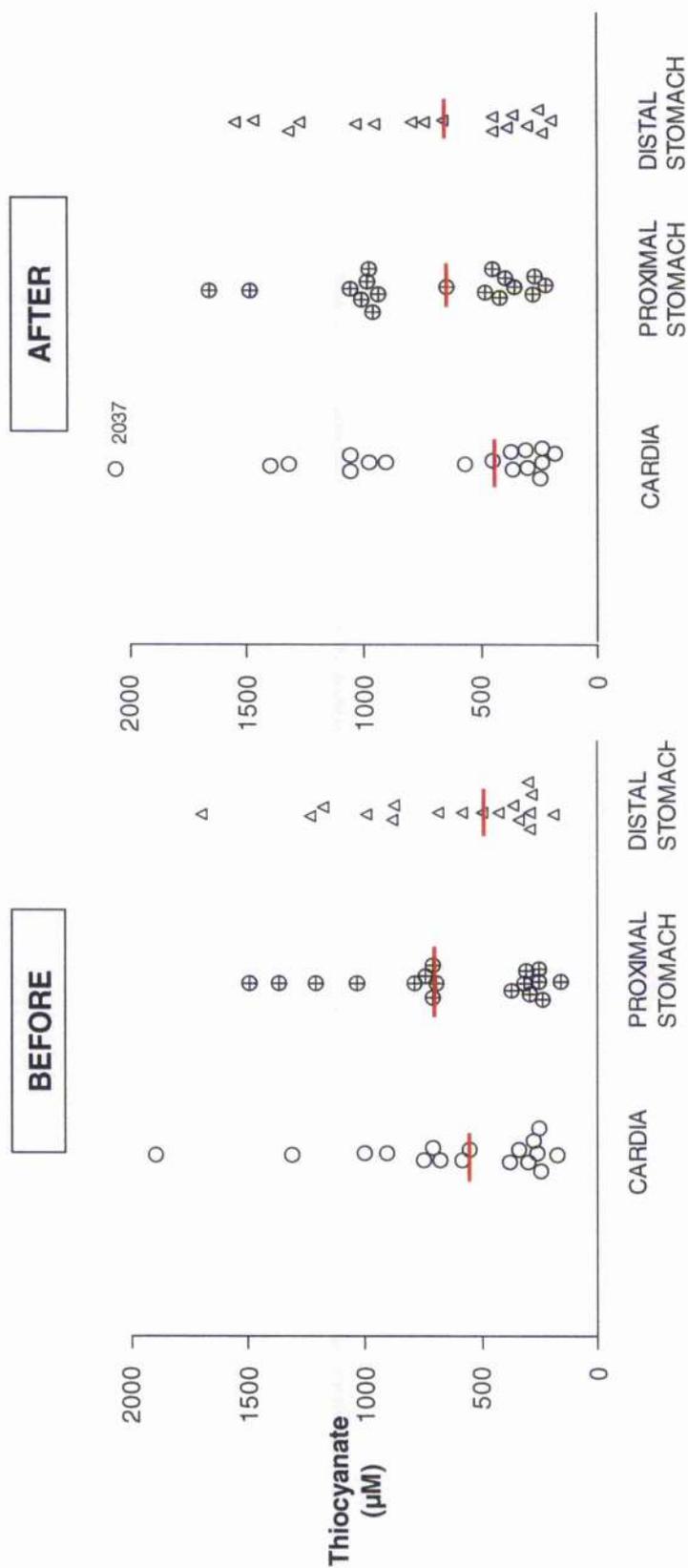


Figure 10.7: Concentrations of SCN^- in the stomach before and after nitrate administration. Horizontal line represents mean concentrations. Outliers are indicated with values next to symbols. No significant differences in SCN^- concentrations in different gastric regions before or after the nitrate meal.

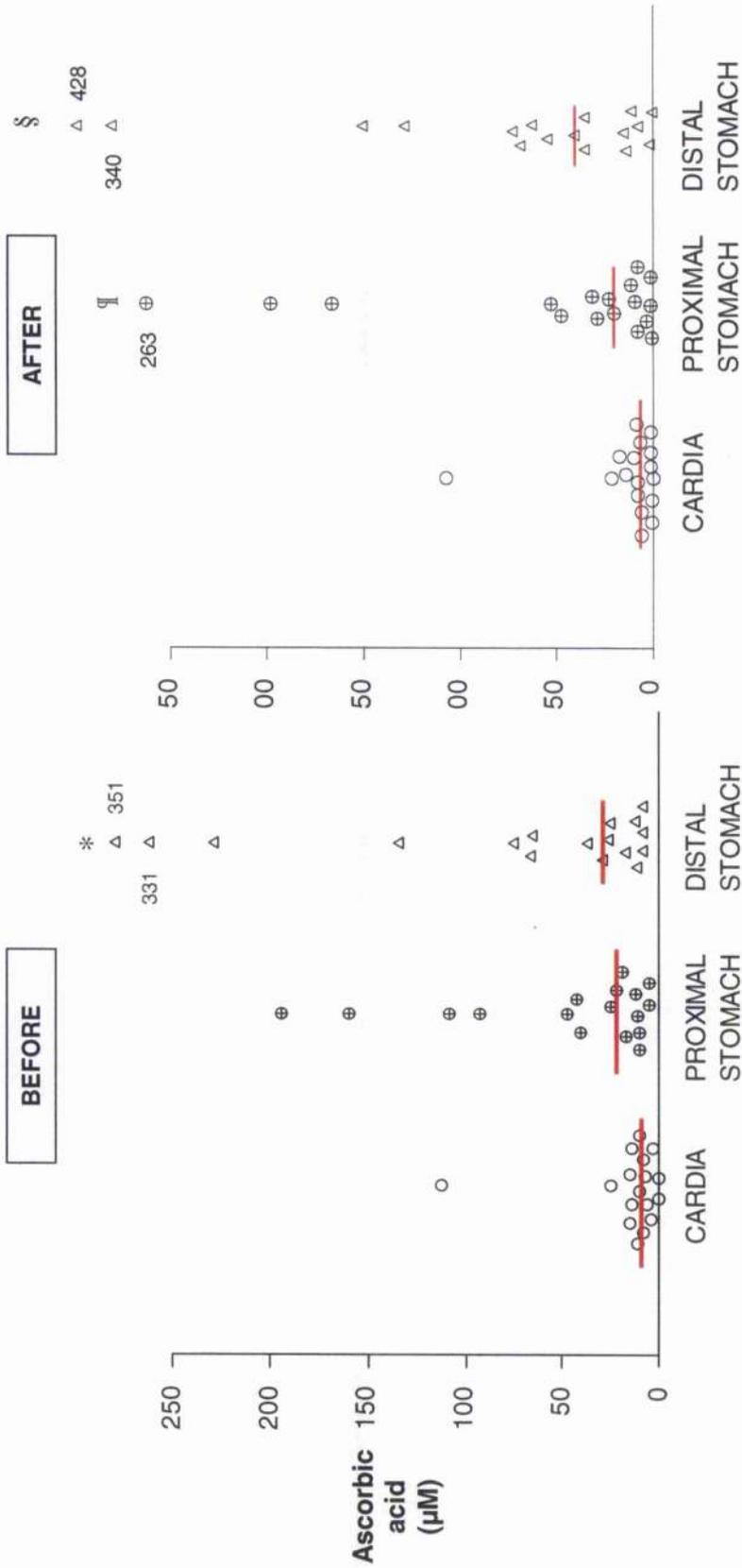


Fig 10.8: Concentrations of ascorbic acid in the stomach before and after nitrate administration .

Horizontal line represents median concentrations. Outliers are indicated with values next to symbols.

* $p < 0.01$ vs cardia before nitrate; ¶ $p < 0.018$ vs cardia after nitrate; § $p < 0.01$ vs cardia after nitrate.

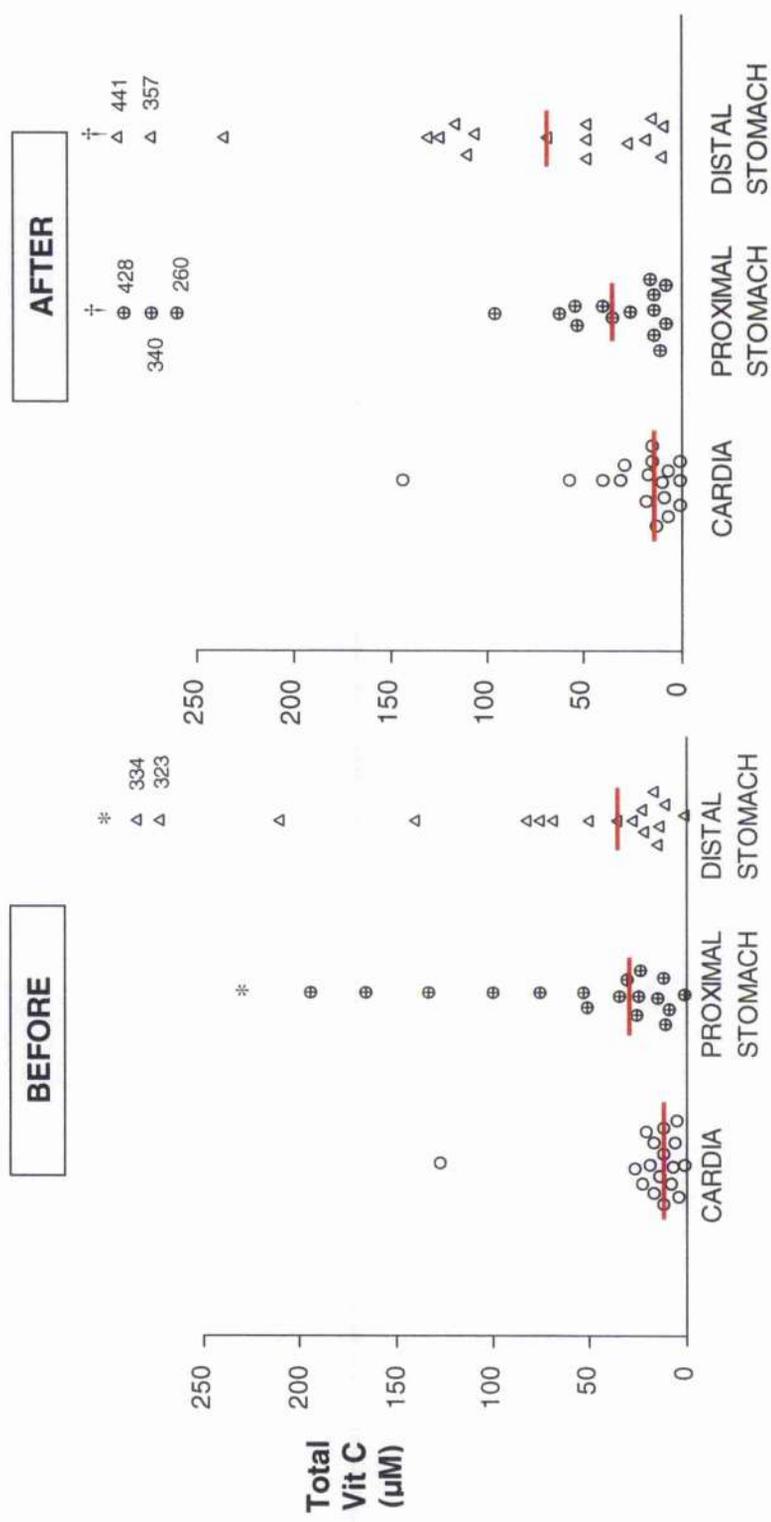


Fig 10.9: Total vitamin C in the stomach before and after nitrate administration.
 Horizontal line represents median concentrations. Outliers are indicated with values next to symbols.
 * $p < 0.01$ vs cardia before nitrate; † $p < 0.01$ vs cardia after nitrate.

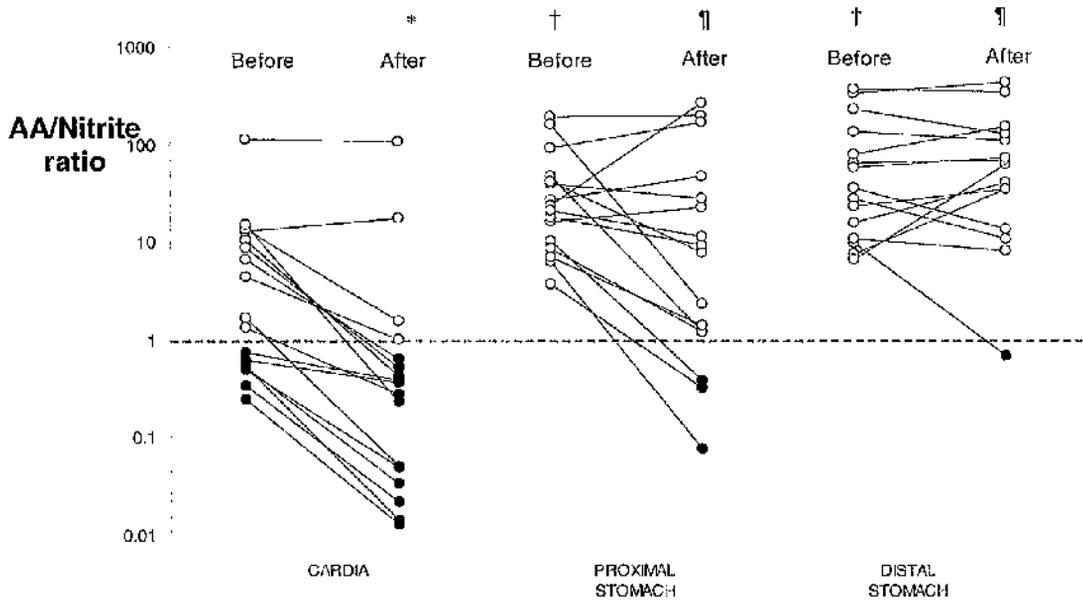


Fig 10.10: Graph showing nitrosation potential in different regions of the stomach before and after nitrate administration.

Interrupted line at AA/nitrite ratio of 1. Points above this line (○) AA is in excess of nitrite and condition does not favour nitrosation;

points below the line (●) nitrite is in excess of AA and condition favours nitrosation.

* $p = 0.018$ compared to pre-nitrate meal.

† $p < 0.01$ compared to cardia pre-nitrate meal.

‡ $p = 0.001$ compared to cardia post-nitrate meal.

	OESOPHAGUS	CARDIA	PROXIMAL STOMACH	DISTAL STOMACH
pH	7.0 (6.6 – 7.6)	2.6 (2.5 – 3.6)	1.8 (1.6 – 2.9)	1.7 (1.4 – 3.0)
Nitrite (μM)	17.6 (13.1 – 34.3)	0 (0 – 14.2)	0	0
Ascorbic acid (μM)	11.6 (2.4 – 16.7)	9.3 (4.5 – 13.9)	20.8 (9.5 – 69.3)	27.7 (10.3 -104.2)
Total Vitamin C (μM)	14.2 (3.7 – 17.1)	11.9 (5.7 – 18.7)	29.8 (12.4 – 87.8)	35.0 (15.0 – 111.2)
Thiocyanate (μM)	540 (398 – 1096)	554 (267 – 833)	697 (286 – 914)	486 (289 – 933)

Table 10.1: Median (IQR) pH, nitrite, ascorbic acid, total vitamin C and thiocyanate concentrations in the different regions of the upper gastrointestinal tract before nitrate administration.

	OESOPHAGUS	CARDIA	PROXIMAL STOMACH	DISTAL STOMACH
pH	7.0 (6.6 – 7.6)	2.8 (2.5 – 3.7)	1.7 (1.5 – 3.0)	1.5 (1.4 – 2.2)
Nitrite (μM)	165.5 (103.4 – 237.6)	30.9 (12.4 – 57.9)	0 (0 – 18.7)	0
Ascorbic acid (μM)	6.9 (1.5 – 15.5)	6.9 (1.3 – 12.0)	19.9 (5.5 – 50.6)	40.4 (12.3 – 101.0)
Total Vitamin C (μM)	7.8 (5.4 – 16.1)	14.9 (7.1 – 30.1)	35.1 (13.5 – 79.9)	68.4 (22.7 – 128.1)
Thiocyanate (μM)	629 (438 -1237)	469 (273 – 1057)	651 (381 – 1009)	650 (325 – 1139)

Table 10.2: Median (IQR) pH, nitrite, ascorbic acid, total vitamin C and thiocyanate concentrations in the different regions of the upper gastrointestinal tract following nitrate administration.

10.6 Discussion.

Previous studies have regarded the gastric lumen as a homogeneous compartment with respect to acid-catalysed nitrosation and examined the concentrations of chemicals relevant to luminal nitrosation in saliva and aspirated mixed gastric juice^(327, 328). We examined the luminal concentrations in different anatomical regions of the upper gastrointestinal tract. This showed that the most proximal cardia region of the stomach has the most favourable chemical conditions for luminal generation of *N*-nitroso compounds. The concentrations of both nitrite and thiocyanate were found to be high in the distal oesophagus. Thiocyanate concentration in the distal oesophagus was approximately half of the concentration in saliva whereas nitrite concentration in the distal oesophagus was similar to that in the saliva. Nitrite concentration in the oesophagus increased approximately nine-fold following nitrate ingestion consistent with it originating from the enterosalivary recirculation of nitrate and its reduction by buccal bacteria^(286 - 288, 325, 326). An interesting finding was that approximately 50% of the subjects had a higher concentration of nitrite in their distal oesophagus than in the salivary samples. The high concentrations of nitrite and thiocyanate in the oesophagus are largely due to swallowing saliva and diffusion distributing these anions produced in the mouth throughout the oropharyngeal-oesophageal cavity. However, nitrate and thiocyanate are secreted by many exocrine glands^(283, 329) which likely includes oesophageal submucosal glands which are similar to salivary glands⁽³³⁰⁾. The fact that the oesophageal thiocyanate concentration was lower than that of the saliva whereas approximately 50% of the subjects had oesophageal nitrite concentration higher than that of saliva suggests that more nitrite was either being secreted in the oesophagus, or more likely, an on-going reduction of nitrate to nitrite was occurring within the oesophageal lumen by the bacterial nitrate reductase activity present in the saliva. Nitrate reduction has been demonstrated outside the oral cavity in expectorated saliva⁽³³¹⁾ and nitrate reduction is unlikely to be confined to the oral cavity. Concentrations of both nitrite and thiocyanate measured in the saliva sample obtained prior to insertion of the orogastric tube were approximately two times higher than those measured in saliva when the tube was in place. This can be explained by the presence of the tube stimulating buccal secretions. Stimulation of salivary secretion has been shown to reduce the

concentration of nitrate and nitrite in saliva ⁽³³²⁾. However, the *output* of nitrate increases on stimulation and more nitrate may be reduced to nitrite during mechanical stimulation ⁽³³²⁾.

Our studies have also demonstrated that there are marked differences in the concentrations of nitrite and ascorbic acid in the different anatomical regions of the stomach. Under fasting conditions, little nitrite was detected in the stomach. However, following nitrate ingestion, the nitrite concentration increased in the most proximal cardia region of the stomach but showed no significant rise in the more distal regions of the stomach. Consequently, the nitrite concentration after nitrate ingestion was highest in the gastric cardia. The observation that the nitrite concentration was highest in the cardia region of the stomach is consistent with the fact that nitrite enters the acid secreting stomach via the oesophagus which opens into the gastric cardia. When this nitrite enters the stomach the acidic gastric juice containing ascorbic acid rapidly converts nitrite to nitrous acid and subsequently to nitric oxide, the latter will rapidly diffuse into the surrounding epithelium ^(302, 333).

The total vitamin C concentration, and to some degree the ascorbic acid concentration in the distal stomach, increased non-significantly following the administration of nitrate. In 6 out of the 17 subjects the total vitamin C concentrations were lower at all gastric sites, including the cardia, during the pre-nitrate period compared to the post-nitrate period. A possible explanation is that immediately after inserting the microdialysis assembly, the salivary flow may have been greater compared to the post-nitrate period of the study when the subjects were accustomed to the presence of a foreign object in the upper gastrointestinal tract and therefore may have been producing less saliva and hence less dilution of gastric juice vitamin C. The concentrations of ascorbic acid and total vitamin C also showed marked intragastric regional variations both being lower in the cardia region than in the proximal or distal stomach. This pattern was apparent both before and following nitrate ingestion. The reason for the concentration of ascorbic acid and total vitamin C being lowest in the most proximal cardia region of the stomach is also unclear. Vitamin C present in fasting gastric juice is believed to be due to its active secretion by the gastric mucosa ^(274, 275). The cells responsible for secreting the vitamin into gastric juice are not known but biopsies from different regions of the stomach have shown that the mucosa levels of

vitamin C are highest in the antrum⁽²⁶⁵⁾. In contrast with nitrite and ascorbic acid, there was no gradient in the intragastric concentration of thiocyanate with substantial concentrations being present throughout the stomach. The concentration of thiocyanate in the stomach was similar to that in the oesophagus and 50% of that in saliva.

The fact that the cardia region of the stomach had both the highest nitrite concentration and lowest ascorbic acid concentration meant that the ratio of ascorbic acid to nitrite was markedly lower in the cardia than in the more distal stomach. Before nitrate administration, the median ascorbic acid to nitrite ratio at the cardia was 1.5 versus 20.8 in the proximal and 27.7 in the distal stomach. After nitrate, the ratio in the cardia was only 0.3 compared with 7.8 in the proximal and 40 in the distal stomach. The ratio of ascorbic acid to nitrite is a critical determinant of acid nitrosation^(257, 259, 334). When ascorbic acid is in excess of nitrite, acid nitrosation is prevented^(257, 259). These observations indicate that the potential for intragastric acid catalysed luminal nitrosation will not be uniform throughout the length of the stomach but will be maximal in the most proximal cardia region. At this location, intragastric nitrite is maximal, intragastric ascorbic acid minimal, and thiocyanate is available to catalyse the nitrosation process. The clinical significance of our observations is that the anatomical location of maximal potential for acid catalysed luminal nitrosation corresponds with the location of the highest incidence of epithelial mutagenesis within the healthy acid secreting stomach^(116, 201, 335). It is tempting to propose that luminally generated *N*-nitroso compounds may be contributing to the high incidence of metaplasia and neoplasia at the gastric cardia. *N*-nitroso compounds can rapidly diffuse into the adjacent epithelium⁽³³⁶⁾ and therein be metabolically activated to powerful DNA alkylating agents^(337, 338). In addition, many *N*-nitroso compounds are unstable⁽³³⁹⁾ and therefore their epithelial effects will be maximal nearest their site of formation. The findings of our current study are consistent with our previous observation that nitric oxide levels are maximal at the gastric cardia⁽²⁷⁸⁾. In the present study, the concentration of nitrite was similar in buccal saliva and down as far as the distal oesophagus but then fell substantially on entering the gastric cardia. The fall in nitrite concentration between the distal oesophagus and cardia can be explained by the acidic gastric juice and its ascorbic acid content converting nitrite to nitric oxide⁽²⁷⁸⁾.

Observations in our earlier study together with those of our present study indicate that two distinct luminal mechanisms both potentially leading to epithelial DNA damage are occurring maximally at the most proximal cardia region of the acid secreting stomach (Fig 6.2). The first involves generation of high luminal concentrations of nitric oxide arising from the reaction between salivary nitrite and gastric juice ascorbic acid ⁽²⁷⁸⁾. This nitric oxide will diffuse into the adjacent epithelium and within the cells form nitrosating species which can directly and indirectly damage DNA ⁽³⁴⁰⁾. The second mechanism which is reported in the present study involves the generation of nitrosating species within the lumen due to the acidification of nitrite in the absence of adequate ascorbic acid. These nitrosating species formed in the lumen may react with nitrogenous compounds present in the lumen to form *N*-nitroso compounds which may then diffuse into the epithelial cells to damage DNA. The first mechanism is promoted by the presence of ascorbic acid whereas the second is promoted by the lack of ascorbic acid. Both mechanisms may operate maximally at this site although not simultaneously. When a bolus of nitrite laden saliva enters the cardia the ascorbic acid will convert much of it to nitric oxide. In the process, ascorbic acid will be consumed leading to the second mechanism now occurring at that same site. A common factor driving both processes is the delivery of nitrate derived nitrite into the acidic cardia region of the stomach. Further studies are required to determine whether this nitrate derived nitrosative chemistry focused at the most proximal cardia region of the acid secreting stomach is contributing to the high incidence of mutagenesis at this anatomical site.

