STUDIES OF DIETARY NITRATE INDUCED NITROSATIVE STRESS IN THE UPPER GASTROINTESTINAL TRACT

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A Thesis Submitted for the Degree of Doctor of Philosophy to the Faculty of Medicine of the University of Glasgow

Division of Medical Sciences

Faculty of Medicine

University of Glasgow

April 2008

Declaration

I declare that this thesis has been compiled exclusively by myself. The work described herein has, unless otherwise stated, been carried out by myself. It has not been submitted for any other degree.

Stuart Paterson

Dedication

I would like to dedicate this thesis to my beautiful wife Audrey, who has provided unwavering love and support throughout the rollercoaster of emotions that this PhD produced, and to my wonderful son Angus, who has provided me with the energy to remain positive during even the most trying of times.

Acknowledgements

I would like to thank the following for their help and support throughout the course of researching and writing this thesis:

Medical Research Council for providing funding through an MRC Clinical Research Training Fellowship.

Professor Kenneth McColl has been an invaluable supervisor of this research providing regular laboratory supervision and patience. Without the invaluable advice, mentorship and support of Professor McColl I would not have been able to embark upon this valuable period of research training. I am also grateful to Dr Tom Preston for providing expert advice on matters relating to *N*-nitrosamine analysis, which have been technically and practically challenging. I would also like to extend my thanks to Professor Kennedy Lees who provided support and useful advice in the role of Educational Advisor of Studies.

I am grateful to all members of the laboratory staff in Lab 305 and 306 who over the last few years have provided practical support and advice. I would in particular like to mention Ms Anne Williams who provided technical assistance in setting up many of the bench top models. I would like to thank colleagues Dr Jack Winter, Dr Harry Suzuki and Dr Jonathan Manning for their useful discussions and comments at our regular research group meetings.

Miss Dorothy Rooney and other secretarial staff in the Division of Medical Sciences in the Faculty of Medicine Department in Glasgow University have provided not only exceptional secretarial assistance but also friendship with times of laughter and genuine care. To Dorothy, in particular, I am extremely grateful.

A special thank you to my mother, father and sister. Over the years of study they have provided me with the emotional and practical support that only a loving family can provide.

Abstract

The incidence of neoplasia at the gastroesophageal junction has increased markedly over the past 20 years, but the mutagen responsible for this remains unknown. Human saliva contains substantial quantities of nitrite and is the main source of nitrite entering the stomach. It is derived from the enterosalivary recirculation of dietary nitrate and its reduction by buccal bacteria. Carcinogenic *N*-nitrosocompounds may be formed from the nitrite in swallowed saliva by bacterially-catalysed nitrosation in the achlorhydric stomach or acid-catalysed nitrosation in the healthy acid secreting stomach. Protection against luminal acid-catalysed nitrosation is provided by the ascorbic acid content of human gastric juice which reduces nitrite to nitric oxide (NO). A high luminal concentration of NO generated from salivary nitrite has been shown at the gastroesophageal junction.

A bench top model has been developed to explore the chemistry occurring in an aqueous phase, representing the lumen at the gastroesophageal junction, after the addition of nitrite. Within this model an adjacent lipid phase has been created. The lipid phase represents the adjacent mucosa to the lumen or the dietary fat shown to sit within the fundus of the stomach. In this two-phase model, addition of physiological concentrations of nitrite to an acidic aqueous phase in the absence of ascorbic acid generated nitrosative stress within the aqueous phase. The addition of physiological concentrations of ascorbic acid to the aqueous phase prevents nitrosative stress within the aqueous phase but in so doing generates nitrosative stress within the lipid phase. This can be explained by the ascorbic acid converting the salivary nitrite to NO which

diffuses into the lipid phase and there reacts with O_2 to form the nitrosating species N_2O_3.

The bench top model has been developed further to include the lipid antioxidants, α-tocopherol, β-carotene and BHT. All three of these antioxidants are effective in inhibiting nitrosative stress within the lipid phase. The concentration of α-tocopherol required to inhibit nitrosation is less than would be expected from the reaction equation. A possible explanation for this is that ascorbic acid recycles the active antioxidant α-tocopherol from its reduced form. Further studies to support recycling of α-tocopherol by ascorbic acid are presented within this thesis.

The design of a model used in vivo to assess for nitrosative stress is discussed. The model consisted of a hydrophobic silastic tubing containing a secondary amine at a neutral pH. The hydrophobic membrane that the tubing is composed of allowed the passage of gaseous NO. Human studies in healthy volunteers were performed using the silastic tubing. The data presented shows that after a nitrate rich drink there is significantly more nitrosative stress within the upper gastrointestinal tract and this is particularly the case in the first part of the stomach where salivary nitrite meets acidic gastric juice containing ascorbic acid.

Finally, the studies explore directly measuring nitrosative stress in biopsy samples from humans and rats. As nitrite has been shown previously to be a marker of nitrosative stress in bench top models biopsy samples were assessed for nitrite and nitrate concentrations. Methods for assessing nitrite and nitrate concentrations within

biopsy samples from the upper gastrointestinal tract are discussed, as nitrite and nitrate have not been analysed from this region previously. Thereafter, upper gastrointestinal biopsy samples from healthy human subjects were analysed after a control drink and a drink rich in nitrate. The studies presented show that biopsy nitrate and nitrite was higher in samples taken from the proximal stomach as compared to the oesophagus and distal stomach. This suggests that there is more nitrosative stress in the biopsies from the same area as where NO is generated after a nitrate meal.

Together the data presented supports a novel mechanism for *N*-nitrosation in the upper gastrointestinal tract of a healthy subject. The *N*-nitrosation is maximal where swallowed salivary nitrite from dietary nitrate meets acidic gastric juice containing the aqueous antioxidant ascorbic acid.

Contents

List of Figures

List of Tables

Abbreviations

ADP	Adenosine diphosphate
ASC	Ascorbic acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
cGMP	Cyclic guanidine monophosphate
CLO	Campylobacter like organism
ClogP	Compound logP
CO_2	Carbon dioxide
COX	Cyclooxygenase
$Cu(NO_3^-)_2$	Copper nitrate
dH_2O	Deionised water
DMA	Dimethylamine
DNA	Deoxyribonucleic acid
DNIC	Dinitrosyl dithiolato iron complexes
DTT	Dithiothreitol
EC_{50}	Median effective concentration
EDTA	Ethylenediamine Tetraacetic Acid
FAD	Flavin Adenine Dinucleotide
Fe-NO	Iron nitric oxide
GC	Gas Chromatography
GI	Gastrointestinal
GO	Gastroesophageal
GORD	Gastroesophageal reflux disease
GSH	Glutathione
GSNO	Nitrosylated glutathione

$H_2NO_2^+$	Nitrous acidium ion
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HO.	Hydroxyl radical
HPLC	High Pressure Liquid Chromatography
IARC	International Agency for Research on Cancer
iNOS	Inducible nitric oxide synthase
KI	Potassium iodide
KNO_2	Potassium nitrite
KNO_3	Potassium nitrate
L-Arg	L-arginine
LDL	Low density lipoprotein
LOO.	Lipid peroxyl radical
MOR	Morpholine
MS	Mass spectrometry
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetroxide
NAC	N-Acetyl Cysteine
NaCl	Sodium Chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NaSCN	Sodium thiocyanate
NDMA	*N*-Nitrosodimethylamine
NDEA	*N*-nitrosodiethylamine

NEDD	N-1-Naphylethylene diamine
NHO_2^-	Nitrous acid
NMOR	*N*-Nitrosomorpholine
NO	Nitric oxide
NO^+	Nitrosonium ion
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
NOSCN	Thiocyanate
NPIP	*N*-Nitrosopiperidine
NPRO	N-nitrosoproline
NSAID	Non steroidal anti-inflammatory drug
O_2	Oxygen
OD	Optical density
$ONOO^-$	Peroxynitrite
pA	Picoamplitude
PIP	Piperidine
pKa	Ionisation constant
PMC	2,2,5,7,8 Pentamthyl-6-chromanol
psi	Pounds per square inch
PTV	Programmable temperature vaporiser
RNA	Ribonucleic acid
RNOS	Reactive nitrogen oxide species
ROS	Reactive oxygen species
TVC	Total vitamin C
UV	Ultraviolet
WPI	World Precision Instruments

Chapter 1 – Introduction

1.1 Overview of adenocarcinoma of the gastroesophageal junction and cardia of the stomach

The first report of an oesophageal adenocarcinoma was over a century ago and is credited to White in 1898. A review of the literature in 1900 revealed only six cases (1). Initially it was thought that this cancer was an extension of gastric tumours into the lower oesophagus. It was not until the 1950s that the existence of a primary adenocarcinoma of the oesophagus was established. While once an uncommon tumour, it is now one of the cancers with the fastest increasing incidence in the developing world. In the US, the rate of increase in adenocarcinoma of the gastroesophageal junction and cardia of the stomach has overtaken the next closest cancer, melanoma, by nearly three times (2-4). Using the European computer database Eurocim, it was found that, when the period 1968-95 was examined, there was an increase in incidence of adenocarcinoma of the oesophagus and gastric cardia throughout most of Europe (5). The interest in cancer of the oesophagus and gastric cardia is such that the recent fourth meeting in the series of international 'From Gene to Cure' congress in 2002 was dedicated to the topic. Scotland, in particular, has a high and increasing incidence of adenocarcinoma at and around the gastroesophageal junction. The incidence here has increased two to three fold in the last three decades. The incidence of cancer in Scotland, from all anatomical sites combined, has not changed much over the time period 1975-99. However, when

broken down into regions of the body, there are some striking differences. Areas of the body which are exposed to high luminal nitrite have shown significant increases in the incidence of cancer between the years 1975-99. These areas include the oral cavity (+151%), oesophageal adenocarcinoma (+195%), oesophageal squamous carcinoma (+60%) and the cardia region of the stomach which has the highest increase in incidence over this time period with a percentage increase of 230% (6).

In contrast to the increasing incidence of cardia gastric cancer, there is a progressive fall in incidence of distal gastric cancer (7). These opposing trends would suggest that there is a different aetiology behind these cancers. In keeping with this, non cardia cancer is associated with *Helicobacter pylori* induced atrophic gastritis and hypochlohydria (8,9), whereas, gastric cardia and gastroesophageal junction cancers occur in individuals with healthy acid secreting stomachs, and are not associated with *Helicobacter pylori* (8,10). The decreasing incidence of non cardia gastric cancer may be explained by the decreasing incidence of *Helicobacter pylori* and associated atrophic gastritis. However, the cause of the increasing incidence of gastroesophageal junction and gastric cardia adenocarcinoma remains unclear. Patients diagnosed with oesophageal cancer have a poor prognosis, with 5 year survival ranging between 5% and 20% in patients amenable to surgery. An understanding of the causes of this cancer and its progression is therefore important, to try and decrease the incidence of this disease and also to help direct therapeutic interventions.

1.2 Epidemiology of Gastroesophageal Junction Adenocarcinoma

The incidence of oesophageal adenocarcinoma is usually higher in men than women and increases over the age of 35 years, although the rate of rise differs markedly between different regions, probably in association with differences in risk factor patterns. Long-standing gastroesophageal reflux, Caucasian race and obesity are well recognised associations with this cancer. While tobacco smoke has been shown in some studies to be a risk factor the evidence of this is not universal and some studies have shown no risk. This is in contrast to non cardia gastric cancer where there is a strong association with cigarette smoking. Long-standing gastroesophageal reflux disease may induce Barrett's oesophagus, which is a pre-malignant condition where the squamous cells of the lower oesophagus are replaced by columnar epithelial metaplasia. The rate of conversion from Barrett's oesophagus to oesophageal adenocarcinoma is 0.5-1% per year. There may be a protective effect from infection of the stomach with *Helicobacter pylori* and it is known that *Helicobacter pylori* is itself associated with achlorhydria. There was a nested case control study from Norway based on over one hundred thousand individuals where it was shown that *Helicobacter pylori* was associated with a 5 fold reduction in risk of cardia cancer and a five fold increase in risk of non cardia cancer (11,12). Similar, although less dramatic, results have been shown elsewhere (13) and meta-analysis of this data shows a negative association between *Helicobacter pylori* infection and cardia gastric cancer (14).

The pattern of increase would make it unlikely that the increasing incidence in oesophageal adenocarcinoma is related to genetic factors and there does not seem to be any link with heredity. This would therefore suggest that acquired factors are more likely to be involved. The demonstration of a 'birth cohort effect', with higher incidence in younger cohorts, would support the idea that exposure to environmental factors in early life is an important determinant of risk (15). A potential source of the acquired risk factors would be gastroesophageal reflux disease. Perhaps surprisingly, there is not reliable data to suggest a correlation between GORD and oesophageal adenocarcinoma. It is also of note that long term acid suppressive therapy does not remove the risk of malignant complications of the disease (16,17). Acid reflux exposure has however been shown to cause non-specific DNA damage (18-20). There has been one large case control study that has demonstrated a positive association between use of lower oesophageal sphincter relaxing medication and the risk of oesophageal adenocarcinoma (21). It has been postulated that the increased abdominal pressure caused by central obesity, a sedentary position and wearing of tight belts may be a factor for gastro-oesophageal reflux (22). The pattern of incidence of adenocarcinoma, the rapidity of its increase and the marked male preponderance are inconsistent with the increased body mass index seen in the western world being related to GORD and subsequent adenocarcinoma.