CHAPTER ELEVEN

**NITRITE CONCENTRATIONS
IN THE UPPER
AERO-DIGESTIVE TRACT
IN HEALTHY VOLUNTEERS**

11.1 Introduction.

The major source of nitrate found in the human saliva is derived from the diet with approximately 1 mmol per day of nitrate being derived endogenously^(90, 279, 284). The nitrate is taken up by the salivary gland where it is concentrated and is then secreted in the saliva into the oral cavity where nitrate is reduced to nitrite^(286, 287). The reduction of nitrate to nitrite in the oral cavity is enzymic as boiled or filtered saliva is devoid of such activity⁽³⁴¹⁾ and it also follows the classic Michaelis Menton kinetics⁽²⁸⁸⁾. The enzyme nitrate reductase is derived from the bacteria found in the oral cavity and ingestion of antibiotics or the use of chlohexidine antibacterial mouthwash greatly reduces/ abolishes the conversion of nitrate to nitrite^(325, 326). The nitrate reductase activity is located on the dorsum of the tongue and the other mucosal surfaces are apparently devoid of such activity⁽²⁸⁸⁾. However, nitrate reduction to nitrite does not necessarily have to take place on the surface of the tongue as studies of expectorated saliva have shown nitrate reducing capability^(331, 341).

Our preceding study revealed a high concentration of nitrite in the distal oesophageal lumen. The concentrations of the chemicals relevant to nitrosation, which include nitrite, nitrate and thiocyanate in the other regions of the upper aero-digestive tract including the nasal cavity and the pharynx are presently unknown and maybe important in the pathogenesis of neoplasia in this region. Our previous study has shown that microdialysis probes are a suitable method for sampling chemicals from multiple sites simultaneously and with slight modifications in assembly of the probes we were able to measure the chemicals within the upper aero-digestive tract.

11.2 Aim.

The aim of this part of the study was to investigate the concentrations of the chemicals relevant to nitrosation in the pharynx, the upper and lower oesophagus as well as the nasal cavity. In addition the study was conducted to elucidate whether there was any concentration gradient for these chemicals in the upper aero-digestive tract.

11.3 Methods.

i) Microdialysis Assembly

Four microdialysis probes were mounted onto a thin nasogastric tube and secured as before. They were spaced such that a microdialysis probe was positioned in the nasal cavity, pharynx (7.5cm distal to the nasal probe), upper and lower oesophagus (10 and 20cm distal to the pharyngeal probe respectively) (Fig11.1). The positions of the microdialysis probes could be estimated beforehand as the endoscopic measurements were available prior to the study. A fine Teflon feeding catheter was secured to the assembly to allow administration of nitrate solution into the distal oesophagus. The microdialysis probes were tested for their recoveries of nitrite and thiocyanate at pH 7 at 37°C prior to human experiments.

ii) Subjects and experimental procedure

Seven asymptomatic, healthy volunteers were studied. The subjects consumed a low nitrate diet for 24 hours and attended having fasted overnight. The probes were passed intra-nasally and the pharyngeal microdialysis probe was observed to be in the correct position by observing the oral cavity during placement of the probe assembly.

The microdialysis probes were perfused with distilled water at 0.15 mL h⁻¹ and allowed to run for 15 minutes to reach equilibrium. The microdialysis products were collected in 20 µL of 1 M NaOH. Collection of the microdialysis samples was performed for 40 minutes under fasting conditions and 40 minutes after 50 ml of 2 mmol potassium nitrate solution administration. A 10-minute time lag was included for starting and ending the collection of microdialysis products to allow for the time the dialysate to travel the length of the microdialysis collecting tube. One half of the product was removed and transferred to another vial and stored at -80°C for later analysis of nitrate by HPLC. Serum nitrate and salivary samples were collected every 15 minutes as described in the previous experiments for microdialysis. The microdialysis probes were decontaminated and their function checked prior to reassembly as mentioned previously.

iii) Chemical analyses

Serum nitrate was analysed using bacterial nitrate reductase to reduce the nitrate to nitrite and then analysed colorimetrically using the Greiss reagent as previously described. Measurement of serum nitrate by HPLC after filtration through a microfilter was attempted but the separating column became rapidly contaminated and hence the samples were analysed colorimetrically.

Salivary nitrite was analysed colorimetrically using the Greiss reagent as described previously. A small number of salivary specimens (4 patients) were also analysed for nitrate and nitrite using high performance liquid chromatography as described below.

Microdialysis sample for nitrite was analysed both colorimetrically using the Greiss reagent and by HPLC as described above. Microdialysis samples of nitrate were analysed using HPLC since collection in NaOH and the small volumes involved (50 μL) made it impossible to use the bacterial nitrate reductase method without 'risking' the loss of the samples during the neutralisation of alkali with acid. Thiocyanate samples were analysed colorimetrically using the Bowler method as described previously.

iv) Nitrate analysis by high performance liquid chromatography

Nitrate concentrations in the microdialysis samples were determined by HPLC using an ultraviolet detector employing the method described by Blanco⁽³⁴²⁾. The instruments comprised of an automated sample injector (Shimadzu SIL-10AD) with a 50 μL loop, a pump (Shimadzu LC-10AT VP), an ultraviolet detector (Shimadzu SPD-6A), a precolumn (Phenomenex Security guard SAX strong anion exchanger 4 x 3 mm (i.d.)) and an analytical column (Partisil SAX 10 μm , 25 cm x 4.6 cm (i.d.)) respectively. The mobile phase consisted of 20 mM NaCl with 1 mM sodium dihydrogen orthophosphate adjusted to pH 7 using 1 mM NaOH. A mixture of 85 % mobile phase and 15 % acetonitrile was used. The mixture was filtered through a 0.45 micron filter and degassed with helium.

Analysis was performed at room temperature. The flow rate was 1.5 mL min^{-1} and the retention time of nitrate was approximately 6 minutes. A stock nitrate solution of 10 mM was prepared every 2 weeks and stored at 4°C. Working standards of 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 μM were prepared by

diluting appropriate volumes of the stock solution on the day of analysis. The autosampler was programmed to inject 20 μL of standards, quality control and samples. Saliva samples were centrifuged at 13000 r.p.m for 2 minutes and diluted with distilled water to a ratio of 1:2 to 1:5 depending on the nitrite concentration as determined colorimetrically. The variable dilution was to ensure that the nitrate in the samples was diluted sufficiently to lie within the 'standard curve'. It was assumed that approximately 30% of the nitrate will be converted to nitrite and hence we derived the approximate nitrate concentration and diluted the samples accordingly so that the samples were neither too concentrated nor too dilute. Microdialysis samples were injected undiluted, unless the microdialysis sample analysed for nitrite suggested further dilution was necessary. Subsequent standards and samples were processed with standards and quality controls occurring every tenth sample. The coefficient of variation for this method was 10.7 % for 15.6 μM and < 5 % for nitrate concentrations > 31.25 μM .

11.4 Data Analyses.

The microdialysis nitrite concentrations are presented using the colorimetrically derived data. The results obtained using the HPLC and the colorimetric methods were very similar in most instances but the HPLC appeared to significantly overestimate the nitrite concentrations on a number of samples where the data was clearly incorrect both in relation to the nitrite as determined colorimetrically and the nitrate concentration as determined simultaneously by HPLC. Since we have found the colorimetric method for nitrite determination to be accurate and reproducible in our experience with the lowest detection limit being 1 μM , and that the higher nitrite concentrations as determined by the colorimetric method was in good agreement with the HPLC method, the nitrite data is presented from the colorimetric analysis. The nitrate data was derived using HPLC as the method of collection for the microdialysis and saliva specimens entailed collecting the samples in NaOH which rendered the nitrate reductase method unsuitable.

The data are presented as median and inter-quartile range unless otherwise stated. Serum nitrate, salivary nitrite, salivary thiocyanate and saliva nitrate concentrations were grouped into pre-nitrate (0 – 45 minutes) and post-nitrate

periods (90 – 135 minutes) and the two periods were compared using the 1-sample Wilcoxon test. Comparisons of salivary nitrite, nitrate and thiocyanate before (-15 minutes) and after (0 – 45 minutes) insertion of the microdialysis probes were performed using the Mann-Whitney U test. For the microdialysis samples, Kruskal-Wallis test was used to analyse the chemicals of interest at the different anatomical regions for both the pre and post-nitrate data. Comparison between pre and post-nitrate microdialysis data was analysed using the 1-sample Wilcoxon test. A two-tailed $p < 0.05$ was considered statistically significant.

The concentrations of thiocyanate, nitrite and nitrate in the pharynx, upper oesophagus and lower oesophagus relative to the concentrations of these chemicals in the saliva were examined. The results are expressed as a percentage and calculated using the equation shown below. Where either the nitrite or the nitrate was undetectable, the data for the ratio was excluded. We did not compare the concentrations of the chemicals in the nasal cavity as the site was not exposed to saliva.

$\frac{\text{Concentration of chemical in the microdialysis sample}}{\text{Mean concentration of the chemical in saliva over the 40 minute collection period}} \times 100 \%$

Nitrite to nitrate ratio was also examined in the different anatomical sites and was expressed as a percentage.

11.5 Results.

Seven subjects were included in the study, six were male. The mean age was 29.1 years (range: 19 - 42).

11.5.1 Serum Nitrate.

The serum nitrate concentration increased from 11.0 μM (9.2 – 13.4) before the administration of nitrate to 34.6 μM (26.7 – 42.3) after the administration of nitrate

($p < 0.001$) with a peak of $38.3 \mu\text{M}$ ($28.7 - 45.5$) at 75 minutes, 25 minutes after nitrate administration (Fig 11.2).

In the four patients who had salivary nitrate measured, the serum nitrate concentration was $10.6 \mu\text{M}$ ($9.3 - 12.3$) before the administration of nitrate and $32.6 \mu\text{M}$ ($22.2 - 38.3$) after the administration of nitrate ($p = 0.001$).

11.5.2 Saliva.

i) Nitrite, nitrate and nitrite to nitrate ratio

The salivary nitrite concentration in the seven subjects before inserting the microdialysis assembly (-15 minutes) was $89.1 \mu\text{M}$ ($38 - 149.3$). Following insertion of the microdialysis probes salivary nitrite concentration fell to $29.7 \mu\text{M}$ ($16 - 41.5$) before the administration of nitrate (0 - 45 minutes) ($p = 0.003$ vs before inserting microdialysis probes) and increased to $259 \mu\text{M}$ ($123 - 329$) following the administration of nitrate (90 - 135 minutes) ($p < 0.001$ vs pre-nitrate) with a peak concentration of $331 \mu\text{M}$ ($139 - 371$) at 75 minutes, 25 minutes after nitrate administration. The nitrite concentration decreased to $121.2 \mu\text{M}$ ($115 - 170$) at the end of the experiment at 135 minutes (Fig 11.3).

In the four subjects that had salivary nitrate measured, the salivary nitrate concentration before the insertion of the microdialysis probes (-15 minutes) was $178.7 \mu\text{M}$ ($100.1 - 246.5$). Following the insertion of the microdialysis probes the nitrate concentration fell slightly to $137 \mu\text{M}$ ($101 - 184$) (N.S. vs before microdialysis insertion) before the administration of nitrate (0 - 45 minutes) and increased to $1237 \mu\text{M}$ ($471 - 1769$) after the administration of nitrate (90 - 135 minutes) ($p < 0.001$ vs pre-nitrate). In these four subjects the salivary nitrite concentrations were similar to the salivary nitrite concentrations of the whole group. The salivary nitrite concentration for the four subjects was $119.2 \mu\text{M}$ ($50.8 - 202.3$) before the insertion of the microdialysis probes (-15 minutes) which fell after the insertion of the microdialysis assembly to $30.4 \mu\text{M}$ ($7.9 - 66.5$) before the administration of nitrate ($p = 0.04$ vs before microdialysis insertion) and increased to $175.3 \mu\text{M}$ ($121.4 - 360.9$) following the administration of nitrate ($p < 0.001$ vs pre-nitrate). The peak nitrite concentration was $268 \mu\text{M}$ ($77 - 619$) at 90

minutes, 40 minutes after nitrate, and this slowly declined to 121.6 μM (115.3 – 195.3) at the end of the experiment at 135 minutes.

The median salivary nitrite to nitrate ratio for the four patients before insertion of the microdialysis assembly (-15 minutes) was 0.87 (0.22 – 1.19). Following the insertion of the assembly the ratios were significantly lower, the ratio was 0.21 (0 – 0.66) before the administration of nitrate ($p < 0.05$ vs before insertion of microdialysis assembly) and 0.23 (0.1 – 0.42) following the administration of nitrate (N.S. vs pre-nitrate).

ii) Thiocyanate

Before the insertion of the microdialysis assembly (-15 minutes) the salivary thiocyanate concentration in the seven subjects was 1833 μM (372 - 3429). The salivary thiocyanate concentration fell to 796 μM (330 – 1602) after microdialysis insertion and before the administration of nitrate (0 – 45 minutes) (N.S. vs before microdialysis insertion). Following the administration of nitrate (90 – 135 minutes) the thiocyanate concentration remained at a similar concentration of 821 μM (312 – 1407) (N.S. vs before nitrate).

11.5.3 Microdialysis samples.

i) Nitrate, nitrite and thiocyanate in the upper aerodigestive tract

The nitrate concentrations in the nasal cavity, pharynx, upper and lower oesophagus before the administration of nitrate for the seven subjects were 8.9 μM (0 – 13.1), 58.9 μM (25.3 – 64.7), 93.9 μM (78.4 - 113) and 89.2 μM (72.7 - 125) respectively ($p = 0.001$ for trend, Kruskal-Wallis). The concentrations following the administration of nitrate were 41.6 μM (34.1 – 51.7), 383 μM (132 - 493), 795 μM (566 - 1221) and 814 μM (365 - 1386) for the nasal cavity, pharynx, upper and lower oesophagus respectively ($p = 0.002$ for trend, Kruskal-Wallis; $p < 0.05$ vs pre-nitrate for all 4 sites) (Fig. 11.4).

The nitrite concentrations for the seven subjects before the administration of nitrate were 0 μM , 6.8 μM (1.6 – 15.8), 21.0 μM (11.5 – 49.7) and 19.9 μM (17.2 – 37.8) for the nasal cavity, pharynx, upper and lower oesophagus respectively ($p < 0.001$ for trend, Kruskal Wallis). Following the administration of nitrate no

nitrite was detected in the nasal cavity whereas nitrite concentrations increased to 93 μM (23.8 – 213), 243 μM (134 - 271) and 196 μM (142 - 289) in the pharynx, upper and lower oesophagus respectively ($p = 0.001$ for trend, Kruskal-Wallis; $p < 0.05$ vs pre-nitrate for pharynx, upper and lower oesophagus) (Fig. 11.4).

The thiocyanate concentrations in the nasal cavity, pharynx, upper and lower oesophagus for the seven subjects before the nitrate administration were 90 μM (72 - 128), 262 μM (110 - 985), 638 μM (223 - 1142) and 725 μM (181 - 1146) respectively ($p = 0.003$ for trend, Kruskal-Wallis). Following the administration of nitrate, concentrations of thiocyanate in the nasal cavity, pharynx, upper and lower oesophagus remained unchanged at 100 μM (42.0 - 109), 326 μM (128 - 845), 633 μM (240 - 1361) and 594 μM (219 - 1432) ($p = 0.003$ for trend, Kruskal-Wallis) respectively (Fig. 11.4).

ii) Nitrite to nitrate ratio in the upper aerodigestive tract

The median (range) nitrite to nitrate ratios, expressed as percentage were 0% (N = 5), 9% (1 - 67) (N = 7), 26% (11 - 97) (N = 7) and 32% (13 - 100) (N = 6) before the nitrate meal in the nasal cavity, pharynx, upper and lower oesophagus respectively ($p = 0.002$, Kruskal-Wallis); the ratios after the nitrate meal were 0%, 19% (5 - 110), 22% (15 - 92) and 28% (14 - 93) for the respective anatomical regions ($p = 0.002$, Kruskal-Wallis; $p = \text{N.S.}$ vs pre-nitrate). In comparison the corresponding salivary nitrite to nitrate ratios, in the 4 subjects where salivary nitrate was available, were 0.21 (0 – 0.66) before nitrate administration and 0.23 (0.1 – 0.42) following nitrate administration.

11.5.4 Thiocyanate, nitrite and nitrate concentrations in the pharynx and the oesophageal lumen relative to salivary concentrations.

i) Thiocyanate

The median (range) ratio of thiocyanate concentration in the pharynx, upper and lower oesophagus relative to the thiocyanate concentration in the saliva expressed as a percentage for the seven subjects before the administration of

nitrate were 43 % (11 – 60), 76 % (46 – 104) and 72 % (31 – 109) ($p = 0.013$ for trend, Kruskal-Wallis) respectively. After nitrate administration they were 44 % (9 -70), 88 % (45 – 102) and 79 % (29 – 103) in the pharynx, upper and lower oesophagus respectively ($p = 0.012$ for trend, Kruskal-Wallis; $p = \text{N.S.}$ pre vs post-nitrate).

ii) Nitrite

The median (range) ratios of the nitrite concentration in the pharynx, upper and lower oesophagus relative to the nitrite concentration in the saliva expressed as a percentage for the seven subjects were 31 % (3 – 49), 81 % (47 – 160) and 92 % (59 – 103) ($p = 0.01$ for trend, Kruskal-Wallis) before nitrate administration; 38 % (3 – 93), 82 % (44 – 160) and 59 % (49 – 190) after nitrate administration in the pharynx, upper and lower oesophagus respectively (N.S. for trend, $p = 0.035$ for pharynx pre vs post-nitrate).

In one of the 7 subjects before nitrate administration and 3 of the 7 subjects after nitrate administration, nitrite concentrations in the oesophagus were greater than that of the saliva. The concentrations of nitrite detected in the upper oesophagus for one subject before nitrate administration was 160% of the salivary nitrite concentrations. The concentrations of nitrite detected from the lower oesophagus for the 3 subjects after nitrate administration were 130%, 160% and 190% of the salivary nitrite concentrations.

iii) Nitrate

The results presented are for the 4 subjects with salivary nitrate data. The median (range) ratios of the nitrite concentration in the pharynx, upper and lower oesophagus relative to the nitrite concentration in the saliva expressed as a percentage were 33% (29– 42), 69 % (36 – 110) and 93 % (36 – 119) (N=3) before nitrate administration; 33 % (22 – 59), 53 % (45 – 104) and 56 % (36 – 118) after nitrate administration in the pharynx, upper and lower oesophagus respectively. No statistical analysis was performed in view of the small sample sizes.

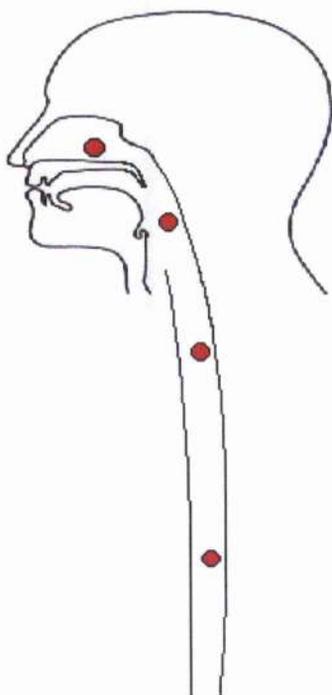


Fig 11.1: Positions of the microdialysis probes.
Red dots indicate the positions of the microdialysis probes in the nasal cavity, pharynx, upper and lower oesophagus.

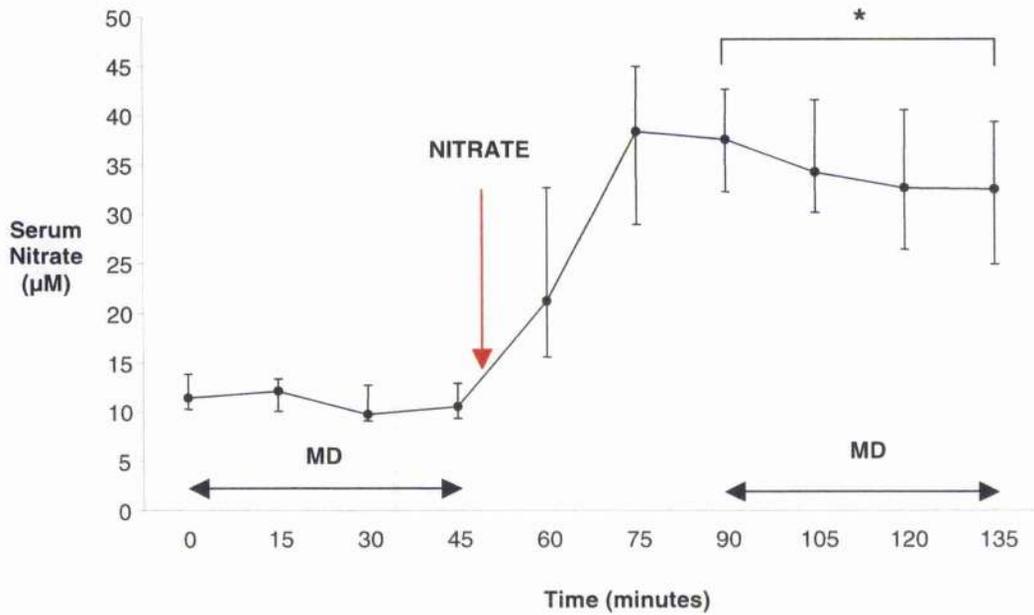


Fig 11.2: Median serum nitrate concentrations before and after nitrate administration.

The error bars represent IQR. Two mmol potassium nitrate administered at 50 mins (red arrow). MD indicates microdialysis sampling periods (black arrows); 0 – 45 minute collection period is before nitrate administration and 90 – 135 minute collection period is after nitrate administration.

* $p < 0.001$ comparing 90 - 135 min (after nitrate) to 0 to 45min (before nitrate).

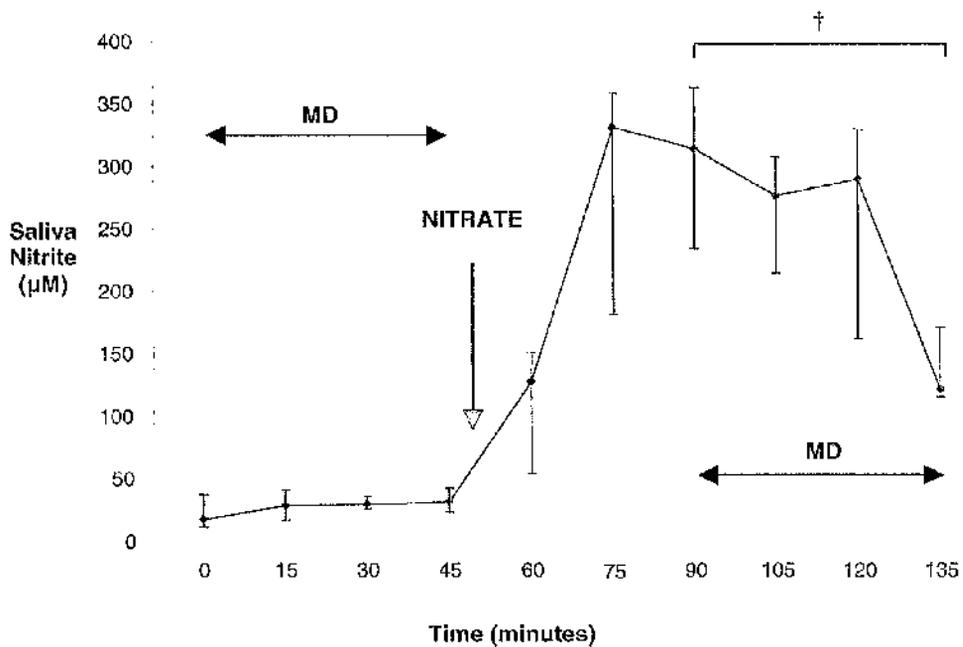


Fig 11.3: Median salivary nitrite concentrations before and after nitrate administration.