As well as the epidemiology discussed above, there have also been some interesting epidemiological findings which could suggest a possible implication of dietary nitrate in the increasing incidence of upper gastrointestinal cancer. After the

Second World War, until the mid 1980s, there was an exponential rise in nitrogen fertiliser usage in the UK. This same picture was seen in the USA (23). In the UK there has been a gradual decline in the nitrate rich fertilisers used in predominately arable farming. Since 1984 there has been a fall of approximately 30% in nitrate fertiliser use (24). It is known that, for many epidemiological factors implicated in cancer development there is a lag period between the cancerous insult and the resulting cancer, an example being cigarette smoking and bronchial carcinoma. This is found in comparing the rise in adenocarcinoma at and around the gastroesophageal junction and nitrogen fertiliser usage. There have been surprisingly few studies looking at the epidemiology of fruit and vegetable intake and cardia/gastroesophageal junction adenocarcinoma. Most of the studies that have been performed have classified upper gastrointestinal cancers into oesophageal and gastric cancers. As we suspect that there may be at least two different mechanisms to gastric cancer, with one involving the healthy acid secreting subject, and the other the hypochlorhydric *Helicobacter pylori* colonised stomach, it is difficult to draw useful relevant epidemiological analysis from most of these studies looking at non cardia cancer. There was, however, a study from the Karolinska Institute in Sweden in 2001, where they evaluated fruit and vegetable consumption in the prevention of oesophageal and cardia cancers. Interestingly, in the authors' conclusions from this paper, they showed a 40% lower risk of oesophageal squamous cell carcinoma but no risk reduction for gastric cardia adenocarcinoma, in individuals in the highest fruit and vegetable exposure quartile. They suggest that the lack of association with gastric adenocarcinoma may be due to etiological differences between these cancers

(25). Looking closely at the data from this study shows that there was a trend toward those with higher fruit and vegetable intake having a higher incidence of adenocarcinoma of the gastric cardia, although this was not statistically significant ($p=0.92$) (25). This raises the possibility that, despite the increased antioxidant provided by fruit and vegetables, there is still a potentially harmful effect from dietary nitrate that will be present within these farmed products.

The possibility, that a luminal, locally acting carcinogen is involved, is highlighted by a study in Iran, looking at the anatomical site of gastric adenocarcinoma (26). This group found that within the Ardabil region of Iran, an area with a very high incidence of gastric cancer, the great majority of the cancers were arising in the right side of the cardia. It has previously been shown that, on entering the stomach, non solid material proceeds down the lesser curvature, following the 'Magenstrasse' formed by the longitudinal mucosal folds. The right side of the cardia will therefore have a particularly high exposure to carcinogens from the lumen. The observation that, within an area where they have the highest incidence of gastric cancers in the world, there are a significant number of the tumours located in the same anatomical region where non solid material passes through the stomach, makes it highly likely there is a luminally acting carcinogen.

1.3 Dietary Nitrate and Nitrite

The dominating dietary source of nitrate is the ingestion of vegetables (27) and this in part relates to the use of nitrogen rich fertilisers. Green leafy vegetables such as spinach and lettuce, but also beetroot, cauliflower and celery, are especially rich in nitrate, as are strawberries, grapes, and a few other fruits (28). Total intake of nitrate is subject to seasonal variations, fertiliser use and cooking procedures, and varies greatly between individuals and regions. Drinking water also contains nitrate and nitrite but the concentrations of these are strictly regulated in Europe and the USA in part due to fears over *N*-nitrosocompounds and also the formation of methaemoglobin in infants. Nitrite in the form sodium nitrite is used as a fixative and preservative during the processing of meat, cheese and fish (29). It is widely used to preserve lunchmeat, ham, sausages, hot dogs and bacon (30). The antimicrobial activity of sodium nitrite is important in controlling the bacterium that causes botulism (31). It is thought that there are more in the way of nitrates in our diet than nitrites, in part due to farming methods and due to regulations that are in place regarding nitrite.

1.4 Barrett's oesophagus

Barrett's oesophagus is the conversion of oesophageal squamous epithelium to columnar-lined epithelium as first described by Norman Barrett from St Marks hospital in London in the 1950s. Due to concerns regarding the incidence of both Barrett's oesophagus and oesophageal adenocarcinoma, The UK National Barrett's Oesophagus Registry (UKBOR) was set up in 1996 (32,33). Barrett's oesophagus can be considered a pre-malignant condition. Between 5-10% of patients with Barrett's oesophagus will develop adenocarcinoma, the annual risk in surveillance programmes being 0.5%-1%, which is 30-125 times that of the general population (3,34,35). Not everyone with Barrett's oesophagus has a similar risk of developing adenocarcinoma and much work has been done to identify risk factors that increase the risk of adenocarcinoma conversion from Barrett's oesophagus (Table 1.1).

Overall it is known that injury to the squamous oesophagus by bile reflux or acid causes chronic inflammation, which results in the evolution of Barrett's oesophagus. The step which causes chronic inflammation to progress to Barrett's oesophagus is as yet unknown. The transition from metaplasia to adenocarcinoma proceeds via low grade and high grade dysplasia with increasing aneuploidy (53). There are many cell signalling pathways that have strong evidence to suggest involvement in oesophageal carcinogenesis: c-myc, TNF, FasL, MAP kinase activation, p53 and p27 cell cycle check point control, cytokine signalling (COX/prostaglandins, NFκB) and gastrin, to name a few. Cyclooxygenase (COX)-2

is reportedly involved in chronic inflammation and epithelial cell growth. It appears to be constitutively expressed in the oesophagus and the level of COX-2 expression seems to increase with progression from Barrett's oesophagus to oesophageal adenocarcinoma. In a study performed by the Triadafilopoulos group they have shown that when Barrett's oesophagus explants are exposed to acid or bile there is a significant increase in COX-2 expression and stimulated proliferation (54,55).

In order for an individual to develop Barrett's oesophagus, and in some cases adenocarcinoma, environmental exposures probably need to interact with genetically determined characteristics that define personal susceptibility (56). It is unlikely that a single gene mutation is to blame, as most cases occur in the absence of a family history. This, therefore, means that inherited genetic factors are likely to be normal variants or polymorphisms in multiple genes, such as has been seen in DNA repair mechanisms, cytokine responses and chemical detoxification (57-59).

The metaplastic conversion to columnar-lined epithelium from oesophageal squamous epithelium could arise from two different categories of cell. One possibility is transdifferentiation, where there is the direct conversion of differentiated cells in the absence of cell proliferation (60). The other possibility is where metaplasia can develop from the conversion of a pluripotent cell to columnar epithelium, either tissue specific stem cells (61) or bone marrow derived stem cells (62), although there is no direct evidence of the latter in Barrett's oesophagus as yet. There are likely to be many determinants of a cell's fate. The triggering

mechanisms whereby specific genes are activated or silenced, leading to the development of Barrett's metaplasia are not yet understood. Given that GORD is implicated in the pathogenesis of Barrett's oesophagus, it is interesting to know how this may affect gene expression. Reflux contents are a complex mixture of variable pH fluid, bile salts and dietary components. The combination of any or all of these may be important as regards genotoxicity. It is with respect to this refluxate that parts of this thesis are related.

The COX-2 enzyme has a role in inducing angiogenesis (63), as well as effects on immune surveillance, tissue growth and cell adhesion. As already discussed, acid and bile have been shown to increase COX-2 expression (54,55). Laboratory and epidemiological data suggest that aspirin and NSAIDs may be chemopreventive, through inhibitory effects on COX-2 (64-66). It has been suggested, by a prospective study, that the incidence of oesophageal cancer and aneuploidy is significantly lower in those people who took aspirin or NSAIDs (67). Based on this COX-2 evidence and evidence suggesting that acid reflux is important in Barrett's oesophagus, the 10 year AspECT chemoprevention trial has been set up to look at the role of high-dose versus low dose esomeprazole, with low dose or no aspirin in affecting the overall mortality of patients with Barrett's oesophagus. Although lacking a placebo limb, this trial offers a look at the effect of anti-secretory therapy in relation to oesophageal adenocarcinoma in Phase III studies. This high profile study highlights the expected importance of the luminal environment, as well as signalling at a cellular level, in Barrett's oesophagus.

Table 1.1: Risk factors for developing adenocarcinoma from Barrett's oesophagus

Risk Factor Category	**Risk Factor**	
Demographic	Male preponderance (M:F 4:1) (36) Obesity (37,38)	
Pathophysiological	Hiatus Hernia length (39,40) Increased acid exposure (40) Decreased lower oesophageal sphincter pressure (40) Duodeno-gastric reflux (41)	
Environmental	Drugs (lowering lower oesophageal sphincter pressure) (38,42) Low fruit intake (37) Dietary nitrate? (43,44,45)	
Histopathological	Length of Barrett's >6cm (39,40,46) Presence of dysplasia (47,50) Ulceration in high grade dysplasia (48,47,49,50,51)	
Molecular Genetics (52)	**Increased Proliferation**	**Increased Invasive potential**
	TGFα, EGF, TGFβ	EGFR, C-erb B2, p27
	COX-2, TNFα, iNOS	PKC
	Gastrin, Bile salts, Acid	c-myc, β catenin
	Ras, Fos, Jun, c-myc	CCK2, COX2, $p38^{MAPK}$, $p44^{ERK}$
	CyclinD1, E, B1, p16	p21
	P53, Bcl-2	
	APC, FGF1&2, E-cadherin	

1.5 Correa Hypothesis for non cardia gastric cancer

The Correa hypothesis was first proposed as early as 1975 (68). This hypothesis postulated that intestinal type gastric cancer is the end result of a series of mutations and cell transformations begun in early life, possibly as early as the first decade. Initially, it was believed that factors such as a high salt diet may be the trigger to superficial gastritis. There is then a change from superficial gastritis to atrophic gastritis. More is known about some of these early steps in this model since the identification of *Helicobacter pylori* infection. It is known that this infectious agent is associated with increased gastric pH and achlorhydria. Once the pH of the stomach is above pH of 4 it can be colonised by denitrifying bacteria. This, thereafter, allows the bacterial generation of *N*-nitrosocompounds, which are known to be carcinogenic by causing DNA damage. Since 1994 the infectious agent *Helicobacter pylori* has been regarded as a class I human carcinogen by the IARC (69). More recently polymorphisms, in both the microbial agent and host, have shown the influence of genetic susceptibility to the development of gastric cancer (70).

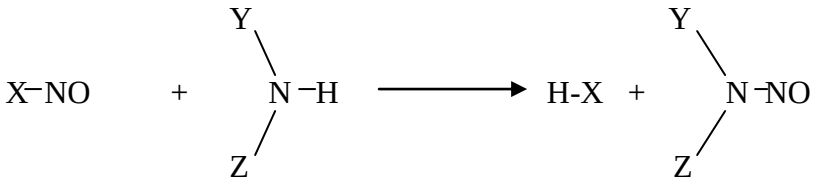
However, this hypothesis only explains the potential stages of development in non cardia gastric cancer and this cancer has been shown to be both decreasing in incidence and related to individuals who are *Helicobacter pylori* infected. The focus of interest in the upper gastrointestinal tract has now shifted to look at gastroesophageal junction adenocarcinoma and proximal gastric cardia tumours.

The Correa hypothesis still provides us with insights into the construction of a model for other cancers of the upper gastrointestinal tract, in particular, the potential of *N*-nitrosocompounds to be implicated in upper gastrointestinal tract tumours. The Correa hypothesis is still directly relevant to non cardia gastric tumours.

1.6 *N*-nitrosocompounds

1.6.1 Introduction

N-nitrosation is one of the oldest organic chemical reactions known. *N*-nitrosamines were first described in the middle of the nineteenth century. *N*-nitrosation is the replacement of hydrogen, attached to nitrogen, by a nitrosogroup as shown in the following reaction (71).



There are groups known as nitrosating agents which form nitrosogroups. The prime candidate for the formation of nitrosating agents *in vivo*, particularly in the acidic environment of the stomach, is nitrous acid (HONO = NHO_2) (pKa 3.3). Nitrous acid is formed from the reaction of nitrite ion (NO_2^-) with protons (H^+ or

H_3O^+). This reaction has been considered to be the prime candidate for the formation of nitrosating agents in the acidic environment of the stomach for some time. There are a number of potentially *N*-nitrosatable substrates in the diet (72) as well as in refluxate, particularly of bile contents. Secondary amines and amides are compounds capable of being nitrosated.

The first validated confirmation of the environmental occurrence of *N*-nitrosamines was provided when traces of *N*-nitrosodimethylamine were found in nitrite preserved herring, in sheep fodder, following an outbreak of toxic hepatosis (73). *N*-nitrosocompounds have been found to produce tumours in many animal species. Studies from more than 130 *N*-nitrosocompounds, obtained from foodstuffs (74), from the environment (75), and from the interaction between drugs and nitrite (76), indicate that about 80% of them are carcinogenic in animals. Although this suggests that *N*-nitrosocompounds may be carcinogenic in humans, there has yet to be an established causal relationship between exposure to *N*-nitrosocompounds and human cancer. In the IARC list of agents causing cancer in humans, there are no *N*-nitrosocompounds mentioned. This is not to say that *N*-nitrosocompounds are not carcinogenic. It can be difficult to demonstrate a direct cause effect relationship when there is no unexposed control group and when the available epidemiological instruments lack the sensitivity to show a relationship between low levels of *N*-nitrosocompounds and individual cancers. The IARC classifies *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-methyl-N-nitrosurea and *N*-ethyl-

N-nitrosourea as probably carcinogenic in humans (Group 2A) and have listed 15 other *N*-nitrosocompounds as being possibly carcinogenic in humans (Group 2B).