Error bars represent IQR. Two mmol potassium nitrate administered at 50mins (red arrow). MD indicates periods of microdialysis sampling (black arrows); 0 to 45 minute collection period is before nitrate administration and 90 to 135 minute collection period is after nitrate administration. *Before* insertion of the microdialysis probes, the nitrite concentration was 89 µM (38 -149).

† $p < 0.001$ comparing 90 - 135 minutes (after nitrate) vs 0 - 45 minutes (before nitrate),

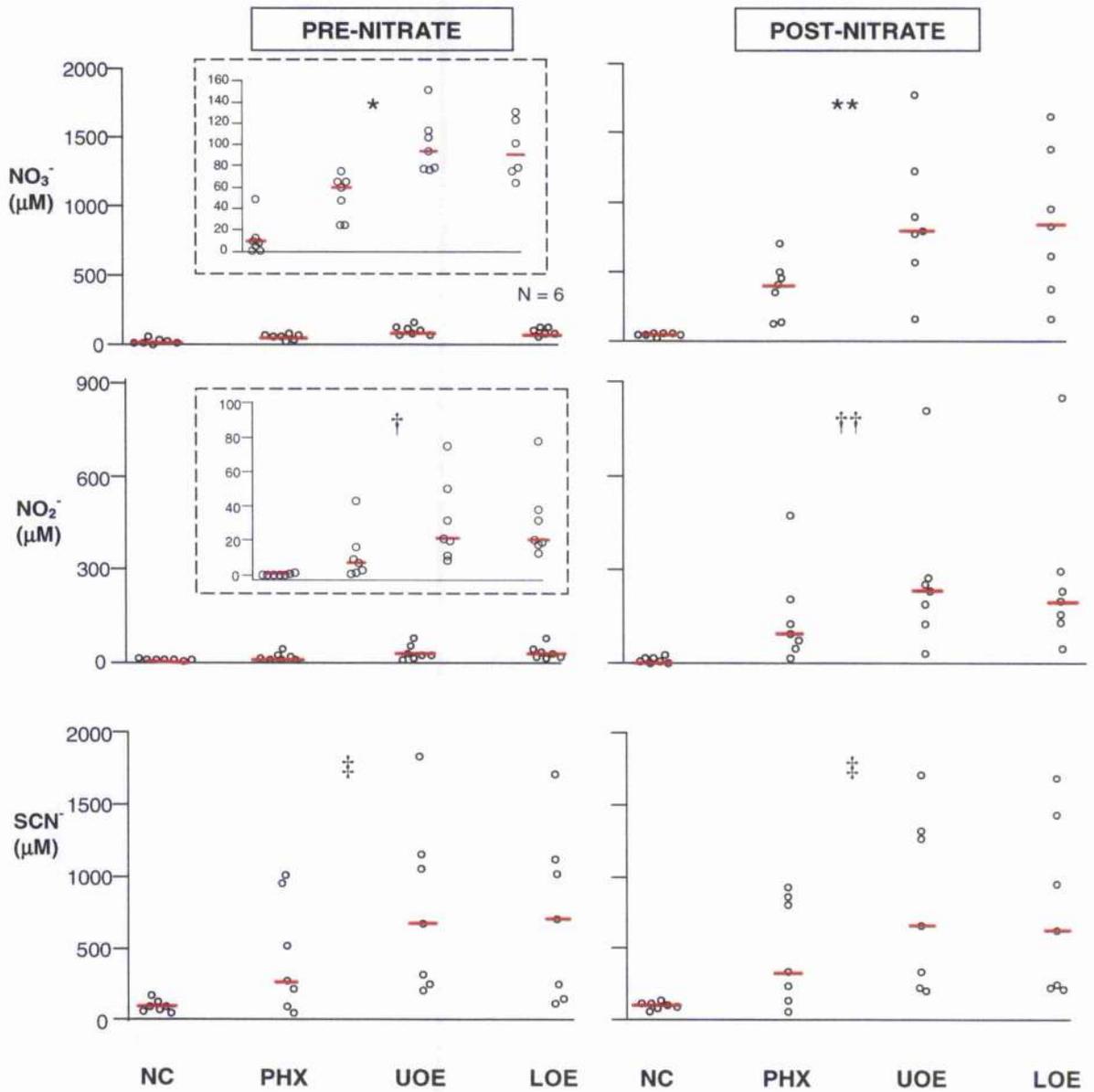


Fig 11.4: Concentrations of nitrate, nitrite and thiocyanate in the upper aerodigestive tract before and after nitrate administration.

The horizontal bars indicate medians. NC, nasal cavity; PHX, pharynx; UOE, upper oesophagus; LOE, lower oesophagus.

NO_2^- , nitrite; NO_3^- , nitrate; SCN^- , thiocyanate.

* $p = 0.001$ for comparing different anatomical regions for pre-nitrate, Kruskal-Wallis.

** $p = 0.002$ for comparing different anatomical regions post-nitrate, Kruskal-Wallis; $p < 0.05$ for all sites vs pre-nitrate.

† $p < 0.001$ for comparing different anatomical regions pre-nitrate.

†† $p = 0.001$ for comparing different anatomical regions post-nitrate; $p < 0.05$ for pharynx, upper and lower oesophagus vs pre-nitrate.

‡ $p < 0.05$ for comparing different anatomical regions pre and post-nitrate.

11.6 Discussion.

We have previously studied the concentrations of the chemicals relevant to nitrosation in the saliva and the lumen of the upper gastrointestinal tract of healthy subjects without gastro-oesophagal reflux (chapter 10). The previous study indicated that significant concentrations of nitrite and thiocyanate were present in the saliva and the distal oesophagus. In the current study, the fasting salivary nitrite concentration of 30 μM increased by over 10-fold to a peak of 331 μM following nitrate administration. The nitrite concentration in the distal oesophagus increased from 20 μM before nitrate administration to 180 μM after nitrate administration. Both the saliva and oesophageal nitrite results were similar to the previous study in subjects without gastro-oesophageal reflux.

In the four patients in whom salivary nitrate was measured, the median nitrate concentration was 137 μM before nitrate administration and increased almost 10-fold to 1237 μM following nitrate administration. This is in comparison to the serum nitrate concentrations of 11 μM and 33 μM respectively. The serum nitrate concentrations following nitrate administration was somewhat lower than in the previous study (17 μM before nitrate administration and 59 μM after nitrate). The substantially higher nitrate concentration in the saliva compared to the serum is due to the active uptake and concentration of the anion by the salivary gland⁽²⁸²⁾. The disproportionately higher salivary nitrate concentration compared to the serum nitrate concentration after nitrate administration in this study is likely to be due to the nitrate reductase method employed for nitrate reduction in the serum may have potentially underestimated the nitrate concentration in the serum⁽³⁴³⁾. There appears to be a non-proteinaceous chemical (serum filtered through a 10 micron microfilter) in the serum which inhibits the bacterial nitrate reductase. We were also unable to utilise the HPLC for serum nitrate measurement due to the contamination of the analytical column by filtered serum which interfered with the assay. These problems however do not detract from our findings as the main aim was to measure the concentrations of the chemicals in the lumen of the upper aero-digestive tract and measurement of the serum nitrate was merely to confirm the increase in serum nitrate following its administration.

Approximately 20 to 30 % of the nitrate is converted to nitrite in the saliva as indicated by the salivary nitrite to nitrate ratio. The ratios ranged from 0 to

100 %. Despite such a wide range, the ratios in an individual patient were consistent throughout the experimental period. Such individual variability has been noted previously^(283, 286, 326, 331). The variability between patients can be explained by variability in the salivary flow rate⁽³³²⁾ and the capacity to reduce nitrate to nitrite^(286, 326, 331). Salivary secretions are devoid of nitrite^(332, 344). Salivary nitrite is derived from the bacterial reduction of salivary nitrate within the oral cavity. Where the saliva flow rate is lower, the time available for nitrate reduction to nitrite by bacterial enzymes is greater and hence nitrite concentration will be greater as a result. The insertion of the microdialysis assembly stimulates the flow of saliva and in so doing reduces the concentration of both nitrate and nitrite. The reduction in the salivary nitrite to nitrate ratio in our study following stimulation is in contrast to the study by Granli where mechanical stimulation increased the nitrite to nitrate ratio in some individuals⁽³³²⁾. Granli's study involved chewing to stimulate salivary production whereas in our patients stimulation was effected by the presence of a foreign material. Chewing is likely to mix the bacteria on the dorsum of the tongue more thoroughly with saliva and therefore make nitrate reduction more efficient and likely account for the difference observed.

Our study is the first to describe the concentrations of nitrate, nitrite and thiocyanate in the nasal cavity, the pharynx and the upper and lower oesophagus by utilising the microdialysis probes which enabled simultaneous measurement of these chemicals at the different sites. Before the nitrate meal, the concentration of nitrate was lowest in the nasal cavity followed by the pharynx and then the proximal and the lower oesophagus (both of the latter had similar concentrations of the chemicals). Due to the design of the microdialysis assembly the secretions in the nasal cavity may not have been in constant contact with the mucosa in the nasal cavity which resulted in the lower concentrations of the various chemicals sampled. What is clear from the data, however, is that there appears to be no nitrate reduction to nitrite within the nasal cavity even after nitrate administration when nitrate concentrations in the nasal cavity increased by 5-fold. The bacterial nitrate reductase catalyses the nitrate reduction to nitrite in the oral cavity. In man the dorsal surface of the tongue appears to be the site of nitrate-reducing activity whereas the inferior surface of the tongue, the buccal surface, the hard and soft palates are devoid of such activity⁽²⁸⁸⁾. Bacterial nitrate reductase is induced

during anaerobic respiration ⁽²⁴⁹⁾. Previously published studies examined the partial pressure of oxygen within the different regions within the oral cavity ^(345, 346). Eskow noted pO₂ to be 117 mm Hg and 86 mm Hg in the air above the anterior and posterior regions of the tongue whereas pO₂ was noted to be between 2 to 3 mm Hg in the buccal pouch ⁽³⁴⁵⁾. Globerman noted the pO₂ was below 10 mm Hg when the oxygen tension was measured directly at the surface of the tongue ⁽³⁴⁶⁾. The deep pits which are present in the dorsal surface of the tongue may provide the anaerobic condition necessary for inducing anaerobic respiration in the nitrate-reducing bacteria ⁽³⁴⁷⁾. The oxygen tension within the nasal cavity has not been studied as far as we are aware but it is unlikely to have a low oxygen tension in the healthy state and may not be suitable for the induction of the bacterial nitrate-reductase since nitrate-reducing bacteria are present within the nasal cavity ^(348, 349). Other potential factors for the difference observed for nitrate reduction to nitrite include differences in the micro-organisms found in the nasal cavity and the oral cavity/tongue ^(350, 351, 352) and the possible differences in the density of the nitrate-reducing bacteria at the two sites since there appears to be a positive correlation between nitrite production and the density of the nitrate-reducing bacteria ⁽³⁴⁷⁾.

Compared to the two oesophageal sites the concentrations of the chemicals were lower in the pharynx. One explanation for such finding may be because the act of swallowing saliva is intermittent and therefore the microdialysis probe in the pharynx may be sampling intermittently within the pharyngeal cavity whereas the probes in the oesophagus lies in a more confined space where the lumen is virtually collapsed ⁽³⁵³⁾. The viscous saliva swallowed is likely to remain adherent on the oesophageal mucosa which will be sampled constantly by the microdialysis probe which would produce a higher concentrations of chemicals compared to the pharynx. This theory is supported by the findings of a lower thiocyanate concentration in the pharynx compared to that of the oesophagus. The thiocyanate concentrations relative to that of the saliva in percentage terms were 43 %, 76 % and 72 % in the pharynx, upper oesophagus and lower oesophagus before nitrate administration. A similar trend was seen for thiocyanate concentrations after the nitrate meal. In the validation study, at pH > 4 (Chapter 8), nitrite recovery was similar to the thiocyanate recovery at approximately 80 - 90 % therefore the low concentrations of thiocyanate, nitrite and nitrate in the pharynx compared to the

oesophageal samples is in keeping with intermittent nature of the microdialysis sampling in the pharynx.

In the current experiment, one of the seven subjects before nitrate administration and three of the seven subjects after the nitrate administration had nitrite recoveries from the oesophagus greater than that of the salivary nitrite concentrations, one patient by as much as 190 % of the nitrite concentration in the saliva. This is likely to be explained by the nitrate being converted to nitrite by the action of the bacterial nitrate reductase while the saliva was adherent to the oesophageal mucosa since the concentration of nitrite in the oesophagus should not exceed that of the saliva as none of the chemicals studied had 100 % recovery in the validation studies. Local secretion of nitrite is an alternative explanation which may account for the higher nitrite recovery in the oesophagus in some individuals. This is unlikely for a number of reasons. Firstly, the submucosal glands in the oesophagus are similar to salivary glands⁽³³⁰⁾ and salivary glands do not secrete nitrite^(332, 344). Secondly, nitrate secreted by the salivary glands is derived from the nitrate in the blood and nitrite concentrations are extremely low in the circulation of healthy individuals as it is rapidly oxidized to nitrate by haemoglobin^(354, 355, 356) therefore there is no source of nitrite unless it is generated within the submucosal glands. Another potential explanation could be due to mechanical stimulation by the presence of the microdialysis tube. A previous study has shown that mechanical stimulation by chewing increased nitrate output and increased the nitrite to nitrate ratio in the saliva⁽³³²⁾. However, our study showed the opposite and the mechanical stimulation of saliva secretion by the presence of a foreign body does not appear to explain the above finding.

The significance of such high concentrations of nitrate and nitrite in the upper digestive tract is three-fold. Firstly, in subjects without gastro-oesophageal reflux and a normal acid-secreting stomach, acid-catalysed nitrosation reaction may occur at the gastro-oesophageal junction, where acidic gastric juice first meets salivary nitrite. Secondly, bacterial nitrosation which has been proposed as a potential mechanism in the development of gastric cancer in the achlohydric stomach occur at neutral pH⁽²¹⁵⁾. This mechanism may be potentially important for the development of neoplasia within the length of the oesophagus since some of the bacteria found in the oral cavity are capable of catalysing the formation of *N*-nitroso compounds in the presence of nitrate and nitrite^(241, 357, 358). Thirdly,

nitric oxide, a highly reactive radical is generated at the gastro-oesophageal junction where salivary nitrite at neutral pH meets acidic gastric juice containing ascorbic acid ⁽²⁷⁸⁾. The nitrite becomes acidified and is rapidly reduced to nitric oxide by the ascorbic acid in gastric juice. The nitric oxide thus produced can diffuse into the surrounding tissues and may lead to detrimental intracellular effects such as deamination of DNA ^(359, 360) or the formation of *N*-nitroso compounds within the cells ^(302, 303) which could cause mutagenesis. The first and the third mechanisms are acid-dependent and therefore require acid gastric juice and are more relevant for cardia and oesophageal adenocarcinoma. Bacterial nitrosation, on the other hand, occurs in the absence of acidic gastric juice at neutral pH and potentially relevant for squamous oesophageal carcinoma and non-cardia gastric cancer. Thus it can be concluded that high concentrations of nitrate and nitrite in the upper gastrointestinal tract have the potential of generating mutagenic chemicals which may be important in the aetiopathogenesis of cancer in the upper gastrointestinal tract.

CHAPTER TWELVE

STUDY OF NITROSATION POTENTIAL

IN BARRETT'S OESOPHAGUS

12.1 Introduction.

The incidences of adenocarcinoma of the oesophagus and cardia in the western nations have increased dramatically over the past 25 years^(3, 361). The cause for the marked increase in these cancers is yet to be identified.

An important factor is gastro-oesophageal reflux, which increases the risk of developing esophageal adenocarcinoma almost 8-fold compared to those individuals without reflux⁽⁵⁹⁾. The increased risk appears to be specific for Barrett's oesophagus which markedly increases the risk for developing esophageal adenocarcinoma compared to uncomplicated reflux⁽³⁶²⁾. Barrett's oesophagus develops in those with duodeno-gastroesophageal reflux^(176, 177) and the presence of Barrett's oesophagus increases the risk for developing esophageal adenocarcinoma 40-fold over the general population with more recent risk estimates suggesting a cancer incidence among patients with Barrett's oesophagus of approximately 1 per 200 patient-years^(123, 124, 141, 142). It is assumed that the reflux of gastric acid, pepsin and bile acids causes mucosal damage leading to the development of intestinal metaplasia, dysplasia and subsequently adenocarcinoma of the oesophagus. However it is unclear whether the mucosal damage induced by the refluxate is sufficient to induce cancer or whether it merely sensitizes the epithelium to luminal carcinogens.

In the previous chapters we have discussed the mechanism of acid-catalysed nitrosation which involves the acidification of salivary nitrite. Acidification of nitrite produces the nitrosating species such as N_2O_3 and NO^+ which react with nitrosatable substrates to generate carcinogenic *N*-nitroso compounds. The major inhibitor of *N*-nitroso compound formation within the gastric lumen is ascorbic acid which is actively secreted into the gastric juice^(274, 275). Ascorbic acid reacts with the nitrosating species to generate nitric oxide and is itself oxidised to dehydroascorbic acid^(257 - 259, 276 - 278). Although this action of ascorbic acid inhibits the luminal generation of *N*-nitroso compounds, the nitric oxide produced in the process can readily diffuse into the adjacent epithelium and within it induce nitrosative stress^(302, 303) as high concentrations of nitric oxide reacts with oxygen to form the nitrosating species N_2O_3 ⁽³⁴⁰⁾. The latter can directly deaminate DNA or indirectly damage DNA via the formation of *N*-nitroso compounds^(340, 359, 360). Nitric oxide has also been shown to inhibit a number of DNA repair enzymes^{(304 -}

³⁰⁶⁾. Nitric oxide in high concentrations is known to be mutagenic and is implicated in neoplasia associated with chronic inflammation ^(363, 364). In chapter 10 we examined the luminal nitrite chemistry following nitrate ingestion in subjects with healthy, acid secreting stomachs and without reflux disease. We found that this chemistry was focused at the gastric cardia where nitrite in saliva first encounters acidic gastric juice. This anatomical location had the chemical conditions most favoring luminal *N*-nitroso compound formation in being the acidic environment with the highest nitrite and lowest vitamin C concentration. Also, in a previous study we found that the peak nitric oxide concentration generated from dietary nitrate occurred at the gastric cardia ⁽²⁷⁸⁾. These observations raise the possibility that the active luminal nitrosating chemistry occurring at the gastric cardia may contribute to the high incidence of mutagenesis occurring at the cardia. In subjects with gastro-oesophageal reflux, the anatomical location where saliva nitrite first encounters acidic gastric juice moves proximally to within the distal oesophagus rather than at the gastric cardia.

In the present chapter, we have examined the anatomical location of luminal nitrosative stress following nitrate ingestion in patients with Barrett's oesophagus. This chapter examines the nitrosation potential within the Barrett's oesophagus using the microdialysis probes and the following chapter examines nitric oxide generation within the Barrett's segment during periods of acid reflux.

12.2 Methods.

12.2.1 Design of the microdialysis probes

CMA flexible microdialysis probes employed in the previous experiments were used to measure the local luminal concentrations of the chemicals relevant to nitrosation (ie nitrite, thiocyanate, ascorbic acid and total vitamin C). Five such probes were attached to a 3.3mm diameter nasogastric tube (Rüsch Manufacturing, Lurgan, UK) as previously described. The probes were located on the tube so that one of the probes would be at each of the following anatomical sites: squamous oesophagus, Barrett's segment, hiatus hernia, proximal stomach and distal stomach (Fig 12.1). The hiatal hernia microdialysis probe was positioned 1cm below the gastro-oesophageal junction (defined by the position of the proximal

gastric fold) and the Barrett's probe 2.5cm above the gastro-oesophageal junction. The gastric microdialysis probes were positioned 5 and 10 cm distal to the hiatal hernia probe. The probe in the squamous oesophagus was positioned 5 to 10cm proximal to the Barrett's probe. These allowed us to simultaneously measure and thus compare the nitrosative chemistry at each of these anatomical locations. Individual tubes were prepared for each subject with the location of the microdialysis probes located at sites corresponding to the patient's individual anatomy. A multi-channel pH catheter (Synectics Medical Ltd., Enfield, U.K.) was mounted alongside the nasogastric tube so that we could monitor the luminal pH at the site of each microdialysis probe. The outputs from the pH sensors were digitalized (DAS 1201 data acquisition board, Keithley Instruments Ltd., Reading, Berkshire, U.K.), monitored on a computer screen during the procedure and stored on computer for subsequent analysis. A fine-bore Teflon tube with its opening at the tip of the nasogastric tube was attached to the assembly for intragastric delivery of the nitrate solution.

Each microdialysis probe was perfused with degassed distilled water by means of a microdialysis pump (Univentor 864 microdialysis syringe pump, Biotech Instruments Ltd., U.K.) at a rate of $150 \mu\text{L hr}^{-1}$. The microdialysis product was collected from each probe for analysis of the various chemicals. The samples for ascorbic acid and total vitamin C were collected into a glass vial (no. 1) containing $20 \mu\text{L}$ of a solution of 2 % metaphosphoric acid and 0.5 % sulphamic acid (M/S) and were further diluted with equal volumes of metaphosphoric / sulphamic acid solution for ascorbic acid and with 12 mg mL^{-1} dithiothreitol in metaphosphoric/sulphamic acid solution (M/S + DTT) for total vitamin C determination. The nitrite and thiocyanate samples were collected into a glass vial (no. 2) containing $20 \mu\text{L}$ 1 M NaOH. The two different collections from each tube were achieved by alternating the microdialysis collecting tube between vials 1 and 2 every two minutes. The vitamin C samples were frozen at -70°C for analysis within four weeks and the nitrite and thiocyanate samples were stored at 4°C for analysis on the same day. Prior to each study, the fully assembled apparatus was calibrated for recovery of each chemical by individual microdialysis probes as previously described.

12.2.2 Subjects and Methods

Patients with endoscopic evidence of Barrett's oesophagus were examined. In each subject, the columnar-lined oesophagus extended at least 3 cm proximal to the upper border of the gastric folds as determined by previous endoscopy. The size of the hiatal hernia was measured from the proximal gastric fold to the point at which the diaphragmatic constriction of the gastric lumen occurred. Biopsies of the columnar-lined oesophagus confirmed intestinal metaplasia in all patients. Each patient was confirmed to be *H. pylori* – negative by histology and rapid urease test of both antral and body mucosal biopsies.

The subjects stopped all anti-secretory medications for at least five days and were instructed to avoid nitrate-rich foods for 24 hours prior to the procedure. They attended in the morning having fasted since the previous evening and underwent an endoscopic examination to confirm anatomic locations and to apply radio-opaque clips (Haemoclips, Olympus HX-600-090, KeyMed, Southend-on-Sea, UK) at the squamo-columnar junction and the gastro-oesophageal junction to delineate the Barrett's segment. Following this the microdialysis probe assembly was passed per orally such that the microdialysis probes were located appropriately using the distance from the incisors as the reference point. The position of the assembly was checked by an abdominal X-ray and final adjustment performed using the Hemoclips as reference points.

The purpose of this study was to examine the nitrosation chemistry during gastro-oesophageal reflux. In order to ensure that reflux occurred throughout the duration of the experiment, the subjects lay in the right-lateral decubitus position⁽³⁶⁵⁾ with or without the head-down position to provoke gastro-oesophageal reflux. A low dose ($0.06 \mu\text{g kg}^{-1}$) of pentagastrin (Cambridge Laboratories, Wallsend, England, U.K.) was infused intravenously to stimulate physiological levels of gastric secretion and acid reflux throughout the study.

The first 15 minute collections of the microdialysis samples were discarded and the following 40 minutes collected for analyses. Following this basal collection, 2 mmol potassium nitrate in 25mL of water was administered intra-gastrically via the fine Teflon feeding tube attached to the nasogastric tube. Forty minutes after administering the potassium nitrate further microdialysis collections were obtained for 40 minutes. The delay between administration of nitrate and

commencing collections is to allow time for the nitrate to be absorbed, secreted in saliva and converted in the mouth to nitrite. Venous blood was obtained from an indwelling intravenous catheter every 15 minutes for determination of serum nitrate. The samples were centrifuged at 3000 r.p.m. at 4°C for 10 minutes and the supernatant for serum nitrate was transferred into a plain test tube and frozen at -20°C until analysis within 4 weeks. Samples of saliva were obtained every 15 minutes for nitrite and thiocyanate determination. This was done by asking the patient to spit into a sterile container and 500µL was immediately added to 50µL of 1 M NaOH in a 1.5 mL Eppendorf tube for nitrite and thiocyanate analysis and stored at 4°C until analysis on the same day. Prior to analysis, the saliva samples were centrifuged at 13000 r.p.m. for two minutes using a microcentrifuge and the supernatant was analysed.

12.2.3 Chemical Analyses

The chemicals of interest, nitrite, thiocyanate, ascorbic acid and total vitamin C were determined as previously. Serum nitrate and salivary nitrite were also determined as previously described.

12.3 Statistical Analyses.

Data are presented as medians and inter quartile range (IQR) unless otherwise stated. Kruskal-Wallis test was used to analyse the concentrations of the chemicals of nitrosation in the different regions of the upper GI tract for both pre and post-nitrate data. Comparison between the pre and post-nitrate microdialysis data was analysed using 1-sample Wilcoxon test.

Serum nitrate, salivary nitrite and thiocyanate data and pH of the different regions of the upper GI tract were analysed using 1-sample Wilcoxon test using the Bonferroni's correction.

For the analysis of ascorbic acid to total vitamin C ratio, if both ascorbic acid and total vitamin C were undetectable from the single sample, the sample was excluded from statistical analysis.

In order to calculate the ascorbic acid to nitrite ratio, any samples with undetectable levels of ascorbic acid or nitrite concentrations were allocated a

concentration of 1 μM , the approximate lower limit of detection for both ascorbic acid and nitrite using our analytical methods. If both ascorbic acid and nitrite were undetectable, these samples were excluded from analysis. Kruskal-Wallis test was used to analyse the ascorbic acid to nitrite ratio in the different regions before and after nitrate ingestion. A two-tailed $p < 0.05$ was considered statistically significant.

12.4 Results

Fourteen subjects with Barrett's oesophagus consented to the study. Four could not tolerate the procedure and therefore results are available for ten subjects. The mean age of the subjects was 65 years (range: 48 - 70) and 4 were females. The median length of Barrett's segment was 5 cm (range: 3-12 cm) for the ten patients in the microdialysis experiment.

12.4.1 Serum

The nitrate concentration in the blood just prior to administration of the nitrate solution at 45 minutes was 13.0 μM (12.3 – 19.8) and the peak nitrate level 50.5 μM (30.5 – 69.6) ($p < 0.01$ vs 45 min) was seen 55 minutes after the nitrate was administered and remained at this level throughout the remainder of the experiment (Fig 12.2).

12.4.2 Saliva

i) Nitrite and thiocyanate

The saliva nitrite concentration before the insertion of the probe assembly was 73.1 μM (61.8 – 216.4) ($n = 9$) and fell to 35.0 μM (26.3 – 78.9) at 15 minutes ($n = 9$) after the insertion of the microdialysis assembly (N.S.). Following the administration of nitrate, the saliva nitrite concentration increased from 56.3 μM (23.2 – 82.6) ($n = 10$) at 45 minutes, just prior to nitrate administration, to a peak of 351.4 μM (107.8 – 405.4) at 135 minutes, 85 minutes after nitrate administration ($p < 0.01$) ($n = 8$) (Fig 12.3).

The saliva thiocyanate concentration just before passing the probe assembly was 794 μM (682 - 1339) ($n = 9$) similar to 770 μM (459 - 1446) ($n = 9$) (N.S.) 15 minutes after the placement of the assembly. The thiocyanate concentrations remained at a similar concentration after the administration of nitrate and fell non-significantly to 491 μM (333-656) ($n = 8$) at 135 minutes.

ii) pH of the individual regions of the upper GI tract

The pH in the squamous esophagus, Barrett's oesophagus, hiatal hernia, proximal and distal stomach were 6.2 (5.8 - 7.0) ($p < 0.05$ vs all other sites), 2.7 (1.6 - 3.3) ($p < 0.05$ vs hiatal hernia, proximal and distal stomach), 1.4 (1.2 - 1.9), 1.4 (1.3 - 1.8) and 1.4 (1.2 - 1.7) respectively before nitrate administration (table 12.1). The respective pH after nitrate administration were 5.7 (4.5 - 6.5) ($p < 0.05$ vs all other sites), 2.7 (1.7 - 3.8) ($p < 0.05$ vs hiatal hernia, proximal and distal stomach), 1.6 (1.1 - 1.9), 1.5 (1.2 - 1.6) and 1.4 (1.2 - 1.6) (table 12.1).

The pH was greater than 4 for 94 % (61.9 - 100), 18.7 % (0.9 - 42.3), 0 % (0 - 0.2), 0 % and 0 % in the squamous esophagus, Barrett's segment, hiatus hernia, proximal and distal stomach respectively before nitrate administration. After nitrate administration the respective percent pH > 4 were 92 % (56.3 - 98.2), 3.6 % (1.2 - 15.3), 0 %, 0 %, 0 % (all N.S. vs pre-nitrate).

12.4.3 Microdialysis Samples

i) Nitrite

The nitrite concentration in the squamous oesophagus before the nitrate meal was 40.1 μM (12.7 - 63.4). In the Barrett's segment, nitrite concentration fell to 0 μM (0 - 5.9); for hiatal hernia, proximal and distal stomach the nitrite concentrations were 0 μM (0 - 0.3), 0 μM and 0 μM respectively ($p < 0.001$ for trend) (Fig 12.4, table 12.1). Following nitrate administration, the squamous oesophageal nitrite concentration was 106.2 μM (75.7 - 141.6) and fell by 98 % to 1.9 μM (0 - 7.4) in the Barrett's segment; the nitrite concentrations in the hiatal hernia, proximal stomach and distal stomach were 0 μM (0 - 1.1), 0 μM and

0 μM respectively ($p < 0.001$ for trend) (Fig 12.4, table 12.1). The nitrite concentrations in the upper GI tract was significantly different before nitrate ingestion compared to after nitrate ingestion only for the squamous oesophagus ($p = 0.03$).

ii) Thiocyanate

The thiocyanate concentration in the squamous oesophagus was the highest at 409 μM (319 - 673) before nitrate administration, and in the Barrett's segment, hiatal hernia, proximal stomach and distal stomach thiocyanate concentrations were 317 μM (192 - 511), 265 μM (239 - 422), 270 μM (231 - 434) and 279 μM (223 - 505) respectively (N.S. for trend) (Fig 12.5, table 12.1). Following nitrate administration, thiocyanate concentrations in the squamous oesophagus, Barrett's segment, hiatal hernia, proximal stomach and distal stomach were 390 μM (262 - 622), 260 μM (171 - 679), 235 μM (207 - 486), 229 μM (151 - 440) and 249 μM (184 - 494) respectively (N.S. for trend; N.S. vs pre-nitrate) (Fig 12.5, table 12.1).

iii) Ascorbic acid and total vitamin C

Increasing ascorbic acid and total vitamin C concentrations were seen moving distally from the squamous oesophagus to the distal stomach. The ascorbic acid concentrations for the squamous oesophagus, Barrett's segment, hiatal hernia, proximal and distal stomach were 3.5 μM (0 - 11.7), 9.9 μM (3.4 - 16.8), 27.7 μM (6.7 - 42.5), 38.4 μM (20.6 - 66.6) and 43.4 μM (24.1 - 68.5) respectively before nitrate administration ($p < 0.001$ for trend) (Fig 12.6, table 12.1). Following nitrate administration, the ascorbic acid concentrations for the squamous oesophagus, Barrett's segment, hiatal hernia, proximal and distal stomach were 1.2 μM (0 - 12.4), 2.9 μM (0.2 - 32.4), 14.7 μM (0 - 42.7), 28.4 μM (9.1 - 45.4) and 48.6 μM (17.0 - 73.6) respectively ($p = 0.009$ for trend) (N.S. vs pre-nitrate) (Fig 12.6, table 12.1).

Total vitamin C concentrations for the respective regions before nitrate administration were 3.8 μM (0 - 14.7), 19.1 μM (5.1 - 32.2), 34.1 μM (10.2 - 47.5), 41.1 μM (24.9 - 77.0) and 58.9 μM (22.6 - 72.8) ($p = 0.001$ for trend) (Fig

12.7, table 12.1). After nitrate administration concentrations of total vitamin C for the respective regions were 5.6 μM (0.4 – 34.3), 23.4 μM (13.4 – 28.4), 33.4 μM (21.5 – 44.9), 35.6 μM (18.3 – 71.2) and 46.5 μM (20.0 – 78.7) ($p < 0.05$ for trend) (N.S. vs pre-nitrate) (Fig 12.7, table 12.1).

The median ascorbic acid to total vitamin C ratios before nitrate administration for squamous oesophagus ($n = 7$), Barrett's segment ($n = 8$), hiatal hernia, proximal and distal stomach were 0.70 (0.4 – 0.78), 0.64 (0.55 – 0.70), 0.78 (0.49 – 0.89), 0.93 (0.86 – 0.97) and 0.85 (0.82 – 0.98) respectively ($p = 0.028$ for trend) (Fig 12.8). After nitrate administration the median ascorbic acid to total vitamin C ratios were 0.27 (0 – 0.4) ($n = 9$), 0.16 (0.02 – 0.63), 0.42 (0.03 – 0.86), 0.67 (0.47 – 0.82) and 0.83 (0.69 – 0.91) ($p = 0.048$ for trend) ($p = 0.018$ for proximal stomach pre-nitrate vs post-nitrate) (Fig 12.8).

iv) Ascorbic acid to nitrite ratio

The ascorbic acid to nitrite ratio provides an indication of the potential to generate carcinogenic *N*-nitroso compounds by luminal acid-catalysed nitrosation. The median (IQR) ascorbic acid to nitrite ratios for the squamous oesophagus, Barrett's segment, hiatal hernia, proximal and distal stomach were 0.1 (0.02 – 0.47), 7.4 (1.6 – 16.8), 26.2 (6.7 – 42.5), 32.3 (15.1 – 65.2) and 32.7 (18.9 – 68.5) respectively before nitrate administration ($p < 0.001$ for trend) (Fig 12.9, table 12.1). The ascorbic acid to nitrite ratios after nitrate administration for the respective regions were 0.02 (0.01 – 0.13) ($p = 0.032$ vs squamous oesophagus pre-nitrate), 3.4 (0.14 – 12.4) ($n = 9$), 29.2 (1.5 – 47.9) ($n = 8$), 25.0 (8.5 – 48.5) ($n = 9$) and 34.2 (13.8 – 64.3) ($p < 0.001$ for trend) (Fig 12.9, table 12.1).

Although the squamous oesophagus had the lowest ascorbic acid to nitrite ratio both before and after nitrate administration, acid-catalysed nitrosation does not normally occur due to the high pH at this anatomical site. The only anatomical sites favouring luminal acid-catalysed nitrosation were in the Barrett's segment where 2 of the 10 subjects before nitrate and 4 of 9 after nitrate had acidic nitrite in excess of ascorbic acid and within the hiatus hernia in 1 of the 10 before nitrate and 2 of the 8 studied after nitrate. In the proximal and distal stomach, all subjects had ascorbic acid in excess of nitrite.

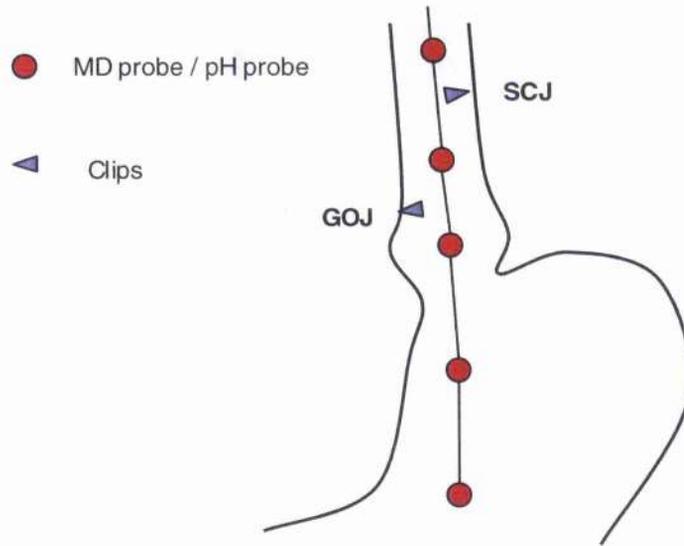


Fig 12.1: Positions of the microdialysis and pH probes in subjects with Barrett's oesophagus.

From proximal to distally: squamous oesophagus, Barrett's oesophagus, hiatal hernia, proximal and distal stomach. Metallic clips were attached to the squamo-columnar junction (SCJ) and gastro-oesophageal junction (GOJ) to delineate the Barrett's segment.

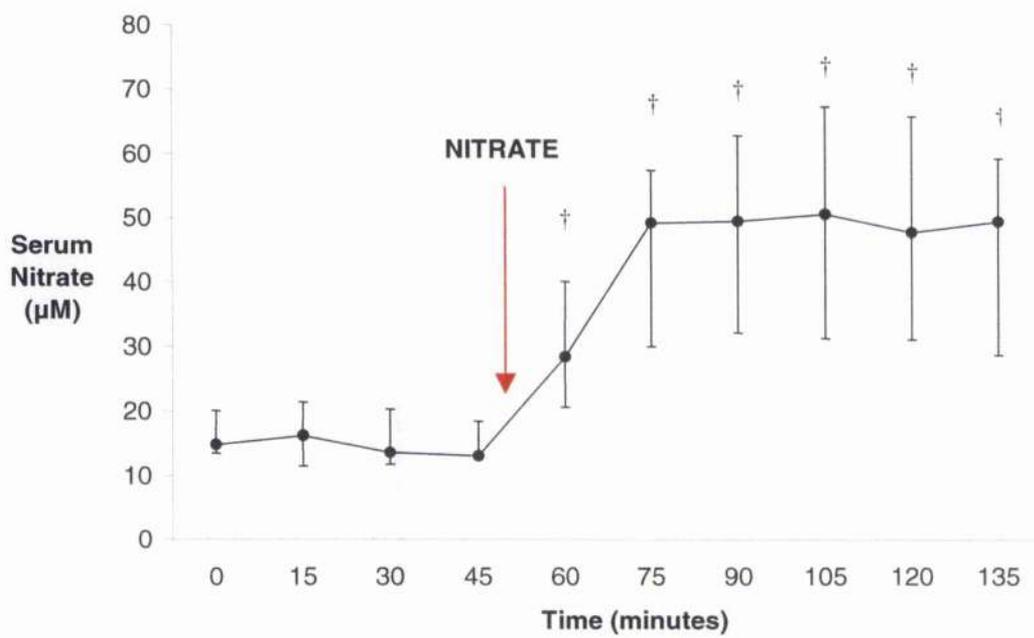


Fig 12.2: Median serum nitrate concentrations before and after nitrate administration.

The error bars represent the IQR. Potassium nitrate (2 mmol) was administered at 50 minutes, indicated by the red arrow.

† $p < 0.01$ vs 45 minutes, just prior to nitrate administration.

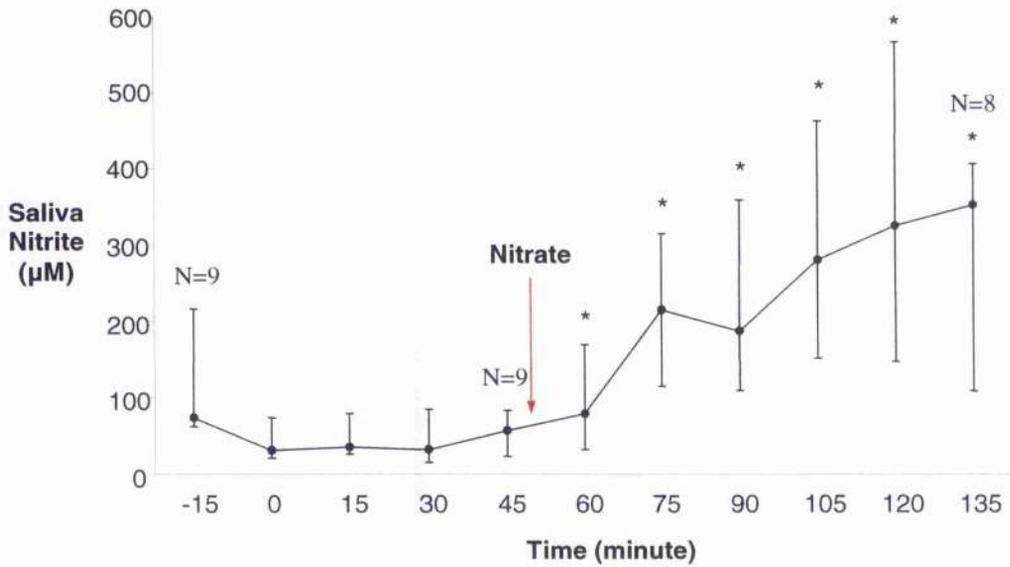


Fig 12.3: Median salivary nitrite before and after nitrate administration. The error bars represent the IQR. N=10 for all time points unless indicated otherwise. Potassium nitrate (2 mmol) was administered at 50 minutes, indicated by the red arrow. -15 minutes indicates 15 minutes before insertion of microdialysis probe assembly.
 * $p < 0.05$ vs 45 minutes (just prior to nitrate administration).

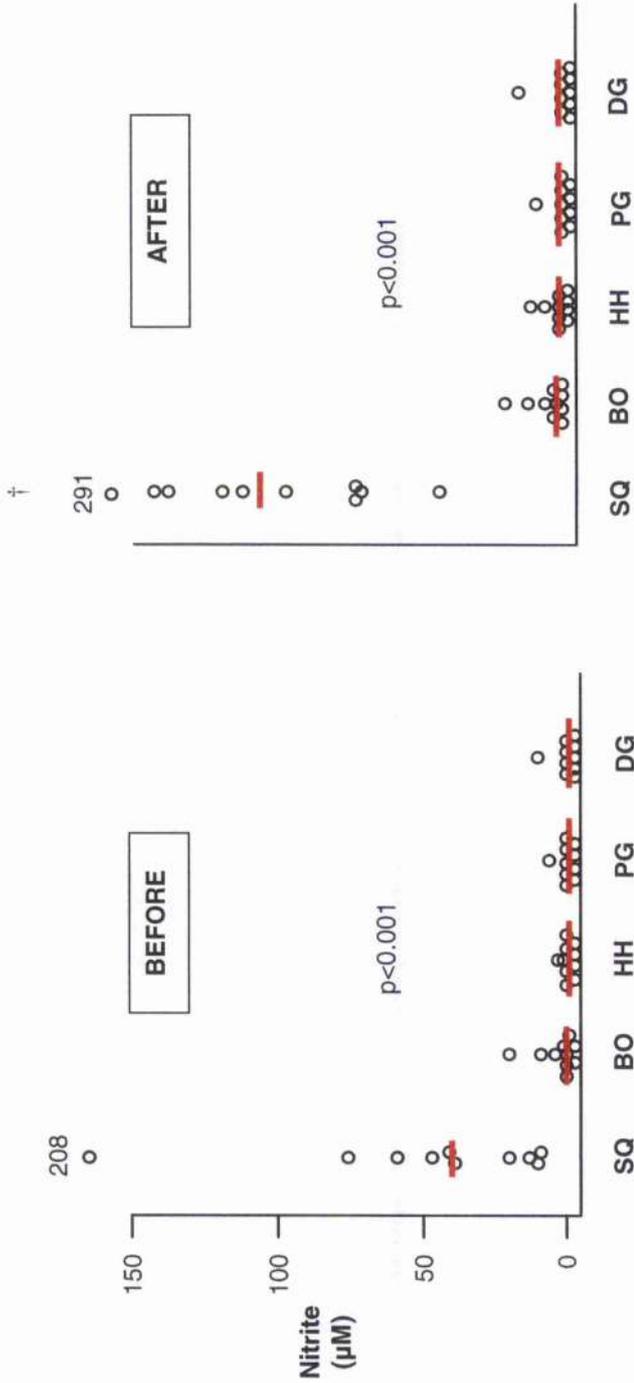


Fig 12.4: Concentrations of nitrite in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.

SQ = squamous oesophagus, BO = Barrett's oesophagus, HH = hiatal hernia, PG = proximal stomach, DG = distal stomach. Pre = pre-nitrate, Post = post-nitrate.

P-values indicate Kruskal-Wallis analysis comparing the different anatomical sites pre- and post-nitrate. Significant difference in nitrite concentrations was observed in the different regions of the upper GI tract both pre- and post-nitrate.

† p = 0.03 vs pre-nitrate for squamous oesophagus.

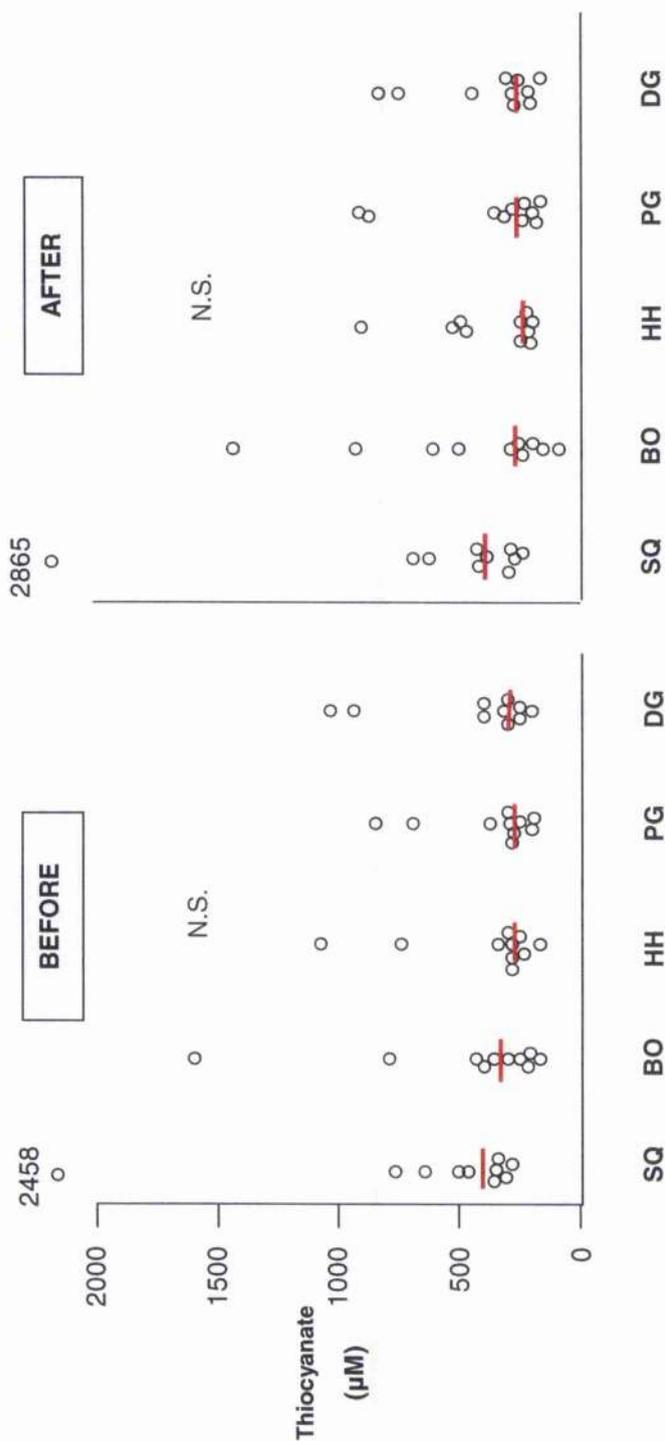


Fig 12.5: Concentrations of thiocyanate in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.

Key to abbreviations as for fig 12.4.

N.S. indicate that the Kruskal-Wallis analysis comparing the different anatomical sites showed no significant difference pre- and post-nitrate administration.