Epidemiological studies across several countries have shown evidence relating elevated intake of environmental nitrate being associated with stomach cancer. Sander and Buerkle first demonstrated that the reaction between ingested secondary amine and nitrite could occur *in vivo* and could produce carcinogenic nitrosamines in laboratory animals (77). An effective and simple method for demonstrating the endogenous formation of *N*-nitrosoproline (NPRO) in humans was later designed (78) and was widely used to assess for the importance of nitrosation *in vivo*. In a typical example of this technique, sequential oral doses of nitrate and proline were administered to a subject and the resulting NPRO measured in a 24 hour urine collection. This method is effective because NPRO is not metabolised, is not carcinogenic and can be quantitatively measured in urine (79). Patients with an achlorhydric stomach have suitable conditions for the intragastric formation of nitrosamines because of the presence of denitrifying bacteria and subsequent nitrosation of amino precursors. In a field study of Polish inhabitants, from areas of low (urban) versus high (rural) risk for gastric cancer, the urinary excretion of *N*-nitrosamino acids and nitrate was significantly higher in inhabitants of high risk areas than in inhabitants of low risk (80). A similar study was performed in Japan (81), where three different 24 hour samples of urine were collected, from each of the inhabitants of a high risk area (Akita) and a low risk area (Iwate). NPRO concentrations were measured before and after the ingestion of the nitrosatable

amine proline. After the intake of proline, the NPRO concentration increased significantly only in the subjects who were from the high risk area. The increase over urinary background NPRO in high versus low risk areas was 4 versus 1.4 fold. In both the Japanese and Polish studies ascorbic acid intake inhibited the increase in urinary *N*-nitrosocompounds. In a study in Costa Rica, in an area of high risk of gastric cancer, children have a significantly higher urinary NPRO level after proline intake compared with results for children in a low risk area (82). In a separate study in a high risk group for gastric cancer, the results, when taken as a whole, suggest that gastric carcinogenesis, in high risk areas, is more likely to be related to intragastric *N*-nitrosocompounds formed by acid-catalysed nitrosation, whereas, in low risk areas it is more likely to be related to intragastric *N*-nitrosocompounds formed by biologically catalysed intragastric nitrosation (83).

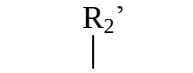
Oesophageal cancer is a prevalent disease in northern China and earlier studies suggested that environmental factors such as *N*-nitrosocompounds and their precursors may be important (84). After this time a study was performed to look at urinary NPRO concentrations before and after oral proline intake, comparing high and low risk populations for oesophageal cancer. Ingestion of proline led to increased urinary NPRO in inhabitants from both areas but the concentrations were twice as high in the high risk area (85). This suggests that endogenous nitrosation may occur to a larger extent when appropriate amine precursors are present from ingestion of foodstuffs and also that the nitrosation seems to occur more in those who are living in the high risk group. There have been further studies in China

indicating that there was a significant mortality rate associated with urinary excretion of NPRO and *N*-nitrosoarcosine (86). There are also results that implicate *N*-nitrosocompounds with colorectal cancer (87), brain tumours (88), nasopharyngeal (89), pancreatic (90) and bladder cancers (91).

There has been considerable interest in the role of *N*-nitrosocompounds and upper gastro-intestinal cancer for many years, in part based on the Correa hypothesis for gastric cancer. The thought being that, at a pH greater than 4, there is the potential for denitrifying bacteria to colonise the stomach and therefore generate *N*-nitrosocompounds. There has also been interest in *N*-nitrosocompounds within the diet, where they would potentially exert a carcinogenic effect on the environment in which they are first situated for a prolonged period of time. Little work was done on the role of nitrosamines in the upper gastrointestinal tract after the identification of *Helicobacter pylori* and the recognition of the microbe's role in gastric cancer. However, we now propose a model by which *N*-nitrosocompounds may still be implicated in cancer of the upper gastrointestinal tract at and around the gastroesophageal junction but in those who are *Helicobacter pylori* negative. This mechanism is discussed in more length later in the introduction.

1.6.2 N-nitrosamines

N-nitrosamines are the *N*-nitrosocompounds formed by the nitrosation of amines. Amines can be divided into primary, secondary and tertiary as shown below.

Primary amine	*Secondary amine*	*Tertiary amine*
		

Amines are nucleophiles in that the lone electron-pair on the nitrogen atom in amines makes the N-atom a nucleophilic centre and is therefore an electron-pair donor. The kinetics of secondary amine nitrosation are such that the rate of formation of secondary amines is proportional to the stoichiometric concentration of the amine and the square of the nitrite concentration. It is only the unprotonated form of the amine that reacts, as is shown by the fact that the overall rate of reaction is dependent on the pH.

Nitrosamines have been known for a number of years to be carcinogenic and because of this were used for the specific purpose of generating animal models of certain cancers. Barnes and Magee first showed, in 1954, the hepatotoxicity of nitrosamines in the rubber industry before going on to note that nitrosodimethylamine was a potent carcinogen in rodents (92). It was not until 1967 that the same two researchers suggested that if you choose a suitable nitrosamine and test animal, cancer could be induced at any site in the body (93). At that time, there was great interest in the formation of nitrosamines as possible carcinogenic agents but, as research into this area was proving difficult, there was a shift away

from studying this important group of compounds. It has only been more recently that there has been renewed interest in these carcinogenic compounds.

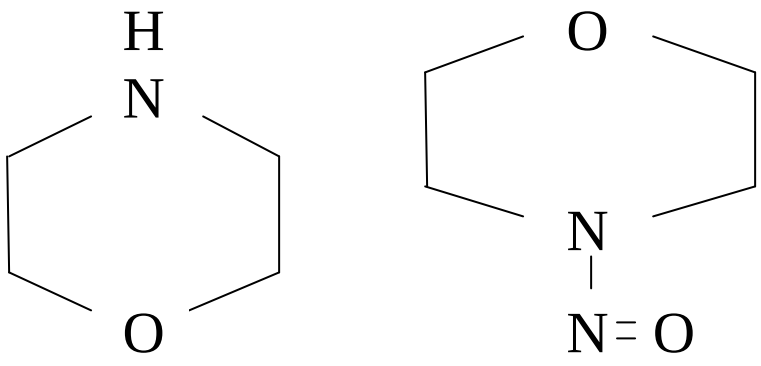
N-Nitrosamines are carcinogenic in that they act on DNA as alkylating agents. *N*-nitrosamines, *per se*, are stable under physiological conditions and require metabolic activation by cytochrome P450-dependent hydroxylation, at the carbon adjacent to the *N*-nitroso group, to form an α-hydroxynitrosamine. This can then form an alkyldiazohydroxide by spontaneous elimination of an aldehyde ($R^{-}CHO$). From the alkyldiazohydroxide an electrophilic alkyl diazonium ion, a strong carcinogen, is produced, which can react at nucleophilic sites of various cellular constituents. The alkylation of DNA is generally considered to be the critical cellular target for carcinogens in the initiation of cancer (73). Alkylation of DNA by dialkylnitrosamines results in a range of modified bases. Not all base modifications are biologically important. The most abundant modified base produced by dialkylnitrosamines is alkylation of the seventh position of guanine and this shows no correlation with carcinogenic activity. A significant proportion of alkylated DNA products are repaired. The formation and persistence of O^6-alkylguanine (94) and O^4-alkylthymine (95) are considered to be important base modifications that result in miscoding, in RNA or DNA synthesis.