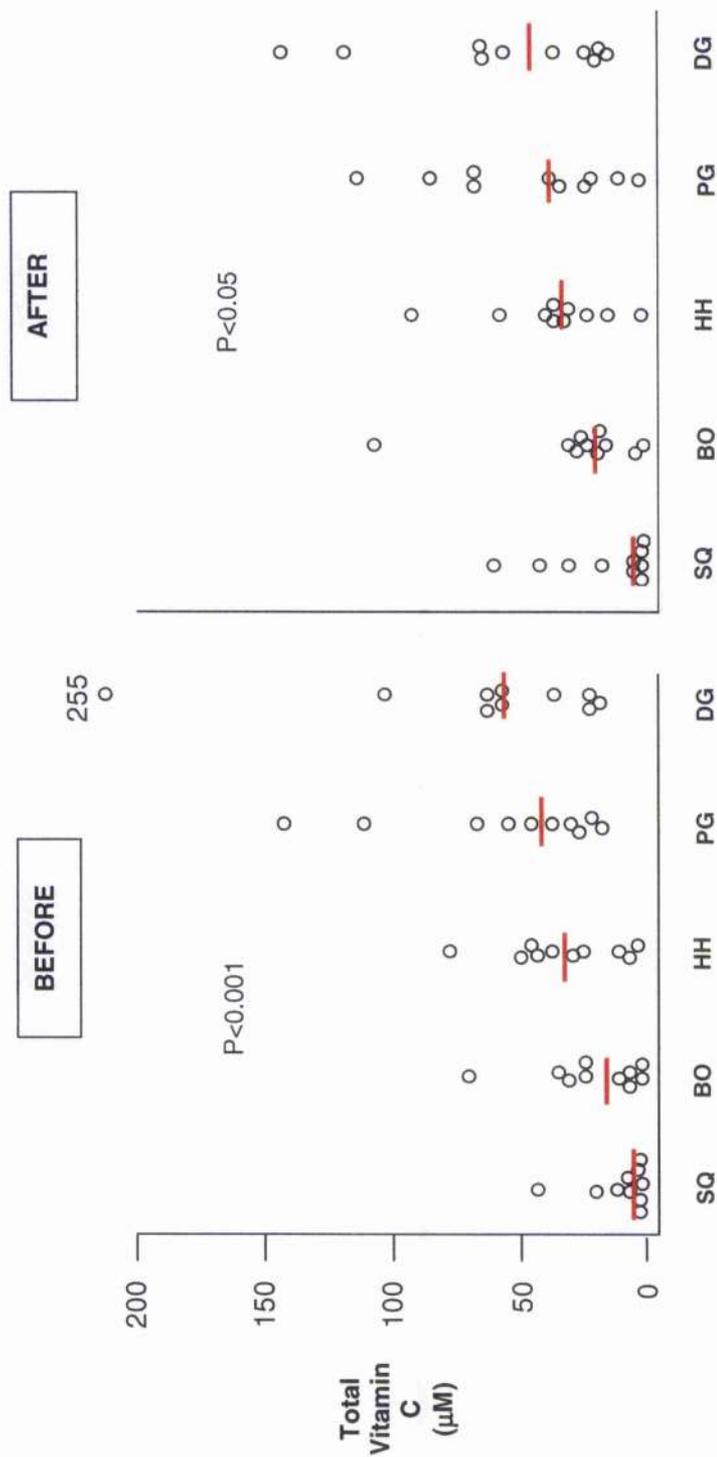


Fig 12.7: Concentrations of total vitamin C in the upper GI tract of subjects with Barrett's oesophagus before and after nitrate.

Key to abbreviations as for fig 12.4.

P-values indicate Kruskal-Wallis analysis comparing the different anatomical sites both before and after nitrate administration. Significant differences in total vitamin C concentrations were observed in the different regions of the upper GI tract both pre- and post-nitrate.

SITE	pH		Nitrite (µM)		Thiocyanate (µM)		Ascorbic acid (µM)		Total vitamin C (µM)		AA / Nitrite	
	PRE	POST	PRE ‡	POST †	PRE	POST	PRE ‡	POST †	PRE ‡	POST §	PRE ‡	POST †
SQ	6.2* (5.8-7.0)	5.7* (4.5-6.5)	40.1 (12.7-63.4)	106.2 † (75.7-141.6)	409 (319-673)	390 (202-622)	3.5 (0-11.7)	1.2 (0-12.4)	3.8 (0-14.7)	5.6 (0.4-34.3)	0.1 (0.02-0.47)	0.02 + (0.01-0.13)
BO	2.7# (1.6-3.3)	2.7# (1.7-3.8)	0 (0-5.9)	1.9 (0-7.4)	317 (192-511)	260 (171-679)	9.9 (3.4-16.8)	2.9 (0.2-32.4)	19.1 (5.1-32.2)	23.4 (13.4-28.4)	7.4 (1.6-16.8)	3.4 (0.14-12.4)
HH	1.4 (1.2-1.9)	1.6 (1.1-1.9)	0 (0-0.3)	0 (0-1.1)	265 (239-422)	235 (207-486)	27.7 (6.7-42.5)	14.7 (0-42.7)	34.1 (10.2-47.5)	33.4 (21.5-44.9)	26.2 (6.7-42.5)	29.2 (1.5-47.9)
PG	1.4 (1.3-1.8)	1.5 (1.2-1.6)	0	0	270 (231-434)	229 (151-440)	38.4 (20.6-66.6)	28.4 (9.1-45.4)	41.1 (24.9-77.0)	35.6 (18.3-71.2)	32.3 (15.1-65.2)	25.0 (8.5-48.5)
DG	1.4 (1.2-1.7)	1.4 (1.2-1.6)	0	0	279 (223-505)	249 (184-494)	43.4 (24.1-68.5)	48.6 (17.0-73.6)	58.9 (22.6-72.8)	46.5 (20.0-78.7)	32.7 (18.9-68.5)	34.2 (13.8-64.3)

Table 12.1: Nitrite, thiocyanate, ascorbic acid, total vitamin C, ascorbic acid / nitrite ratio and pH in the different regions of the upper GI tract in subjects with Barrett's oesophagus before and after nitrate.

Data presented as medians and IQR. SQ: squamous oesophagus; BO: Barrett's oesophagus; HH: hiatus hernia; PG: proximal stomach; DG: distal stomach; AA: ascorbic acid. * p<0.05 vs all other sites; † p<0.05 vs hiatal hernia, proximal and distal stomach; ‡ p = 0.03 vs pre-nitrate; + p=0.032 vs squamous oesophagus pre-nitrate; § p<0.001 / ¶ p=0.009 / § p<0.05 (Kruskal-Wallis test comparing different regions of the upper GI tract).

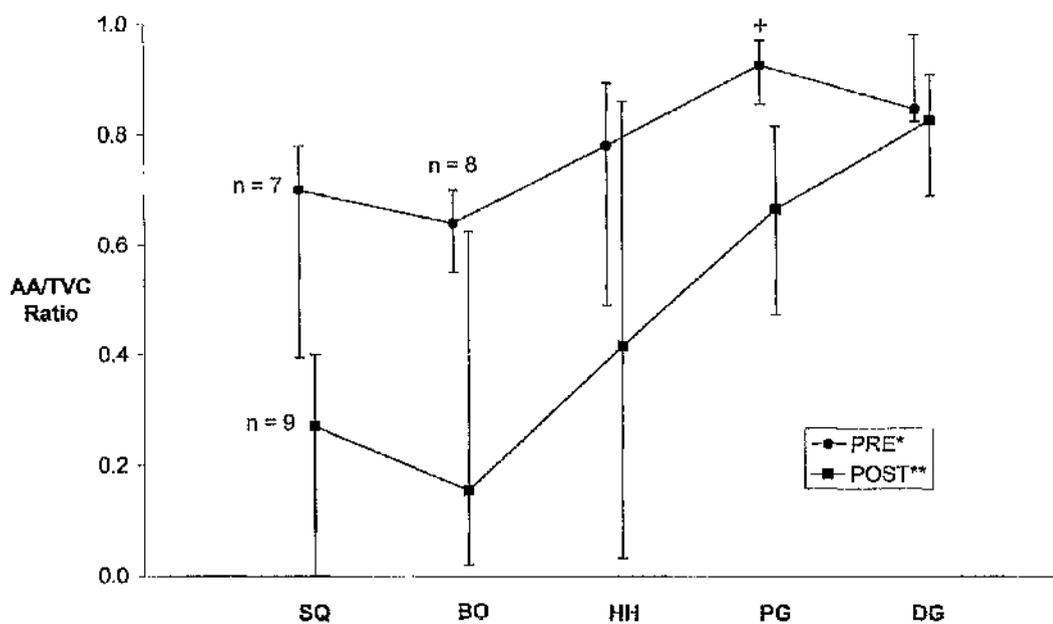


Fig 12.8: Median (IQR) ascorbic acid to total vitamin C ratio before and a after nitrate.

N = 10 unless otherwise indicated.

PRE: before nitrate, POST: after nitrate.

SQ: squamous esophagus, BO: Barrett's oesophagus, HH: hiatus hernia, PG: proximal stomach, DG: distal stomach.

* p = 0.028 for trend. ** p = 0.048 for trend. ⁺p = 0.018 pre vs post-nitrate at proximal stomach.

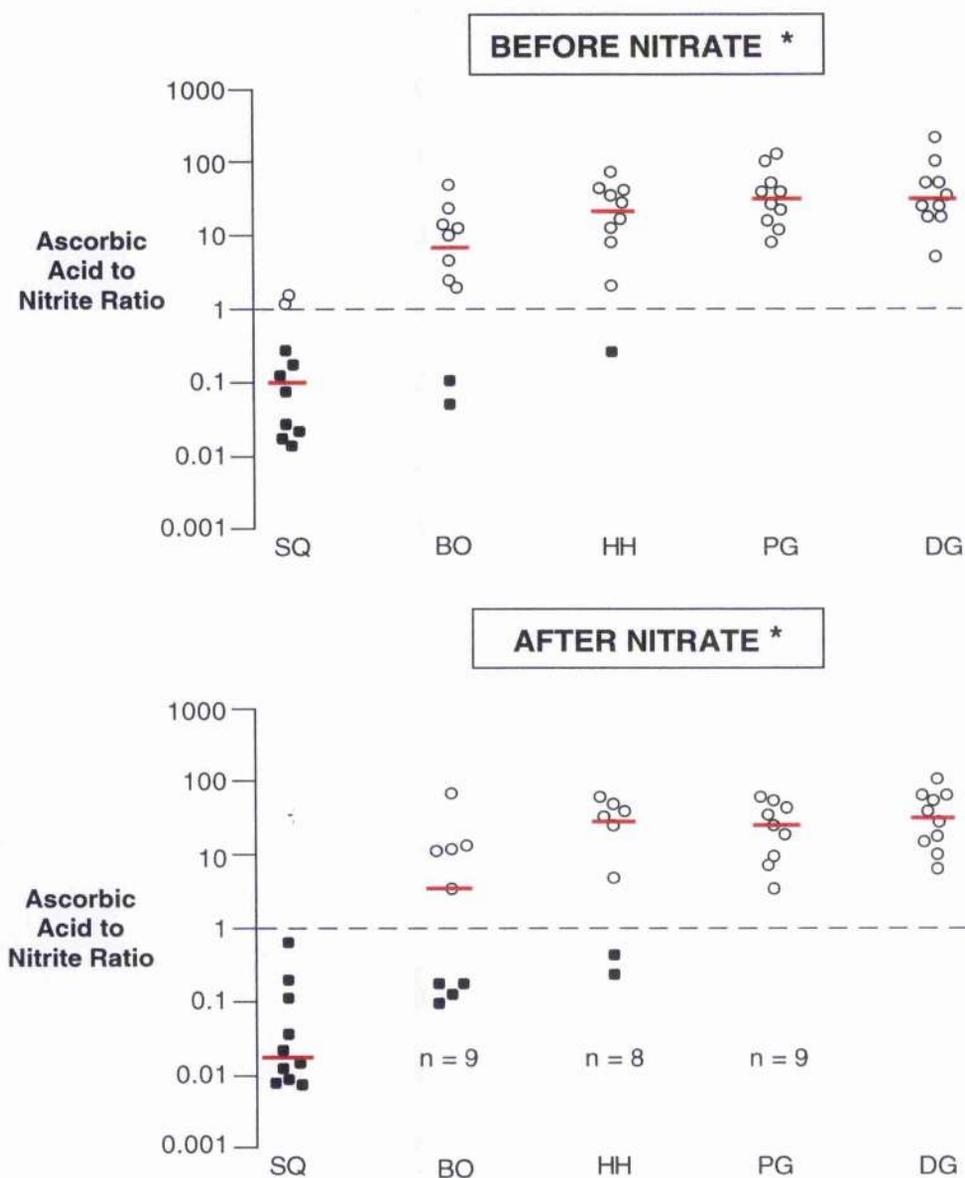


Fig 12.9: Ascorbic acid to nitrite ratio before and after nitrate administration.

SQ: squamous oesophagus; BO: Barrett's oesophagus; HH: hiatal hernia; PS: proximal stomach; DS: distal stomach.

○ condition unfavourable for acid-catalysed nitrosation.

● condition favourable for acid-catalysed nitrosation.

Note that acid-catalysed nitrosation does not occur in the squamous oesophagus where pH is high despite demonstrating the lowest ascorbic acid to nitrite ratio.

$p < 0.01$ for trend both before and after nitrate administration.

† $p = 0.032$ vs pre-nitrate administration.

* $p \leq 0.001$, Kruskal-Wallis test comparing different regions before and after nitrate.

12.5 Discussion.

In the current study examining the nitrosation chemistry in Barrett's oesophagus during periods of gastro-oesophageal reflux, the most notable finding was the fall in luminal nitrite concentration on passing from the more proximal squamous oesophagus into the Barrett's segment exposed to acidic gastric refluxate (Fig 12.4, table 12.1). In chapter 10 we examined the nitrite concentration in the oesophagus and stomach of normal volunteers without gastro-oesophageal reflux. In such subjects, high concentrations of nitrite were found throughout the length of the oesophagus and the marked fall in concentration occurred on entering the acidic environment of the gastric cardia. Previous work indicated that the nitrite concentration in the gastric lumen was similar to that of saliva where high intragastric pH was achieved in subjects treated with omeprazole⁽²⁶⁶⁾. Thus the nitrite concentration seems to fall at the point where neutral luminal pH first drops to acidic luminal pH. During periods of gastro-oesophageal reflux in subjects with Barrett's oesophagus, the transition from neutral to acid pH was occurring more proximally within the Barrett's segment and consequently the nitrite was disappearing more proximally at this site. This suggests that nitrite is either being absorbed by the surrounding Barrett's epithelium or being converted to another chemical. When the nitrite passes from the neutral pH of the proximal oesophagus into the acidic environment of refluxing gastric juice in the Barrett's oesophagus, it will be converted immediately to nitrous acid. However, this in itself cannot explain the marked fall in measured nitrite within the Barrett's oesophagus as the microdialysis probes do not distinguish between nitrous acid and nitrite (both measured as nitrite). Nitrous acid is less polar than the nitrite anion and the former may be more readily absorbed. However, the ability to absorb substances depends upon their polarity at the mucosal surface. Columnar mucosa has a mucus layer and this, along with its bicarbonate secretion, maintains a neutral surface pH⁽³⁶⁶⁾. Consequently, nitrous acid would be in the form of the nitrite anion at the epithelial surface and therefore not fully absorbed. Therefore it seems unlikely that the disappearance of nitrite within the Barrett's segment can be attributed to any significant extent to its absorption of nitrite or nitrous acid. A more plausible explanation for the disappearance of the nitrite on entering the acidic lumen of the Barrett's segment is its conversion to nitric oxide and the absorption of the dissolved gas into the

Barrett's epithelium. The microdialysis probes do not detect nitric oxide (see chapter 8). At pH less than 3.5, approximately 1% of nitrous acid exists as nitric oxide⁽²⁶²⁾. However, in the presence of acid and reducing agents such as ascorbic acid, most of the nitrous acid is rapidly reduced to nitric oxide. Stoichiometrically, 1 mole of ascorbic acid reacts with 2 moles of nitrous acid forming 2 moles of nitric oxide and 1 mole of dehydroascorbic acid⁽³³⁴⁾. The changes in the ascorbic acid to total vitamin C ratio within the Barrett's segment are consistent with nitrous acid reacting with ascorbic acid to form nitric oxide. Prior to nitrate administration, the majority of the vitamin C was ascorbic acid at all the sites examined (Fig 12.8). The Barrett's segment had the lowest ascorbic acid to total vitamin C ratio both before and after nitrate administration. Following the nitrate administration and consequently increased delivery of salivary nitrite, there was a marked fall in the ratio of ascorbic acid to total vitamin C affecting all regions of the upper gastrointestinal tract except the distal stomach where the ratio remained relatively constant above 82 % (Fig 12.8). The lowest ratio of ascorbic acid to total vitamin C was noted in the Barrett's segment which is consistent with nitrite in swallowed saliva reacting with ascorbic acid within the acidic Barrett's segment resulting in the nitrite being reduced to nitric oxide and the ascorbic acid to dehydroascorbic acid. The fall in ascorbic acid to total vitamin C ratio in the regions distal to the Barrett's oesophagus can be explained by the increased nitrite delivery to these regions. The acidified nitrite is incompletely reduced in the Barrett's segment as a result of ascorbic acid depletion and / or due to the rapid transit of unreacted salivary nitrite and passes to more distal locations until it is reduced to nitric oxide. The fall in the ratio of ascorbic acid to total vitamin C in the squamous oesophagus after nitrate despite the median pH of 5.2 was unexpected. A possible explanation for such finding is that ascorbic acid is unstable at the higher luminal pH of the squamous oesophagus and oxidizes to dehydroascorbic acid. However, if this was the explanation, then the ascorbic acid to total vitamin C ratio should remain constant both before and after the nitrate meal since nitrite does not oxidise ascorbic acid at neutral pH. The most likely explanation for the increased ascorbic acid oxidation in the squamous oesophagus following the nitrate meal is that the ascorbic acid originating from the stomach is being oxidized by the acidified nitrite as it refluxes past the Barrett's segment to reach the proximal squamous mucosa. This explanation is supported by the observation in the

squamous oesophagus of some individuals maintaining pH > 4 virtually 100% of the time, whilst in others there were clear increases in the percentage of time pH < 4 in the squamous oesophagus. In the latter group of subjects, greater ascorbic acid and total vitamin C concentrations were noted in the post-nitrate period than in the pre-nitrate period indicating that the gastric juice ascorbic acid refluxed proximally into the squamous oesophagus.

We also examined the ascorbic acid to nitrite ratio which indicates the potential to generate the carcinogenic *N*-nitroso compounds. The conditions required for generating *N*-nitroso compounds by acid nitrosation are pH of less than 4, nitrite in excess of ascorbic acid (low ascorbic acid to nitrite ratio) and thiocyanate which catalyse the reaction ^(236 - 238, 257, 259, 283, 333). A ratio of ascorbic acid to nitrite greater than 1 indicates that the concentration of ascorbic acid is greater than the concentration of nitrite and the conditions are unfavourable for the generation of *N*-nitroso compounds; a ratio less than 1 indicates that nitrite is in excess of ascorbic acid and conditions favour the generation of *N*-nitroso compounds. Before nitrate administration the ascorbic acid to nitrite ratios were 0.1, 7.4, 26.2, 32.3 and 32.7 in the squamous oesophagus, Barrett's segment, hiatal hernia, proximal and distal stomach respectively (Fig 12.9); the respective ratios after nitrate administration were 0.02, 3.4, 29.2, 25 and 34.2 (Fig 12.9). On closer inspection of the individual data we found that the conditions favouring the generation of *N*-nitroso compounds are confined to the Barrett's segment and to a lesser extent within the hiatus hernia during acid reflux. Within the Barrett's segment, 2 of the 10 subjects had acidified nitrite in excess of ascorbic acid before nitrate and 4 of 9 after nitrate administration (Fig 12.9). Within the hiatus hernia 1 of 10 had acidified nitrite in excess of ascorbic acid before nitrate and 2 of 8 after nitrate. In the proximal and distal stomach, ascorbic acid was always in excess of nitrite. In the more proximal squamous oesophagus nitrite was present in considerable excess of ascorbic acid but nitrosation does not normally occur in this region as the pH is too high for such reaction to occur. However, there may be conditions suitable for generating *N*-nitroso compounds during periods of acid reflux where acidified nitrite exists in excess of ascorbic acid particularly in the distal regions of the squamous oesophagus. In contrast to the study in subjects without gastro-oesophageal reflux, we did not specifically study the transitional region where the neutral pH of the squamous oesophagus became acid in patients

with Barrett's oesophagus. We provoked gastro-oesophageal reflux in patients with Barrett's oesophagus which is a dynamic event and it would have been impossible to position the probes accurately with each reflux episode compared to subjects without reflux where this transition zone of the pH was fixed at the gastro-oesophageal junction.

In summary, the current studies demonstrated high nitrite concentrations in the squamous oesophagus in subjects with Barrett's oesophagus which fell considerably within the Barrett's segment during periods of gastro-oesophageal reflux. This can be explained by the reduction of acidified nitrite by the ascorbic acid, present in the gastric refluxate, to nitric oxide. The Barrett's segment demonstrated the lowest ascorbic acid to total vitamin C ratio which indicates the high oxidative stress in this region. This was due to the acidified Barrett's segment being the most proximal region encountering the high nitrite load delivered. We also demonstrated that the Barrett's segment had the conditions most favourable for the formation of *N*-nitroso compounds, that is, the lowest ascorbic acid to nitrite ratio in the acidic environment within the upper gastrointestinal tract which became less favourable in the more distal anatomical regions. Throughout the upper gastrointestinal tract examined the thiocyanate ion, a catalyst for acid-nitrosation, was in abundance. These findings indicate that during periods of acid reflux into the Barrett's oesophagus, carcinogenic nitrosamines may be generated due to the presence of conditions which favours the formation of such chemicals.

We chose to study subjects with Barrett's oesophagus, a group of people with the most severe form of gastro-oesophageal reflux as they form the group of people most at risk of developing adenocarcinoma of the oesophagus and therefore from the present studies we cannot say whether the luminal chemistry observed during reflux in the Barrett's patients differs from that in non-Barrett's patients with an equivalent degree of reflux.

In the next chapter we studied the generation of nitric oxide in the Barrett's segment during gastro-oesophageal reflux.

CHAPTER THIRTEEN

NITRIC OXIDE GENERATION

IN

BARRETT'S OESOPHAGUS

13.1 Introduction.

Nitric oxide is generated in the lumen of the upper gastrointestinal tract by the reaction of acidified nitrite and ascorbic acid secreted in gastric juice. Nitrite is derived from dietary nitrate which undergoes enterosalivary circulation. Nitrate secreted into the oral cavity by the salivary glands is reduced by the enzyme nitrate reductase derived from the bacteria on the dorsum of the tongue. The nitrite in saliva meets acidic gastric juice and becomes protonated to form nitrous acid which generates the nitrosating species such as N_2O_3 and NO^+ . The NO^+ can also combine with thiocyanate which is also secreted in saliva to form another nitrosating species nitrosothiocyanate (NOSCN) and effectively catalyse the nitrosation reaction. The nitrosating species reacts with ascorbic acid in the gastric juice which is secreted by the gastric mucosa to form nitric oxide. In the process ascorbic acid is oxidised to dehydroascorbic acid. In the absence of ascorbic acid, the nitrosating species react with nitrosatable substrates such as secondary amines and amides to generate the carcinogenic *N*-nitroso compounds. Ascorbic acid has a higher affinity for the nitrosating species and reacts to form nitric oxide thereby preventing the formation of *N*-nitroso compounds. Although this action of ascorbic acid inhibits the luminal generation of *N*-nitroso compounds, the nitric oxide produced in the process can readily diffuse into the adjacent epithelium where it can combine with oxygen to form N_2O_3 and thereby induce nitrosative stress intracellularly^(302, 367).

The previous chapter investigated the nitrosative chemistry in patients with Barrett's oesophagus and the results indicated that during periods of acid reflux large amounts of nitrite disappeared within the Barrett's segment. Previous study in healthy volunteers without gastro-oesophageal reflux indicated that nitric oxide was generated at the interface where salivary nitrite at neutral pH entered the acidic gastric juice at the gastro-oesophageal junction⁽²⁷⁸⁾, which was also the site where condition for the generation of *N*-nitroso compounds was optimal. These studies suggested that nitric oxide is likely to be generated within the Barrett's oesophagus during periods of acid reflux.

We measured nitric oxide within the Barrett's segment using a miniturised nitric oxide probe and found that substantial concentration of nitric oxide was generated during periods of gastro-oesophageal reflux. Attempt was made to

measure nitric oxide generation in five patients since the pH probe was refurbished prior to use and subsequently malfunctioned after five subjects were studied.