There are several inhibitors of nitrosation. The most effective of these and the one most relevant to our understanding of adenocarcinoma of the gastroesophageal junction is the powerful reducing agent ascorbic acid. Ascorbic acid is found in

gastric juice at high concentrations (96). These high concentrations can be assumed to be required as a luminal antioxidant. It has previously been shown that this will potentially prevent the luminal generation of *N*-nitrosocompounds. In acting as a reducing agent ascorbic acid requires O_2 and forms NO. O_2 has been shown to be rapidly consumed in the presence of NO generation from nitrite by ascorbic acid in an acidic environment. Other inhibitors of nitrosation are phenols and in a number of *in vitro* systems biologically occurring thiols (cysteine and glutathione) inhibit nitrosamine synthesis (97). Nitrosamine formation is catalysed by thiocyanate. At acidic pH with nitrite present and in the presence of high thiocyanate concentrations, the intermediate product NOSCN is a powerful and the most important nitrosating agent (98,99).

1.6.3 *N*-nitrosomorpholine, *N*-nitrosopiperidine and *N*-nitrosodimethylamine

There have been three nitrosamines that have been looked at closely in this thesis, in *in vitro* experiments simulating the gastroesophageal junction. The first of these is *N*-nitrosomorpholine (NMOR). It is formed from the nitrosation of the secondary basic amine morpholine. Morpholine's (Tetrahydro-1,4-oxazine) structure is shown in figure 1.1. It has a relatively low pKa, for an amine, of 8.3. Another secondary amine measured in the following experiments is piperidine (pKa 11.1), which forms *N*-nitrosopiperidine (NPIP) when nitrosated. The most commonly found nitrosamine in the body is the volatile nitrosamine *N*-nitrosodimethylamine (NDMA) and this was also examined in the following studies. NDMA is formed by nitrosation of the secondary amine dimethylamine (pKa 10.3).



Morpholine

C_4H_9NO

***N*-nitrosomorpholine**

$C_4H_8N_2O_2$

Figure 1.1: The chemical structures for the secondary amine morpholine (left) and the *N*-nitrosated version of morpholine, *N*-nitrosomorpholine (right) are shown.

1.7 NO

1.7.1 Introduction to NO

NO is a small chemically simple and highly toxic gas, which has attracted much interest over the past two decades. In 1992, it was the Molecule of the Year. Nearly every cell is capable of synthesising it by one of three NO synthase isoforms. It is also formed without enzymatic function in the body such as in the cardia of the stomach and through interaction with drugs with nitrogen related compounds. The effects of NO depend on the site where it is formed, the amount formed and the local environment in which it is present.

It was shown, in the 1970s, by Tannenbaum et al that nitrate was formed in humans (100) and this was later confirmed to be the final product of NO metabolism (101). As well as generation of NO from nitrite, as outlined above, in gastric juice, NO can be synthesised from L-arginine through the L-Arg-NO pathway by NO synthase with NADPH (102) and O_2 (103) as cosubstrates. NO consists of seven electrons from the nitrogen atom and eight electrons from the O_2 atom. Therefore NO has the paramagnetism of a molecule with one unpaired electron and is highly reactive in the gas phase with O_2, to form nitrosating agents such as N_2O_3.

The chemical biology of NO has been defined as, ‘the pertinent chemical reactions which occur in biological systems’ (104). These reactions can be

categorised into direct and indirect effects. The reactions where NO interacts directly with biological molecules can be considered to be direct effects. NO is also known to form RNOS and the chemical reactions of RNOS with biological molecules are known as indirect effects. Direct effects are generally seen at lower levels of NO (<1μM), such as those seen through constitutively generated nitric oxide, whereas indirect effects are generally seen at higher concentrations (>1μM) (105). Indirect effects can be further broken down into nitrosative, oxidative and nitrative chemistry (105).

NO is known to directly interact with metalloproteins such as haem containing enzymes but does not react directly with molecules containing thiol or amines. However, under aerobic conditions, NO can react with O_2 or superoxide to form reactive nitrogen oxide species such as peroxynitrite. One of the modes of chemistry of these nitrosating agents is to nitrosate amines and thiols to form *N*-nitrosamines and S-nitrosothiols (106). This is known as nitrosative stress. Peroxynitrite, under certain circumstances, can exert oxidative stress as well as nitrative stress (such as the formation of nitrotyrosine from $ONOO^-$). At a neutral pH, $ONOO^- \rightarrow$ HOONO and this is a reactive intermediate, which can oxidise various biological substrates or simply isomerise to nitrate (107). Although able to exert oxidative stress, the oxidation mediated by peroxynitrite is influenced by the relative amounts of NO and superoxide formed (108,109). In the presence of excess NO or superoxide, peroxynitrite is converted to nitrogen dioxide (1) (109) and this can rapidly react with NO to form the nitrosating species N_2O_3 (2).

$$H + ONOO^- + NO \rightarrow H + NO_2 + NO_2^- \quad (1)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (2)$$

Thus the concentrations and relative amounts of NO and superoxide will impact on the chemistry of peroxynitrite *in vivo*.

NO reacts with O_2 to produce NO_2, which exists in equilibrium with the nitrosating agents N_2O_3 and N_2O_4 (110). Both N_2O_3 and N_2O_4 are capable of reacting with water to form NO_2^- and NO_3^-. Nitrite is oxidised to nitrate in the presence of haemoglobin and is the urinary excretion product of nitric oxide. The reaction of NO with O_2 in both gaseous and aqueous phases is a complicated process. The reaction rate is first order in O_2 and second order in NO (111). Because of the second order NO dependency, the half-life of NO is inversely proportional to its concentration (111). The formation of N_2O_3 and N_2O_4 are favoured in solution compared to the gaseous phase. The intracellular half-life of NO may be affected by reaction with compounds such as haem-Fe^{2+}. As well as reacting with molecular O_2, NO also reacts rapidly with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$). The half life of NO in biological systems is estimated to be 4-50 seconds (112). In a similar fashion to O_2, NO can migrate in and out of cells in part because it is a highly lipophilic molecule (113). This means that there should be

time for NO to travel several cell diameters to reach target sites before being consumed by molecular O_2.

It is also known that biological carriers, such as S-nitroso thiols exist *in vivo* and these allow NO to move via the circulation to tissues distant from the origin of the NO.

One of the direct effects of NO is the reaction with metal complexes (114). NO reacts with some transition metals to form stable metal nitrosyl complexes, such as Fe-NO complexes. One of the most facile NO reactions with metalloproteins is that of NO reacting with proteins containing haem moieties. The most relevant reactions in biological systems include those with haem proteins such as guanylate cyclase, cytochrome P450 and NO synthase. The reaction between NO and guanylate cyclase produces an Fe-nitrosyl complex that becomes activated to form cGMP. The concentration of NO required to activate guanylate cyclase is relatively low, with an EC_{50} of 100nM (115). cGMP is a key messenger mediating numerous regulation functions (116). A significant number of biological effects of NO are mediated through guanylate cyclase. Another haem/NO interaction is of NO with cytochrome P450. Cytochrome P450 activity is inhibited by NO in both a reversible and irreversible manner. Reversible inhibition occurs when NO binds to haem to prevent O_2 binding. Irreversible inhibition is mediated through RNOS formed from NO. As cytochrome P450 is involved in the synthesis and catabolism of numerous biomolecules, inhibition can result in important pathophysiological sequelae. NO

synthase can also be inhibited by NO in a similar fashion to that in which cytochrome P450 is inhibited. NO has also been shown to react directly with various metal-oxygen complexes. An important direct effect of NO is the reaction with oxyhaemoglobin to form methaemoglobin and nitrate (117) as shown in the reaction (3):

$$Hb(Fe\text{-}O_2) + NO \rightarrow metHb(Fe(III)) + NO_3 \qquad (3)$$

This reaction is the primary mechanism by which movement and concentration of NO are controlled *in vivo*. Due to the high concentration of oxyhaemoglobin and its rapid reaction with NO, this is one of the primary metabolic fates of NO (118).