13.2 Aims.

We sought to establish whether nitric oxide was being generated within the Barrett's segment during periods of gastro-oesophageal reflux to explain the disappearance of nitrite in the Barrett's segment during acid reflux.

13.3 Subjects.

The subjects consisted of 5 patients with Barrett's oesophagus who underwent the microdialysis procedure previously. All were *H. pylori* negative on histological examination and rapid urease test of both gastric antral and body mucosal biopsies.

13.4 Methods.

13.4.1 Nitric Oxide Sensor.

We employed a miniaturized custom-made nitric oxide sensor (World Precision Instruments Inc., Sarasota, Florida, U.S.A.) to allow measurement of luminal nitric oxide concentrations throughout the upper gastrointestinal tract as previously described⁽²⁷⁸⁾ (Fig 13.1). An antimony pH sensor (Synetics Medical Ltd., Enfield, England) was attached to the nitric oxide sensor using waterproof taping (Sleek, Smith and Nephew, Hull, England) with the pH sensor positioned adjacent to the tip of the nitric oxide probe. A fine-bore Teflon feeding tube was also attached to the assembly to allow intra-gastric instillation of the nitrate solution. The nitric oxide sensor was connected to a dissolved nitric oxide meter (ISO-NO Mark II, World Precision Instruments, I.N.C., Sarasota, Florida, U.S.A.) which displays the reading as a redox current (pA). The output from the pH sensor was attached to a pre-amplifier. Both the outputs from the nitric oxide sensor and the pH sensor were digitalized (DAS 1201 data acquisition keyboard, Keithley

Instruments Ltd., Reading, England) and displayed and stored on computer for subsequent analysis. The position of the sensors was recorded digitally. All electrical equipments used for the study were attached to the mains electrical supply via an isolation unit to comply with standard clinical safety procedures.

The nitric oxide probe and pH sensor were calibrated at 37°C prior to each study as per manufacturer's instructions (see above). During transfer of the patients to the radiology department for confirming the position of the sensors with an abdominal X-ray, the nitric oxide sensor was attached to a portable unit to maintain their polarization (ISO-NO Activator, World Precision Instruments, Sarasota, Florida, U.S.A.). After use, the nitric oxide sensors and pH probe were detached and cleaned using Sterilox ®.

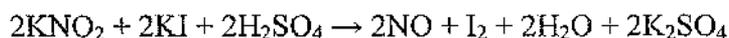
13.4.2 Calibration of nitric oxide probe *in-vitro*.

Stock KNO_2 solutions of the following concentrations were made: 2.02, 4.04, 6.06, 8.08 and 12.12 mM. The solutions were used for calibrating the nitric oxide (NO) probe to 20, 40, 60, 80 and 120 μM respectively. Reaction solution was made up of concentrated sulphuric acid 800 μL added to 120 mL distilled water. To this solution 2.2 g potassium iodide was added just before calibration of the NO probe. The reaction solution was synthesised in a fume cupboard and the calibration process described below was also performed in the fume cupboard.

Five scintillation glass vials each containing 5 mL of the reaction solution were placed in a waterbath set at 37°C and stirred continuously with a small magnetic stirrer. The tip of the NO probe was lowered carefully into a vial containing the reaction solution so as to avoid contact with the magnetic stirrer. Care was also taken to avoid dropping the NO probe on its tip as this may lead to the rupture of the membrane covering the tip of the probe. The nitric oxide sensor was connected to the dissolved nitric oxide meter (ISO-NO Mark II, World Precision Instruments, I.N.C., Sarasota, Florida, U.S.A.). The baseline current (μA) in the reaction solution was recorded.

Two hundred microlitres of the stock 2.02 mM KNO_2 solution was added to the reaction solution and the peak current recorded was noted. The NO probe was then removed, washed and placed in distilled water. The process was repeated using 200 μL of the 4.04, 6.06, 8.08 and 12.12 mM KNO_2 solutions. These

solutions represented the 20, 40, 60, 80 and 120 μM standards of NO respectively. A 'standard curve' was obtained from which the NO concentration was obtained. The NO probe was ready for use. The chemical reactions which lead to the generation of nitric oxide is summarised as follows:



13.4.3 Clinical procedure using the nitric oxide probe.

The experimental conditions were similar to that for microdialysis experiments. The patient avoided nitrate-rich food for 24 hours and fasted overnight. The nitric oxide sensor assembly was inserted per orally to a point 10 cm distal to the gastro-esophageal junction predetermined endoscopically. A plain abdominal X-ray was taken to check the sensor position. The subjects were positioned in the right-lateral decubitus position with or without the head-down position to provoke gastroesophageal reflux throughout the study. Pentagastrin $0.06 \mu\text{g kg}^{-1}$ was administered intravenously to stimulate acid secretion throughout the study. The sensor was withdrawn 1cm every 2 minutes until it was in the squamous esophagus. We have previously noted that the NO production was maximal where saliva first meets acidic gastric juice. In subjects without gastro-oesophageal reflux this was at the cardia⁽²⁷⁸⁾. In subjects with Barrett's oesophagus NO generation was anticipated to occur at the point where salivary nitrite first came into contact with refluxed gastric juice in the distal oesophagus. To study the NO generation within the Barrett's esophagus, the NO sensor was positioned where gastro-oesophageal reflux occurred as indicated by a fall in pH < 4 on the pH meter adjacent to the NO probe. The NO sensor was maintained in this position for several minutes to study NO generation within the Barrett's segment. Following insertion of the sensor, salivary nitrite and serum nitrate were collected every 15 minutes and processed as previously described. Once the recordings were complete in the squamous oesophagus, the sensor was re-inserted to 10cm distal to the gastroesophageal junction and 2 mmol of potassium nitrate in 50mL sterile water was administered intra-gastrically. Previous experiments with healthy volunteers have shown that administering the same total amount of nitrate in 50mL or 25mL produced comparable serum nitrate levels (See chapter

10 and ref. 278). Twenty minutes after nitrate was administered, the NO probe was withdrawn 1cm every 2 minutes as before. The probe was again maintained in the Barrett's segment for several minutes at a point where reflux occurred as described above. The procedure was terminated when recording was completed from the squamous esophagus.

Serum nitrate and salivary nitrite was treated and analysed as previously described and will not be detailed further.

13.5 Statistical Analyses.

All data are presented as medians and interquartile range (IQR). Serum nitrate and salivary nitrite concentrations were grouped into pre and post-nitrate periods and analysed by the 1-sample Wilcoxon test. Since only a small number of patients completed the investigation for NO measurement, statistical testing was not performed. The descriptive data are presented before and after nitrate administration for individual patients.

13.6 Results.

The median (range) length of Barrett's segment was 5cm (4 -10). 3 subjects were females.

13.6.1 Serum

The median serum nitrate concentration during the 45-minute interval before nitrate administration was 16.2 μM (14.6 – 21.1). The median serum nitrate after nitrate administration was significantly higher at 57.3 μM (41.2 – 63.4) ($p < 0.001$) (Fig 13.2).

13.6.2 Saliva

The median salivary nitrite increased almost 7-fold from 43.5 μM (28.4 – 101) during the 45-minute interval before nitrate administration to 287 μM (104 –

419) during the 45-minute interval, 15 minutes after nitrate administration ($p = 0.001$) (Fig 13.3).

13.6.3 Nitric oxide

In two individuals no nitric oxide was detected in the stomach or within the Barrett's segment before or after nitrate administration despite evidence of clear gastro-oesophageal reflux into the Barrett's segment. We suspect this occurred due to technical problems. The data for the three remaining patients are thus presented. The peak nitric oxide concentrations (range) in the Barrett's segment were $7.6 \mu\text{M}$ ($2.6 - 13.4$) before nitrate administration and $20.5 \mu\text{M}$ ($3.4 - 56.5$) after nitrate administration.

The first patient was a 69 year old male with a 5 cm Barrett's mucosa. Before nitrate administration $24.5 \mu\text{M}$ of nitric oxide was detected in the proximal stomach which coincided with a slight rise in pH due to swallowed saliva. In the Barrett's segment nitric oxide attained concentrations of $7.9 \mu\text{M}$ as pH rose from 1.3 to 2.1 at 39 cm (from the incisors) due to swallowed saliva and $7.4 \mu\text{M}$ as pH fell from 6.5 to 1.4 coinciding with a reflux episode at 35 cm (Fig 13.4a). After nitrate administration $7.1 \mu\text{M}$ and $7.2 \mu\text{M}$ of nitric oxide was generated at 49 cm in the proximal stomach and at 41 cm, just distal to the gastroesophageal junction (Fig 13.4b). Within the Barrett's segment, $4.6 \mu\text{M}$ and $3.5 \mu\text{M}$ of nitric oxide were generated at 39 cm and 37 cm respectively and a small pH increment can be noted which indicated swallowed saliva. At 35 cm, pH baseline was 7 interrupted by repeated acid reflux episodes and small concentrations of $1.5 \mu\text{M}$ of nitric oxide was generated at this site. With the nitric oxide probe placed again more distally at 39 cm, $7.5 \mu\text{M}$ of nitric oxide is detected as pH falls from 4.2 to 1.8, coinciding with gastroesophageal reflux, and $13.5 \mu\text{M}$ of nitric oxide is detected as pH rises from 1.5 to 3 (Fig 13.4b).

The second patient was a 65 year-old female with a 3cm Barrett's segment. Before nitrate administration, virtually no nitric oxide was generated in the stomach or the Barrett's segment despite an episode of reflux with the pH falling from 6 to 3.5 (Fig 13.5a). This particular patient initially found some difficulty producing sufficient amount of saliva and it is likely that nitrite was not available in the Barrett's segment to react with the gastric juice ascorbic acid at the time of

gastro-oesophageal reflux. After nitrate administration, up to 8.2 μM of nitric oxide was generated in the proximal stomach between 44 cm and 38 cm. At 33 cm pH reached 6.6 and as the pH fell to below 4 with each reflux episode, up to 56.5 μM of nitric oxide was generated instantaneously. Further generation of nitric oxide up to 53 μM was seen during episodes of gastro-oesophageal reflux at this level.

In the third patient, the Barrett's segment extended from 40 cm to 36 cm. Before nitrate administration between 3.4 μM to 7.7 μM of nitric oxide was generated between 46 cm in the proximal stomach and 41 cm, just distal to the gastro-oesophageal junction (Fig 13.6a). When pH was above 5 within the Barrett's segment, no nitric oxide was generated. Gastro-oesophageal reflux into the Barrett's segment was accompanied by a simultaneous generation of 13 μM of nitric oxide. In the squamous oesophagus at 35 cm to 33 cm reflux episodes were not accompanied by nitric oxide generation. Following nitrate administration, higher concentrations of nitric oxide was generated both in the proximal stomach and the Barrett's segment (Fig 13.6b). Within the gastric lumen, 5 μM of nitric oxide was generated at 46 cm. Just distal to the gastro-oesophageal junction, indicated by a step-up in pH at 41 to 42 cm, 11.5 μM of nitric oxide was generated. Within the Barrett's segment no nitric oxide was generated while pH remained above 4. Two reflux episodes were followed simultaneously by the generation of approximately 20 μM of nitric oxide.

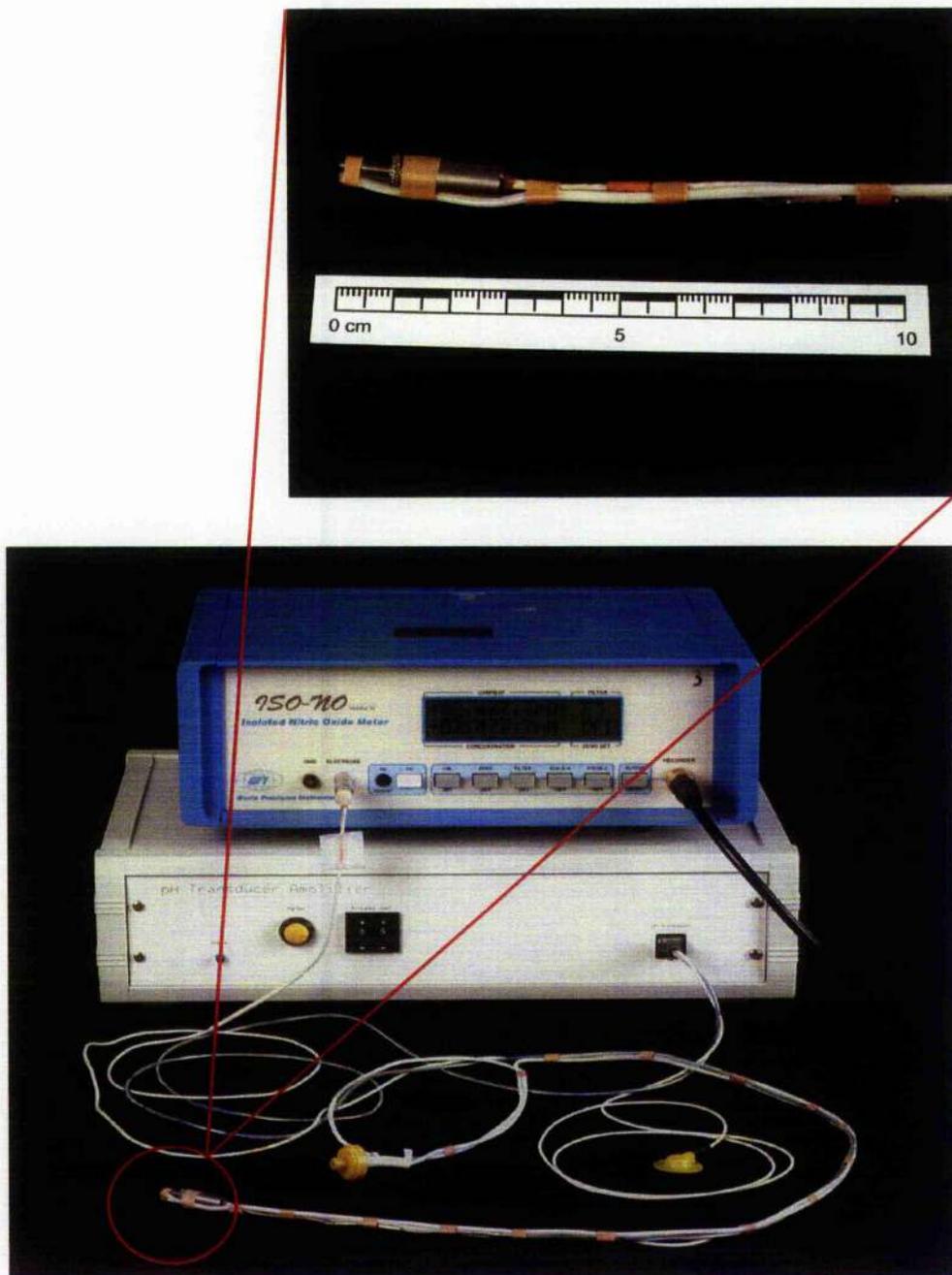


Fig 13.1: Nitric oxide probe and nitric oxide meter (ISO-NO Mark II).
The insert shows the NO probe attached to a pH sensor and a Teflon feeding catheter as used in the human subjects.

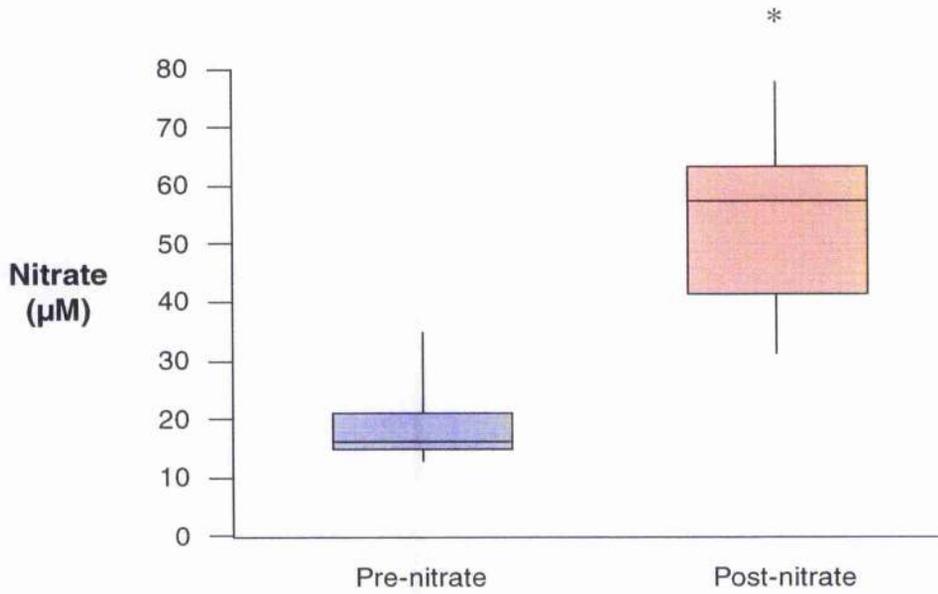


Fig 13.2: Serum nitrate before and after nitrate.
Data presented as medians. Box represents IQR and whiskers represent range.
* $p < 0.001$ pre vs post-nitrate.

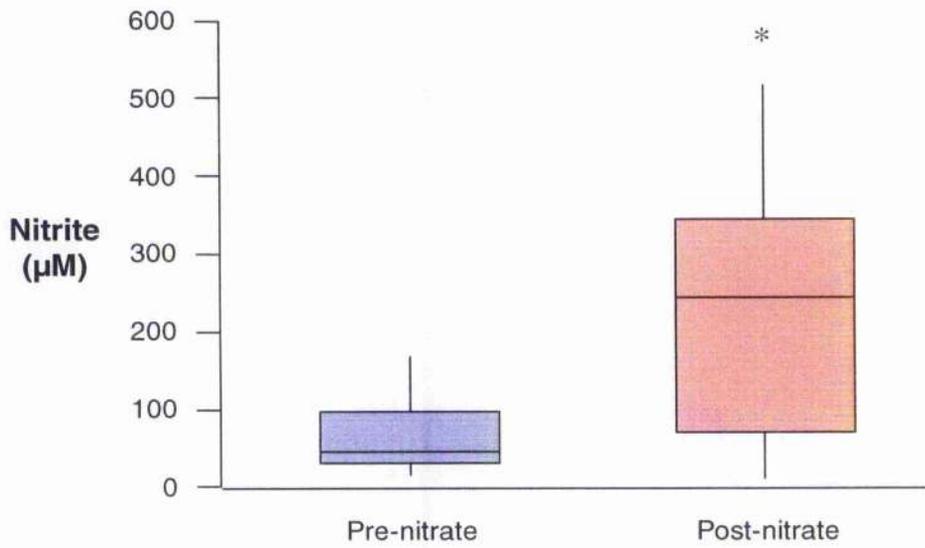


Fig 13.3: Saliva nitrite concentrations before and after nitrate.
Data presented as medians. Box represents median and IQR.
Whiskers represent range.
* $p = 0.001$ pre vs post-nitrate.

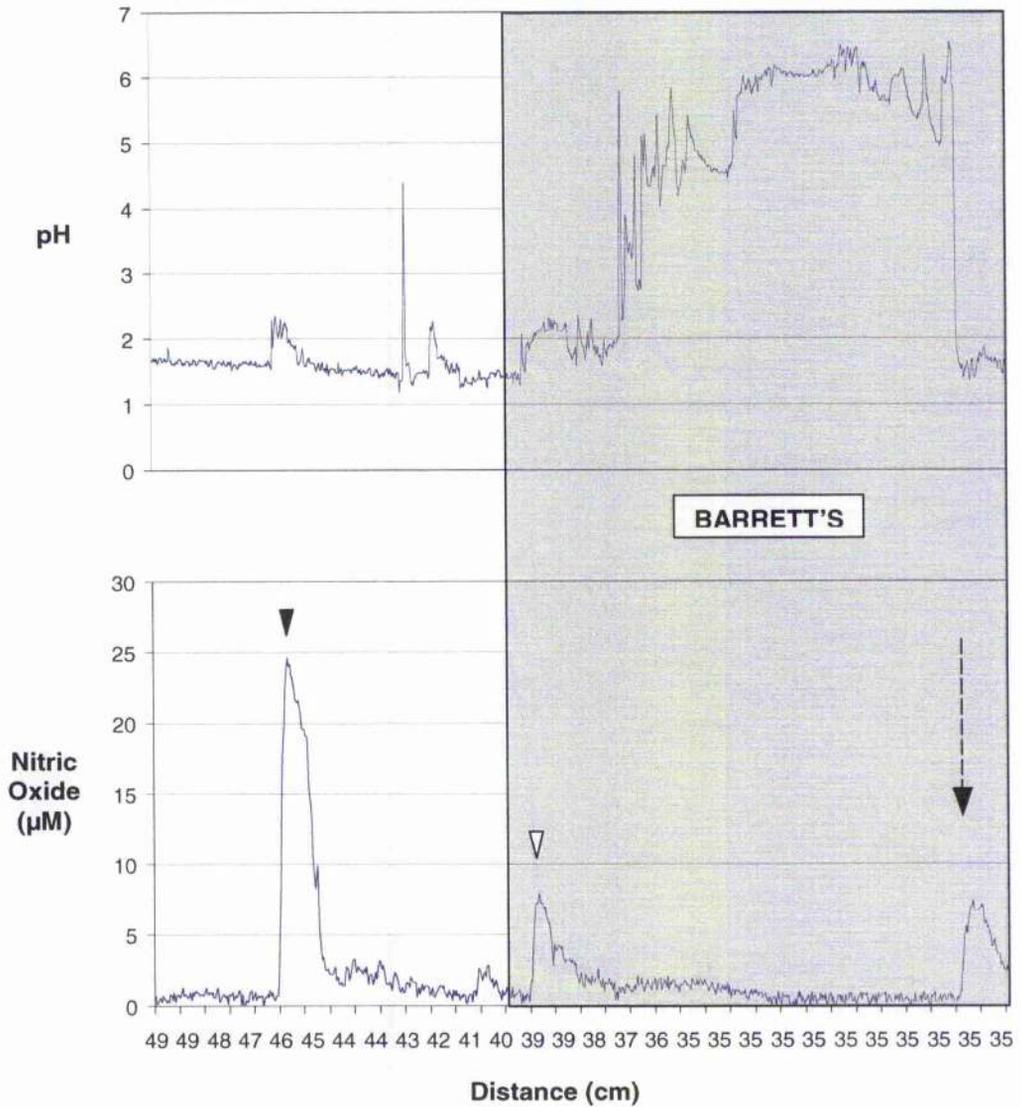


Fig 13.4a: Simultaneous recording of pH and nitric oxide *before* nitrate in patient one.

Distance indicates distance (cm) from incisors to NO probe tip. Barrett's segment extends from 35 – 40 cm (shaded grey). Nitric oxide generation is seen in proximal stomach at 45 – 46 cm (solid arrowhead) coinciding with a slight rise in pH due to swallowed saliva. Smaller concentrations of nitric oxide generated in Barrett's segment, one coinciding with slight rise in pH due to swallowed saliva (open arrowhead) and another during acid reflux in a segment with a neutral pH (broken arrow).

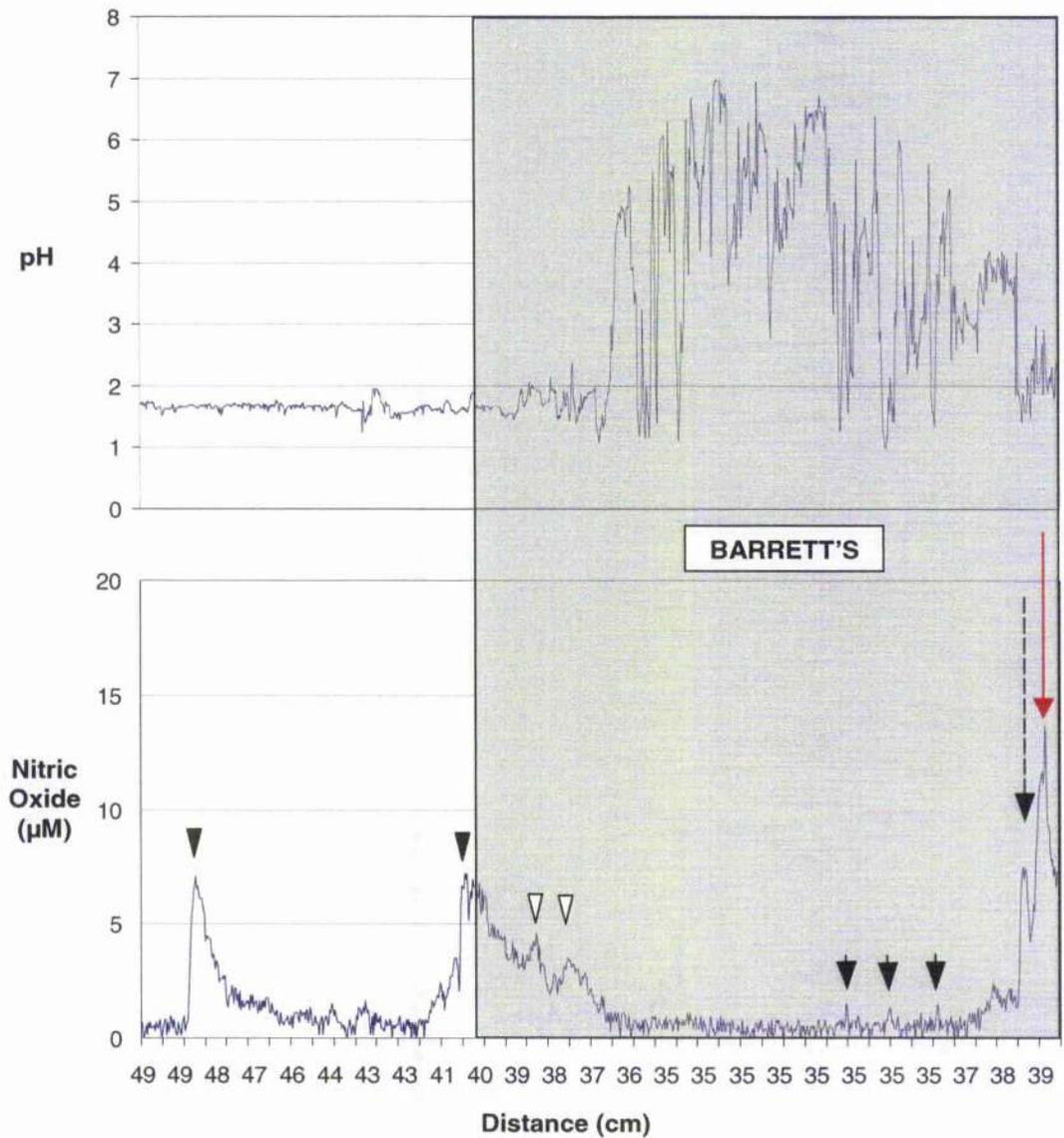


Fig 13.4b: Simultaneous measurement of pH and nitric oxide concentration after nitrate in patient one.

Distance indicates distance (cm) from incisors to NO probe tip. Nitric oxide generation is seen in the stomach from 49 cm to 41 cm (arrowheads). Small amount of nitric oxide is generated within the Barrett's segment (shaded grey) at 35 cm coinciding with reflux episodes (solid arrows) as well as at 39 cm to 37 cm coinciding with slight rise in pH due to swallowed saliva (open arrowheads). A reflux episode at 39 cm generates $7.5 \mu\text{M}$ of nitric oxide (broken arrow). Swallowed saliva at 39cm generates $13.5 \mu\text{M}$ nitric oxide (red arrow).

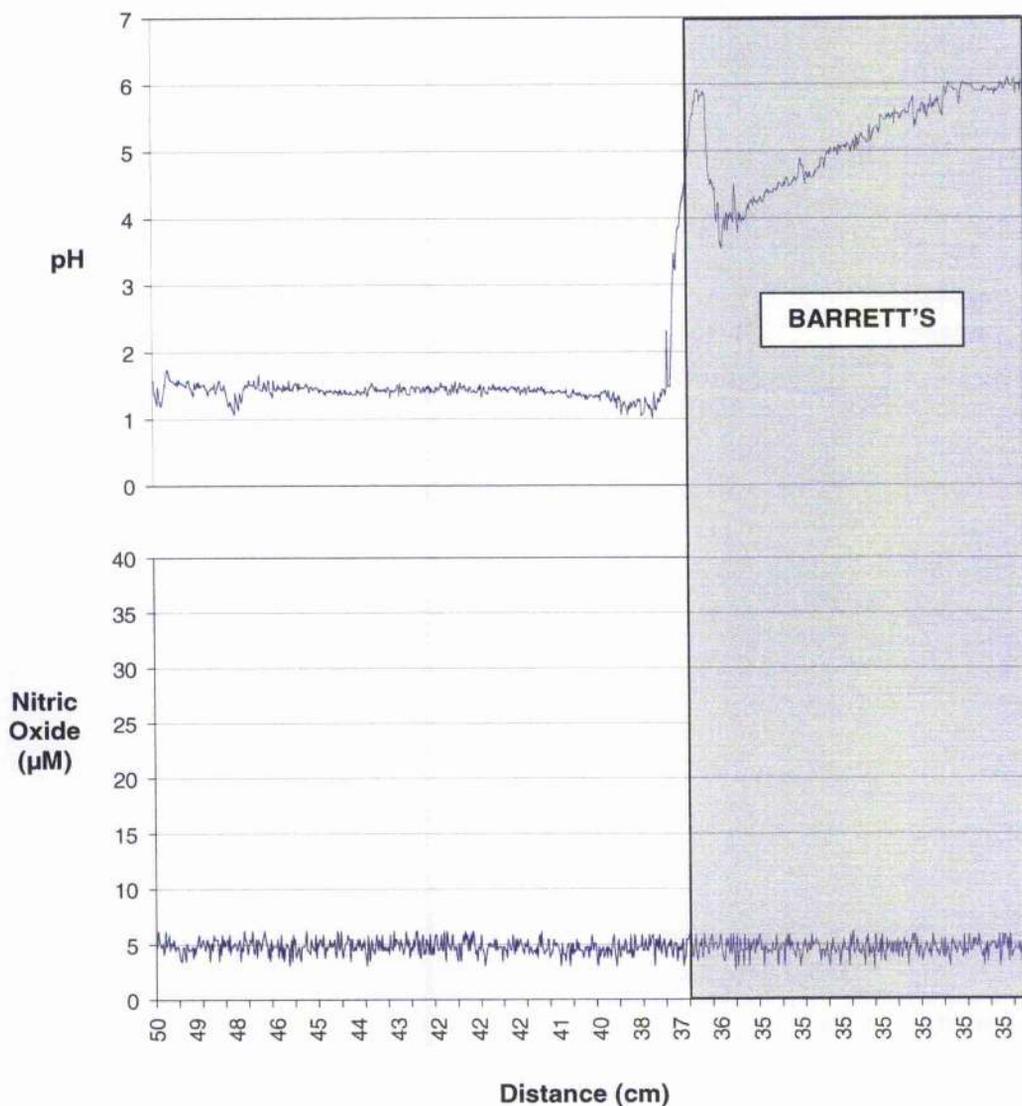


Fig 13.5a: Simultaneous recording of pH and nitric oxide before nitrate in patient two.

Distance indicates distance (cm) from incisors to NO probe tip. Barrett's segment (shaded grey) extended from 36 cm to 30 cm (36 – 33 cm shown). The background 'noise' producing nitric oxide concentration of $5\mu\text{M}$ was not corrected. No nitric oxide production seen in stomach or in the Barrett's segment. A brief episode of reflux did not generate nitric oxide in the Barrett's segment.

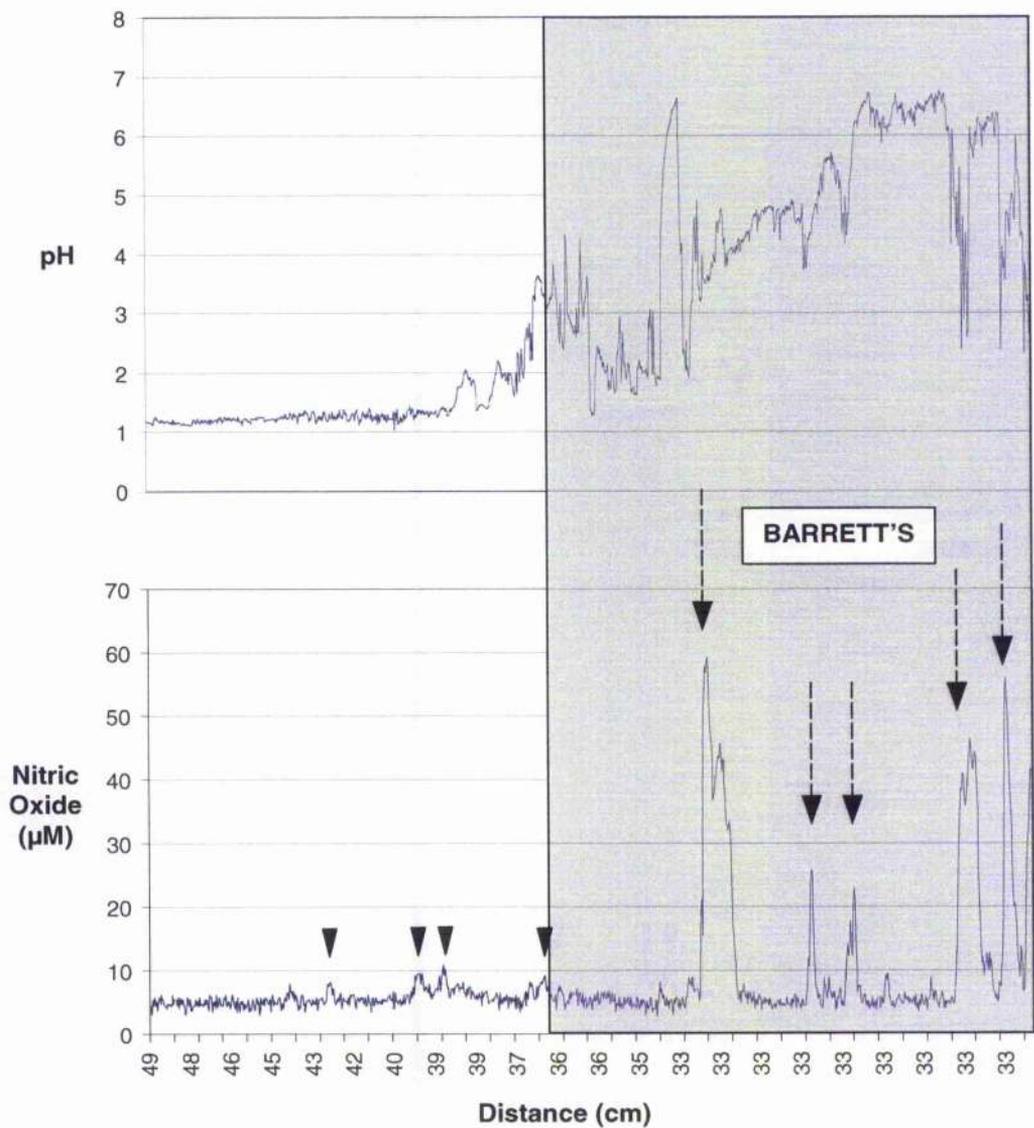


Fig 13.5b: Simultaneous measurement of pH and nitric oxide *after* nitrate in patient two.

Distance indicates distance (cm) from incisors to NO probe tip. Small amount of nitric oxide is generated in the proximal stomach (arrowheads). At 33 cm, within the Barrett's segment (shaded grey), up to $56.5 \mu\text{M}$ of nitric oxide is generated when acid reflux occurs (arrows).

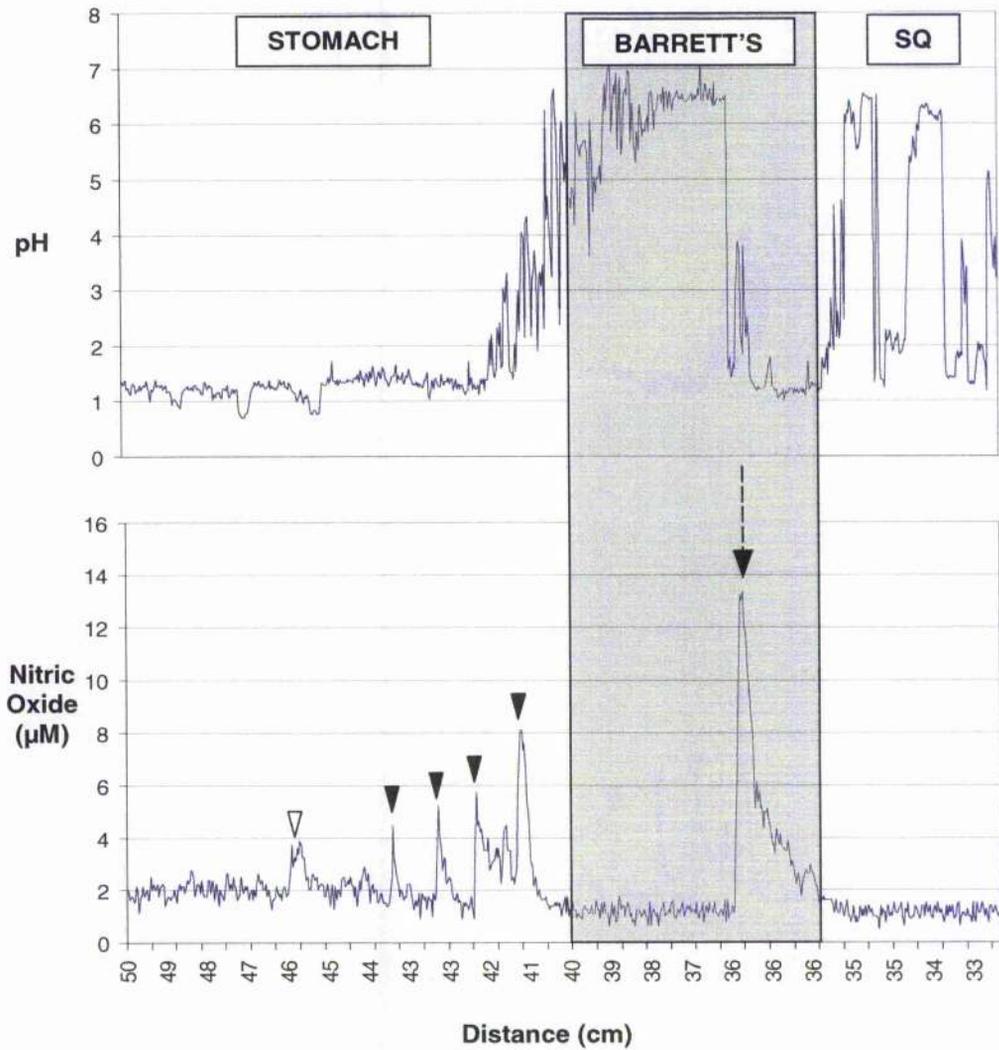


Fig 13.6a: Simultaneous recording of pH and nitric oxide before nitrate in patient three.

Distance indicates distance (cm) from incisors to NO probe tip. The shaded area indicates recordings within Barrett's segment (40 - 36 cm). SQ = squamous oesophagus. There is a small amount of nitric oxide generated in the proximal stomach at 46 cm (open arrowhead). Nitric oxide is generated just distal to the gastro-oesophageal junction (solid arrowheads) indicated by the step-up in pH. A reflux event in the Barrett's segment generates 13 µM of nitric oxide as pH falls from 6.5 to 1.5. No nitric oxide is generated in the squamous oesophagus during the three reflux episodes.

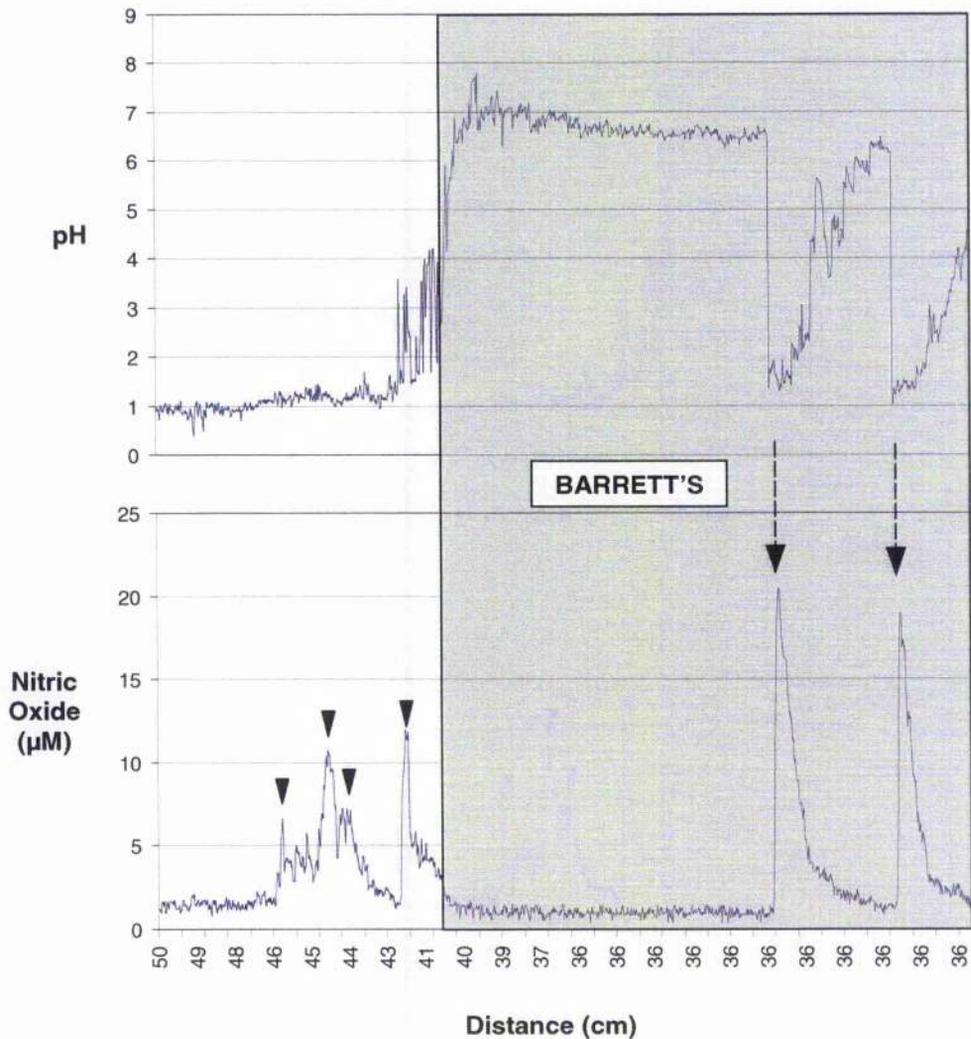


Fig 13.6b: Simultaneous recording of pH and nitric oxide after nitrate in patient three.

Distance indicates distance (cm) from incisors to NO probe tip. The shaded area indicates recordings within Barrett's segment. (Nitric oxide is generated just distal to the gastroesophageal junction (42 cm – 41 cm) and in the proximal stomach (46 cm – 44 cm) (arrowheads). No nitric oxide is produced in the Barrett's segment (shaded gray) between pH 6 to 7. Two acid reflux episodes into the Barrett's at 36 cm generate 20 µM of nitric oxide (broken arrows).

13.7 Discussion.

The previous chapter examined the concentrations of nitrite in Barrett's oesophagus and we found that in most subjects the nitrite delivered in the saliva disappeared as nitrite entered the Barrett's segment under conditions of acid reflux. Our previous study examining nitric oxide production in the lumen of the upper gastrointestinal tract in healthy subjects without gastro-oesophageal reflux demonstrated that nitric oxide is generated at or just distal to the gastro-oesophageal junction as the transition from gastric pH to the neutral pH of the oesophagus occurred ⁽²⁷⁸⁾. We have already discussed the potential mechanisms to explain the loss of nitrite in the Barrett's segment in the previous chapter. These included the absorption of nitrite ion, the absorption of nitrous acid (a less polar molecule formed by the protonation of nitrite by gastric acid) and the reduction of nitrous acid / nitrosating species to nitric oxide by ascorbic acid in the gastric juice. The first two mechanisms were thought unlikely and that the most plausible explanation for the loss of nitrite in the Barrett's segment during acid reflux was likely to be due to the latter mechanism in which nitric oxide was generated from the reduction of nitrous acid / nitrosating species by gastric juice ascorbic acid. We were able to confirm the generation of nitric oxide in Barrett's oesophagus during periods of acid reflux using a real-time miniaturised nitric oxide probe.

In the absence of gastro-oesophageal reflux, nitric oxide was not detected in the Barrett's oesophagus. However, as gastric acid refluxed into the Barrett's segment, immediate generation of very high concentrations of nitric oxide occurred within the Barrett's segment in some patients (Figs. 13.4 -13.6). This is consistent with the ascorbic acid in acidic gastric juice converting the nitrite in the saliva within the oesophageal lumen to nitric oxide. In a proportion of the acid reflux episode, very little or no nitric oxide was detected in the Barrett's segment. A number of reasons may explain the variation in intra-oesophageal nitric oxide generated with acid reflux. The nitric oxide probe only detects the concentration of nitric oxide at its tip and thus within a very localized region. Also the probe only detects nitric oxide in solution and as such, if the probe tip is not immersed in the refluxed gastric acid, it may not detect nitric oxide even if it formed within the vicinity of the probe. This is probably the reason why no nitric oxide was detected in the two subjects who participated in the study. Thirdly, the site of

nitric oxide generation within the oesophagus will be maximal where the refluxing acid meets the saliva and this is likely to vary depending upon the extent of the reflux episode and the amount and site of saliva within the oesophageal lumen. In addition, preceding reflux episodes may deplete local concentration of ascorbic acid and or nitrite and thus prevent nitric oxide generation at that site.

Following its generation in the oesophageal lumen, nitric oxide, unlike nitrite or nitrous acid, would rapidly diffuse across the lipid membranes into the Barrett's epithelium and oesophageal mucosa. A study in rats demonstrated the luminal generation of nitric oxide at the gastro-oesophageal junction (where neutral pH of the oesophagus changed to acidic pH of the stomach) when nitrite and thiocyanate were administered into the oesophagus and ascorbic acid at pH 2 was administered intragastrically. Entrapment of nitric oxide was seen in the epithelium at the gastro-oesophageal junction with a reduction in the antioxidant glutathione indicating an increased nitrosative stress intracellularly at this anatomical site⁽³⁶⁷⁾. A previous study in Barrett's oesophagus has also demonstrated a reduced concentration of glutathione and glutathione S-transferase activity compared to the squamous mucosa in the same subjects within the metaplastic epithelium⁽³⁶⁸⁾. These studies indicate that the Barrett's epithelium is exposed to substantial concentrations of nitric oxide despite having lower concentrations of glutathione and is therefore exposed to greater nitrosative and oxidative stress.

Nitric oxide has diverse physiological roles in the mammalian physiology⁽³⁶⁹⁾, however, prolonged mucosal exposure to nitric oxide has been implicated in the development of cancer in chronically inflamed tissues^(363, 364). In rats administered morpholine, the oral co-administration of nitrite plus ascorbic acid significantly enhanced tumour development in the forestomach, an effect which did not occur when nitrite was administered with morpholine or nitroso-morpholine alone was administered⁽³⁷⁰⁾. In another study, rats pre-treated with *N*-nitroso compound followed by co-administration of nitrite and ascorbate increased the yield of malignant forestomach tumors. Even without the pre-treatment with the nitroso compound, the co-administration of ascorbic acid and nitrite produced papillomas in the forestomach whereas nitrite or ascorbic acid alone did not⁽³⁷¹⁾. The combined administration of nitrite plus anti-oxidant will produce high concentrations of nitric oxide and may be responsible for the

findings noted in such studies. More recent studies in mice have observed that the intragastric administration of single large doses of nitrite plus ascorbic acid produces DNA damage within the cells of the gastric epithelium and this effect was again attributed to the generation of nitric oxide within the gastric lumen⁽³⁷²⁾.