1.7.2 Carcinogenesis of Nitric Oxide

The major nitrosating agents produced from nitrite under aqueous acidic conditions are N_2O_3, NO^+ and $H_2NO_2^+$, none of which reacts with protonated amines (119). Only unprotonated amine can be nitrosated. Nitrosation by N_2O_3 and N_2O_4 at neutral pH is rapid because of the relatively high concentration of free amine. As has been discussed previously *N*-nitrosocompounds are themselves carcinogenic. Thus NO can exert a carcinogenic effect through *N*-nitrosocompounds by reacting with O_2 to form the nitrosating compound N_2O_3 (120).

Another mechanism through which NO may be carcinogenic is through DNA damage mediated by RNOS such as N_2O_3 and peroxynitrite. Nitrosation of primary amines results in deamination. Acidic nitrite can deaminate purines, pyrimidines and various forms of RNA and DNA, through a pathway in which protonation of nitrite leads to formation of N_2O_3. N_2O_3 reacts with unprotonated amino groups of DNA in the presence of water (121). In the early 1990s NO was reported to accelerate base deamination *in vitro* (122) and cause deamination-induced genetic changes in living cells, but only in the presence of O_2 (123). Changes in DNA resulting from deamination are consistent with the types of mutations seen in many cancers. Deamination of cytosine will result in uracil formation. Removal of uracil by uracilglycolase without restoration of cytosine would leave an abasic site, a lesion often misrepaired by insertion of adenine opposite the site during replication. If the cytosine is methylated, the deamination of methylcytosine would result in the formation of thymine in the DNA chain. This change is especially important for mammalian DNA where methylation patterns are critical to gene regulation and differentiation. Nguyen et al took mammalian cells of TK6 human lymphoblastoid cells *in vitro* and added high concentrations of NO gas to the medium. There were 39 and 47 fold increases in xanthine and hypoxanthine bases formed within these cells. These bases are the product of deamination of guanine and adenine, respectively (122). There have been other *in vitro* experiments performed showing mutagenicity of NO (124,125). One of the criticisms of these experiments, at the time, was of the high concentrations of NO used in the experiments and that these did not represent physiological conditions. While this may be the case for the many

areas of the human body, it has been observed that the concentrations of NO at the gastroesophageal junction are the highest found in the body (43). There have also been studies, such as that by Delaney et al in 1993, where they reported stimulated NO formation in rat Islets of Langerhans cells, which was shown to cause DNA damage, as measured by the 'comet' assay (126).

As well as exerting nitrosative stress through *N*-nitrosocompound formation, there may be the potential for oxidative stress from nitric oxide. The potent oxidative species peroxynitrite ($ONOO^-$) and hydroxyl radical ($HO^{\cdot}$) are formed from NO after its reaction with superoxide as shown in equations (4) - (6) below:

$$NO + O_2^- \rightarrow ONOO^- \quad (4)$$

$$ONOO^- + H^+ \rightarrow ONOOH \quad (5)$$

$$ONOOH \rightarrow OH^{\cdot} + NO_2^{\cdot} \quad (6)$$

Peroxynitrite has strong oxidising properties towards biomolecules including thiols, deoxyribose and membrane phospholipids (127). The hydroxyl radical reacts with DNA, leading to several types of DNA damage, including strand breaks, cross links, abasic sites and modified bases (128). 8-Hydroxyguanine, one of the products of base oxidation, can be used as a marker of oxidative DNA damage (128). Although it should be pointed out that the amount of peroxynitrite which reacts with

the biological target is minimal if the NO flux is in excess. In fact, in some studies, it has been shown that NO has abated XO and peroxide mediated damage to DNA (129).

RNOS have a particularly high affinity for amino acids containing thiol residues (130). This suggests that enzymes which have thiol residues critical to their function may be inhibited. It is thought that very often this effect is caused indirectly by NO through the RNOS N_2O_3. Through this mechanism NO can inactivate DNA repair enzymes such as O^6-alkyltransferase (131). Another mechanism through which NO can affect DNA repair mechanisms is by affecting DNA interacting proteins that contain a zinc finger motif. Zinc finger motifs contain either two or four cysteine residues. It has been suggested that N_2O_3 nitrosates the thiol residue, resulting in the ejection of the zinc, allowing degradation of the structural integrity of the protein. This has been shown for the DNA repair enzymes formaminodopyrimidine DNA-glycolase (Fpg protein) which repairs alkylation damage due to guanine (132). In this way the Fpg protein was shown to be inactivated in the presence of aerobic NO (133). Whether this leads to any damage will depend on whether luminally generated NO reaches the adjacent cells.

1.8 Ascorbic acid

Ascorbic acid is a potent aqueous antioxidant. It has been recognised that the healthy stomach actively secretes ascorbic acid and its concentration in fasting gastric juice is several times that in plasma (96,134-136). It is a hydrophilic

molecule. In its reduced form it is present as ascorbic acid and in its oxidised form it is present as dehydroascorbic acid.

Ascorbic acid inhibits the formation of *N*-nitrosocompounds by competing for the nitrosating agents formed from nitrite. When ascorbic acid reacts with a nitrosating agent, such as N_2O_3, it is oxidised to dehydroascorbic acid. For each mole of ascorbic acid oxidised, two moles of NO are formed (137). This reaction is rapid and there is therefore the potential for effective removal of nitrosating agents. However, in the presence of O_2, NO may be reconverted to nitrosating species, thereby negating the inhibitory effect. Net removal of nitrite equivalents occurs only upon oxidation of N_2O_4 to nitrate (NO_3^-), or by physical processes such as the *in vivo* absorption of nitrite across the gastric mucosa, or *in vitro* by the loss of volatile species such as NO. The reaction of ascorbic acid with nitrite in an acidic media can be shown by the equation (7):

$$ASC + NOX \rightarrow DHA + 2NO + H^+ + X^- + H_20 \qquad (7)$$

Where NOX = N_2O_3, NO^+, NOSCN or NOCl

In the presence of thiocyanate and chloride both exert catalytic activity. This can be attributed to formation of their nitrosyl species and their reaction with ascorbic acid (138-140). Thiocyanate is a much better catalyst and this can be attributed to differences in the equilibrium constants of reactions (138,140). The