Nitric oxide is known to be mutagenic by several mechanisms (Fig 13.7). A number of genetic alterations seen in Barrett's oesophagus with or without dysplasia and adenocarcinoma may be explained by the direct or indirect effect of nitric oxide. Nitric oxide can directly deaminate primary amines, such as those of DNA which can lead to base substitutions and affect subsequent base-pairing^(359, 360, 373). Exposure of *Salmonella typhimurium* and human lymphoblastoid TK6 cells to nitric oxide produced G:C → A:T transition mutations that can be explained by nitrosative deamination effected by nitric oxide under aerobic conditions^(359, 374, 375). In patients with oesophageal adenocarcinoma mutational analysis at exon 5 - 8 of the *p53* tumor suppressor gene showed that the majority of the mutations involved a single base substitution, G:C → A:T base transition at CpG dinucleotides⁽³⁷⁶⁾. This is consistent with deamination of 5'-methylcytosine, found at CpG sites of exon 5 - 8 of the *p53* gene⁽³⁷⁷⁾ to thymine resulting in C → T replacement, changes which may be produced by base deamination by nitric oxide in the presence of oxygen⁽³⁵⁹⁾.

Once inside the cells, nitric oxide can also reform nitrosating species in the presence of oxygen and form *N*-nitroso compounds intracellularly. An *in vitro* study suggested that physiological concentrations of the ascorbic acid, nitrite, and thiocyanate in acid pH found in the human upper gastrointestinal tract prevents luminal generation of *N*-nitroso compound but the nitric oxide formed by ascorbic acid and acidified nitrite rapidly diffuses across the cell membranes and generates *N*-nitroso compounds at concentrations 20-fold greater than that generated in the lumen in the absence of ascorbic acid⁽³⁰²⁾. *N*-nitroso compounds thus formed intracellularly may lead to DNA alkylation. The main premutagenic adduct generated by methylating *N*-nitroso compounds, such as nitroso-dimethylamine and methylnitrosourea, is *O*⁶-methylguanine. The characteristic mutation produced by *O*⁶-methylguanine is G:C → A:T base transition⁽³⁷⁸⁾ which may be an alternative mechanism by which such mutations arise in *p53* gene mutation in some patients with oesophageal adenocarcinoma. More recent studies have revealed that transcriptional silencing of tumor suppressor gene p16 involves

hypermethylation of the CpG island promoter in patients with Barrett's oesophagus with or without dysplasia and oesophageal adenocarcinoma^(379, 380, 381). A rat model of hepatocellular carcinoma utilising *N*-nitrosornicotine showed that animals exposed to *N*-nitrosornicotine developing hepatocellular carcinoma had hypermethylation of p16 gene at CpG sites whereas control rats did not have such findings suggesting that *N*-nitroso compounds are capable of methylating DNA bases at these critical sites⁽³⁸²⁾. It is tempting to speculate that the hypermethylation at the CpG sites of the p16 gene in patients with Barrett's oesophagus and oesophageal adenocarcinoma may perhaps be a result of exposure to *N*-nitroso compounds derived from the nitric oxide generated in the oesophageal lumen. In addition, nitric oxide can lead to oxidative damage via the reaction of nitric oxide and superoxide anion to produce peroxynitrite⁽³⁸³⁾. *In vitro* studies have shown that nitric oxide produce DNA strand breaks as a result of oxidative DNA damage in the presence of superoxide anion^(383, 384). The DNA defects described above are normally repaired by enzymes and nitric oxide, in addition to the DNA damage, inhibits key DNA repair enzymes such as *O*⁶-alkylguanine-DNA alkyl transferase⁽³⁰⁴⁾, formamidopyrimidine-DNA glycolylase⁽³⁰⁵⁾ and DNA ligase⁽³⁰⁶⁾. The consequence of the nitric oxide's actions, therefore, not only damages DNA but also prevents DNA repair and genetic defects accumulate leading to mutagenesis and carcinogenesis.

In the past, consideration of the aetiology of oesophageal mutagenesis related to gastro-oesophageal reflux has focused on the potentially noxious effects of acid, pepsin and bile. In this thesis we have demonstrated a couple of potential mechanisms which may cause mutagenesis in patients with Barrett's oesophagus. The generation of carcinogenic *N*-nitroso compounds within the lumen of the Barrett's segment during gastro-oesophageal reflux was discussed in the previous chapter. The current study suggests that potentially mutagenic concentrations of nitric oxide derived from dietary nitrate is formed within the Barrett's segment during periods of acid reflux. From the current studies it is unclear whether similar nitrosative chemistry occurs in the squamous oesophagus. However, nitric oxide generation in the distal oesophagus as a result of acid reflux is unlikely to be confined to Barrett's oesophagus and is likely to occur in other subjects who have gastro-oesophageal reflux. The increased risk for developing oesophageal adenocarcinoma in subjects with acid reflux appears to be confined to those with Barrett's oesophagus⁽³⁶²⁾ and likely to be due to the increased

cellular turnover present in the intestinal metaplastic mucosa present in Barrett's oesophagus^(189, 191, 194, 385, 386) making the mucosa more susceptible to the mutagenic effects of intra luminal carcinogens such as nitric oxide and *N*-nitrosamines.

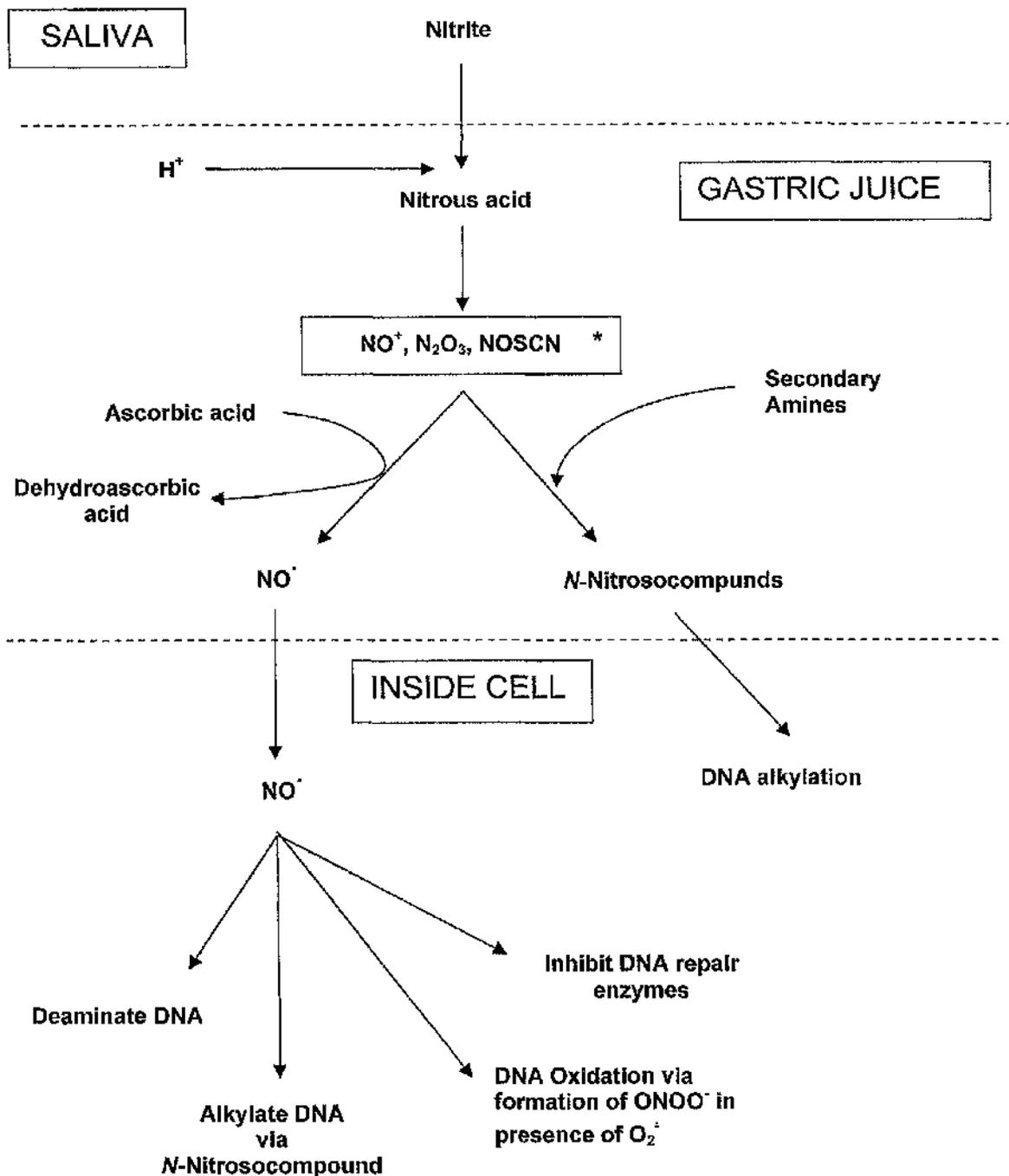


Fig 13.7: Intracellular effects of nitric oxide generated in the lumen of the upper gastrointestinal tract.

Nitrite in the saliva is swallowed and enters the gastric juice where it forms nitrous acid and other nitrosating species (*). Thiocyanate in the saliva and the gastric juice catalyse the acid nitrosation. Ascorbic acid (AA) in gastric juice reduces the nitrosating species to nitric oxide (NO[•]) and the AA is oxidised to dehydroascorbic acid. When AA is depleted, the nitrosating species react with secondary amines to form *N*-nitrosamines. The nitrosamines can diffuse into the cell and alkylate DNA. The NO[•] formed intra-luminally may diffuse into the surrounding epithelium and can cause DNA damage through DNA deamination, DNA alkylation and oxidation as well as inhibiting DNA repair enzymes.

CHAPTER FOURTEEN

GENERAL DISCUSSIONS

14.1 Summary.

The work presented in this thesis describes the novel use of microdialysis probes in the lumen of the human upper gastrointestinal tract to measure chemicals relevant to nitrosation chemistry.

The initial work involved the validation of the microdialysis probes for measuring the relevant chemicals. The major obstacle of the microdialysis experiment was in obtaining sufficient volume of samples for analysis during the stipulated time period of the experiment and utilising a suitable method to analyse the microlitre volumes of the samples produced by the microdialysis probes. The earlier part of the study, not described in the thesis, involved finding the ideal rate of perfusing the microdialysis probes to produce sufficient quantities of microdialysis product (dialysate) without severely reducing the recovery of the chemicals, hence its concentration, which is a consequence of increasing the perfusion rate in order to increase the volume of the dialysate produced. The rate of 0.3 mL hr^{-1} was initially selected for the human study which produced satisfactory recovery and ample volumes to allow duplicate sampling. However, just prior to human study, using fresh microdialysis probes we noted that the recovery became highly variable between each microdialysis probe at 0.3 mL hr^{-1} and that this could be corrected by reducing the perfusion rate to 0.15 mL hr^{-1} . Further validation studies were therefore performed using this rate.

We noted that in $\text{pH} \leq 2.5$ nitrite recovery was much lower than when $\text{pH} > 3.5$. The recovery of nitrite under highly acidic conditions could be increased by increasing the perfusion rate up to a point when the recovery fell once more. This was explained by the acidified nitrite being converted to nitric oxide which was lost through the microdialysis probe and the collecting tubes which are permeable to nitric oxide thereby reducing the measured nitrite concentrations in the dialysate. Increasing the perfusion rate reduced the transit time and the chemical reaction time in the microdialysis probes and therefore the loss of nitrite as nitric oxide was reduced.

We also studied the recovery of the chemicals under dynamic conditions simulating the reaction of salivary nitrite with gastric juice containing ascorbic acid and the microdialysis probe again proved that it was reliable in detecting the

chemicals under the conditions in which ascorbic acid was in excess of nitrite and the reverse condition of nitrite in excess of ascorbic acid.

The human studies involved using multiple microdialysis probes mounted on a nasogastric tube in defined positions to study the chemicals of nitrosation (nitrite, thiocyanate, ascorbic acid and total vitamin C). The initial study examined the nitrosation potential in healthy subjects without gastro-oesophageal reflux. We found that following an overnight fast, there was an increasing concentration gradient of ascorbic acid and total vitamin C at increasingly distal sites. The concentration of nitrite in the distal oesophagus was similar to the nitrite concentration in the saliva and a decreasing concentration gradient was seen at more distal sites with no nitrite detectable in the stomach distal to the cardia. The thiocyanate concentration was relatively constant throughout the upper gastrointestinal tract. Following nitrate ingestion, serum nitrate and salivary nitrite increased indicating that the main source of the nitrite in saliva was dietary. Nitrite in the distal oesophagus and cardia increased following the nitrate load but the reducing concentration gradient more distally was maintained with virtually no nitrite detectable distal to the cardia. The ascorbic acid to total vitamin C ratio, an indication of proportion of ascorbic acid in its reduced and hence its active form was lowest at the cardia and fell further following nitrate ingestion. This indicated that ascorbic acid oxidation was greatest at the cardia and oxidation increased significantly at this site following increased nitrite load. The ascorbic acid to nitrite ratio indicated the potential to generate the carcinogenic *N*-nitroso compounds (the lower the ratio, the greater the nitrosation potential). Whilst this ratio was lowest in the distal oesophagus, the neutral pH at this site made it unlikely for acid-catalysed nitrosation reaction to occur although the condition may be suitable for bacterial nitrosation to occur. The cardia with a pH of approximately 2.5 had the lowest ascorbic acid to nitrite ratio with 44 % of the fasting subjects having nitrite in excess of ascorbic acid, conditions which favour acid-catalysed nitrosation. The proportion of subjects with low ascorbic acid to nitrite ratio increased to 82 % following nitrate ingestion indicating that increased nitrite load following nitrate ingestion made the condition even more favourable for generating *N*-nitroso compounds at the cardia. The ascorbic acid to nitrite ratio also fell at the proximal stomach in 19 % of the subjects following nitrate

ingestion whereas the distal stomach was hostile for acid-catalysed nitrosation to occur even after nitrate ingestion.

Our previous study in subjects without gastro-oesophageal reflux indicated that luminal nitric oxide generation from acidified nitrite and ascorbic acid was maximal at the cardia therefore we can conclude that in subjects without reflux, nitric oxide is generated from acidified nitrite at this anatomical location in the presence of ascorbic acid whereas acid-catalysed nitrosation occurs when ascorbic acid is depleted, both of which produce mutagenic chemicals at the cardia which may provide a potential explanation for mutagenesis and carcinogenesis at this location in subjects without reflux.

The subsequent study in asymptomatic volunteers involved examining the chemicals relevant to nitrosation in the nasal cavity, the pharynx and the proximal and distal oesophagus. We also set up a HPLC method for determining nitrate concentrations. Nitrate was not investigated in the earlier studies as the volume of the microdialysis product was insufficient to allow separate analysis for nitrate without HPLC and even if adequate volumes were available, collection of the dialysate for nitrite analysis in NaOH made it impossible to analyse nitrate using the bacterial nitrate reductase method and HPLC was not set up for measuring nitrate at the time.

We found that the nitrate concentration was lowest in the nose followed by the pharynx and the oesophagus had the highest nitrate concentration. Nitrite concentrations in the saliva, pharynx and oesophagus were approximately 20 to 30 % of the nitrate concentration during mechanical stimulation of saliva which was similar to the nitrite / nitrate ratio in the saliva reported in the literature. Nitrite was not detected in the nasal cavity even if nitrate was readily detectable. This would suggest that nitrate reduction by bacteria did not appear to occur in the nasal cavity which may possibly be related to difference in the partial pressure of oxygen or a different resident microbial flora in the nasal cavity compared to that of the tongue. The study also suggested that there was ongoing nitrate reduction to nitrite in the oesophagus as recovery of nitrite from the oesophageal lumen was higher than the concentration of nitrite in the saliva in some individuals and since the concentration of a chemical recovered using the microdialysis is always lower than that of the sample, recovery rate of nitrite > 100 % would suggest that more nitrite was available at distal sites as nitrate was being reduced to nitrite as saliva

was travelling more distally. The significance of the high nitrite concentrations in the oesophageal lumen is three-fold. Firstly, when salivary nitrite encounters acidic gastric juice, nitric oxide is produced in significant quantities. Secondly, when the ascorbic acid becomes depleted at the gastro-oesophageal junction *N*-nitroso compounds can be generated from acidified nitrite. Thirdly, in the absence of acid for example in the oesophagus and in the achlorhydric stomach, bacterial nitrosation may generate *N*-nitroso compounds. All of these mechanisms may potentially be mutagenic to the surrounding epithelium resulting in carcinogenesis with repeated exposure.

In the third study, patients with Barrett's oesophagus were examined using the microdialysis probes. The increasing ascorbic acid and total vitamin C gradient with increasing distal location seen in healthy individuals without reflux was also seen in subjects with Barrett's oesophagus. The most significant finding was that during gastro-oesophageal reflux into the Barrett's oesophagus, nitrite concentrations fell significantly at this anatomical location despite the increased nitrite load. Recovery of thiocyanate both before and after nitrate ingestion remained unchanged within the Barrett's segment which suggested that the loss of nitrite in the Barrett's oesophagus was indeed correct and that nitrite was being 'consumed' at this site during acid reflux. The ascorbic acid to total vitamin C ratio was lowest in the Barrett's segment both before and after nitrate ingestion suggesting that the oxidation of ascorbic acid due to salivary nitrite was maximal at this location. However, the squamous oesophagus, hiatal hernia and the proximal stomach all had reduced ascorbic acid to total vitamin C ratio following nitrate administration compared to before the nitrate was administered. In the squamous oesophagus this was attributed to the oxidation of ascorbic acid delivered to the oesophagus during gastro-oesophageal reflux. Distal to the Barrett's oesophagus ascorbic acid oxidation was due to the delivery of salivary nitrite which did not react with ascorbic acid at more proximal sites.

Before nitrate ingestion 20% of Barrett's subjects had nitrite in excess of ascorbic acid in the Barrett's segment during acid reflux whereas after nitrate ingestion, 44 % of the patients had nitrite in excess of ascorbic acid. This proportion was higher than the other sites suggesting that the nitrosation potential was greatest in the Barrett's segment during acid reflux, although the numbers did not reach statistical significance. Together, the results of this study and the

previous study in subjects without reflux which showed that nitric oxide was generated at the point where salivary nitrite first encountered gastric juice suggested that in Barrett's oesophagus, the salivary nitrite was being reduced to nitric oxide by the gastric juice ascorbic acid during periods of acid reflux in the Barrett's segment.

The last study in this thesis directly examined nitric oxide generation within the Barrett's oesophagus in order to confirm the hypothesis that the loss of nitrite in the Barrett's oesophagus during acid reflux was due to the generation of nitric oxide. The number of patients examined was limited due to the break down of the nitric oxide probe after examining five patients. The study clearly showed that during periods of gastro-oesophageal reflux into the Barrett's oesophagus, some individuals generated in excess of 50 μM of nitric oxide instantaneously. When reflux did not occur nitric oxide was generated where salivary nitrite first encountered gastric juice just distal to the gastro-oesophageal junction as noted in the previous study in healthy volunteers. Prolonged exposure to nitric oxide is mutagenic and has been implicated in carcinogenesis in inflammatory conditions such as ulcerative colitis. The level of nitric oxide recorded in the Barrett's segment is very much higher than the concentrations of nitric oxide generated enzymatically by intracellular nitric oxide synthetase and provided the substrates for its generation are not depleted, this level of nitric oxide can be repeatedly generated within the Barrett's oesophagus exposing the mucosa to a considerable amount of this highly reactive chemical over a long period of time. The nitric oxide generated in the lumen may diffuse into the surrounding mucosa and can affect the DNA directly or indirectly by reforming nitrosating species which can generate *N*-nitroso compounds thereby exposing the DNA to mutagenic agents. Some of the genetic aberrations seen in patients with adenocarcinoma of the oesophagus and cardia are consistent with the known effects of nitric oxide and *N*-nitroso compounds.

The nitric oxide generation within the oesophageal lumen is unlikely to be unique to subjects with Barrett's oesophagus and is likely to occur in those who have gastro-oesophageal reflux into a normal squamous mucosa but the risk of malignant degeneration is confined to those with the metaplastic mucosa of Barrett's oesophagus and is likely to be related to the higher cellular proliferation

rate in the intestinal mucosa of the Barrett's oesophagus compared to the surrounding squamous mucosa.

14.2 Conclusions.

The work presented in this thesis examined the nitrosating potential in subjects without reflux and those with Barrett's oesophagus who are a subgroup of patients with the most severe gastro-oesophageal reflux. We examined nitrite concentrations in the nasal cavity, pharynx and oesophagus in healthy individuals. Finally we examined nitric oxide generation within Barrett's oesophagus during gastro-oesophageal reflux.

Within the nasal cavity there appeared to be no nitrate reduction to nitrite whereas the pharynx and the oesophagus approximately 30 % of the nitrate was reduced to nitrite. Significant concentration of nitrite was found in the saliva and distal oesophagus at neutral pH in subjects without gastro-oesophageal reflux and in subjects with Barrett's oesophagus before and after nitrate ingestion. Nitrite was converted to nitric oxide on contact with acidic gastric juice in and occurred at the cardia in individuals without reflux and in the distal oesophagus in subjects with Barrett's oesophagus during reflux. Similarly the nitrosating potential was maximal, hence the generation of *N*-nitroso compounds, at the cardia in subjects without reflux and in the Barrett's segment in subjects with Barrett's oesophagus during acid reflux. We therefore conclude that in those with gastro-oesophageal reflux, including those with Barrett's oesophagus, the chemical reactions leading to the generation of nitric oxide and the nitrosation reaction moves more proximally to the distal oesophagus.

There has been a worldwide increase in the use of nitrogenous fertilisers since the Second World War which may have subsequently increased the human exposure to nitrates. The increase in incidence of both oesophageal and cardia adenocarcinoma in the western world has occurred 20 – 30 years following the increased use of nitrogenous fertilisers, consistent with the latent period for the development of cancer following the exposure to a carcinogen / precarcinogen. The chemistry described in this thesis may provide some insight into the pathogenesis of these two cancers.

**PRESENTATIONS AND PUBLICATIONS CONTAINING THE WORK
UNDERTAKEN FOR THIS THESIS.**

Presentations

- 1. *Novel Method for measuring nitrosation potential within localised regions of the upper GI tract.***
Poster presentation, British Society of Gastroenterology Meeting 2002.
Poster presentation at Digestive Diseases Week, San Francisco 2002.
- 2. *Luminal nitrosation potential following nitrate ingestion is maximal at the GO junction.***
Oral presentation, British Society of Gastroenterology Meeting 2002.
Poster presentation at Digestive Diseases Week, San Francisco 2002.
Poster Presentation at UEGW, Geneva 2002.
- 3. *Potential for generation of carcinogenic nitrosamines following nitrate ingestion is maximal at the gastro-oesophageal junction.***
Oral presentation, Glasgow Gastroenterology Club Feb 2002.
Awarded the prize for best paper presented.
Oral presentation at the Scottish Society of Gastroenterology, Ayr 2002.
Awarded the Anne Ferguson Prize for best presentation.
- 4. *In barrett's esophagus, acid reflux generates high concentrations of nitric oxide from dietary nitrate.***
Oral presentation at the British Society of Gastroenterology, Birmingham 2003.
Oral presentation at the Digestive Diseases Week 2003, Orlando, USA.
Oral presentation at the Scottish Society of Gastroenterology, Nov 2003.
- 5. *The oesophageal lumen has the chemical conditions for generating carcinogenic N-nitroso compounds.***
Poster presentation at the British Society of Gastroenterology, Glasgow 2004.
Poster presentation at the Digestive Diseases Week 2004, New Orleans, USA.

Publications

1. ***Conditions for acid catalysed luminal nitrosation are maximal at the gastric cardia.***

H Suzuki, K Iijima, A Moriya, K McElroy, G Scobie, V Fyfe, KEL McColl.
Gut 2003; 52: 1095 - 1101.

2. ***Validation of microdialysis probes for studying nitrosative chemistry within localized regions of the human upper gastrointestinal tract.***

H Suzuki, A Moriya, K Iijima, K McElroy, V Fyfe, KEL McColl.
Scandinavian Journal of Gastroenterology 2003; 38: 856-73.

3. ***Nitrate and nitrosative chemistry within Barrett's oesophagus during acid reflux.***

H Suzuki, K Iijima, G Scobie, V Fyfe, KEL McColl.
Gut 2005; 54: 1527-1535.

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