reaction shown above (7) accounts for all the loss of ascorbic acid, but none of the loss of total nitrite. The nitrite is lost by oxidation to nitrate and transfer of NO and NO_2 to the gas phase. Given that when ascorbic acid reacts with nitrosating species the major product formed is NO, then the loss of nitrite from this system is mainly through transfer of NO in the gas phase. Thus, while ascorbic acid does not remove total nitrite directly, it increases the rate of the removal process. The effective stoichiometry of the reaction between ascorbic acid and nitrite depends on the competition between the recycle of NO back into nitrosating species and the removal of NO via mass transfer (139). The rate determining step in the recycling of NO is the reaction with O_2 producing dinitrogen tetroxide, as shown in equation (8)

$$2NO + O_2 \rightarrow N_2O_4 \qquad (8)$$

The recycling process is therefore second order as regard NO while mass transfer is essentially first order. This means that as the NO concentration is increased, the recycling reaction will increase faster than physical removal. Acidity, thiocyanate and chloride are known to increase the rate of reaction between ascorbic acid and nitrite, and therefore, this tends to favour recycling and increases the effective stoichiometric ratio of ascorbic acid consumption to nitrite removal. The reaction between NO and O_2 is first order with regard to the O_2 concentration. This means that decreasing the O_2 concentration will decrease the rate of recycling, and ascorbic acid will be more effective in inhibiting nitrosation. The equilibrium concentration of dissolved O_2 is an inverse function of temperature, meaning that O_2

concentration is reduced as the temperature is increased. However, increasing the temperature increases the rate of formation of NO from nitrosating species in the presence of ascorbic acid. So, temperature changes can shift the effective stoichemistry between ascorbic acid and nitrite in either direction depending on the conditions (139).

The inhibition of bacterially mediated nitrosation of morpholine by ascorbic acid at neutral pH *in vitro* was noted as early as 1989 (141). In an *in vivo* study, using healthy male volunteer's, inhibition of endogenous NPRO from exogenous precursors requires more than the 2:1 molar ratio of ascorbic acid to nitrite that has been demonstrated *in vitro*. A ten times molar excess of ascorbic acid over the available nitrite concentration in the stomach was required to achieve complete inhibition of endogenous nitrosation of proline in this study (142).

In the human stomach, chemical interaction occurs between the nitrite and thiocyanate delivered in the saliva and the ascorbic acid secreted into acidic gastric juice. Acidified nitrite forms N_2O_3 or reacts with the thiocyanate anion to form NOSCN, both of which can be reduced to NO by ascorbic acid. The ascorbic acid is itself oxidised to dehydroascorbic acid. The NO formed in this way can react with dissolved O_2 to reform nitrous acid. This reformed nitrite can further react with any remaining ascorbic acid. This recycling of nitrite will continue until either all the ascorbic acid is converted to DHA or the O_2 is depleted. Following ingestion of nitrate and subsequent increase in delivery of nitrite to the proximal stomach, there

was a four fold fall in gastric juice concentration of ascorbic acid, due to its conversion to dehydroascorbic acid, as there was accumulation of nitrite within the gastric juice (143). The consumption of ascorbic acid in an acidic environment after the addition of nitrite has been shown to be greater in the presence of dissolved O_2 (144). The increased consumption of ascorbic acid by nitrous acid in the presence of O_2 is thought to be due to recycling of NO generated (144).

1.9 Lipid antioxidants

1.9.1 Overview

In biological systems there are lipid antioxidants as well as aqueous antioxidants. Lipid antioxidants are, in part, present to prevent lipid membrane peroxidation. Three major chain breaking lipid antioxidants are α-tocopherol, β-carotene and butylated hydroxytoluene (BHT). Alpha-tocopherol is the major vitamin E compound found in the lipid fraction of living organisms. It is thought to be one of the most powerful naturally occurring lipid antioxidants known (145). β-carotene is another naturally occurring antioxidant that has been found in many biological systems (146). BHT is a synthetic phenolic lipid antioxidant that is used in food preservatives (147).

1.9.2 Vitamin E

1.9.2.1 Introduction to vitamin E

Eighty five years ago vitamin E was discovered as a micronutrient essential for reproduction in female rats (148). Despite many studies the essential functions in humans are still not fully understood. Deficiencies in vitamin E primarily cause neurological dysfunctions. Vitamin E is an antioxidant and because of this is thought to be important in diseases where oxidative stress occurs such as cancers, chronic inflammatory conditions and cardiovascular disease. Vitamin E refers to one or more of the structurally related phenolic compounds called tocopherols and tocotrienols. These compounds are hydrophobic, peroxyl radical trapping, chain breaking antioxidants, found in the lipid fraction of living organisms. The principle function of vitamin E is thought to be the protection of the lipid material of an organism from the undesirable effects of uncontrolled, spontaneous autooxidation. The most abundant tocopherol found in animal tissue is called α-tocopherol. The relative and absolute effectiveness of chain breaking antioxidants depends primarily on their reactivity towards peroxyl radicals. The most effective tocopherol to undergo this reaction is α-tocopherol. This has been shown by kinetic studies (149). These kinetic studies suggest that this faster rate of reaction can be explained by the stereoelectronic make up of α-tocopherol, whereby the p-types lone pair can overlap with the orbital containing the unpaired electron and so stabilise the phenoxyl radical (149).

The major mechanism of action of α-tocopherol is thought to be the scavenging of lipid peroxyl radicals ($LOO^{\cdot}$) generated during lipid peroxidation, resulting in inhibition of the free radical chain reaction (150). It has been shown that

peroxynitrite can oxidise α-tocopherol predominately by either a two-electron pathway generating α-tocopherone cation or via a minor one electron pathway forming α-tocopheroxyl radical (150). Studies performed in the late 1990s showed that NO reacting with peroxynitrite in the lipid bilayer was preventing lipid peroxidation (151,152). Prior to this there had been suggestions that NO reacted directly with α-tocopherol to generate α-tocopheroxyl radical (153-155). This reaction was examined further by Nagata et al, who examined the reaction between NO and both α-tocopherol and the α-tocopherol analogue 2,2,5,7,8-Pentamethyl-6-chromanol (PMC), in the presence and absence of O_2. At all different ratios of NO to O_2, there was a reduction product of α-tocopherol and this included when NO reacted with PMC or α-tocopherol even in the absence of O_2 (156). There is conflicting evidence in regard to this study (157) and further investigation into the reaction between NO and α-tocopherol concluded that NO does not react with α-tocopherol directly (158). This work suggested that previous experiments showing a direct reaction between NO and α-tocopherol were in fact due to the experiments containing NO gas that may have been contaminated with a little O_2. However, oxidation products of NO in the form of N_2O_3 and related species will oxidise α-tocopherol to both α-tocopheroxyl radical and α-tocopheryl quinone (158). In a paper by Hogg et al, it is suggested that under biological conditions, if α-tocopherol oxidation is due to oxidised metabolites of NO, then the relevance to biological systems is questionable, as the reaction of NO with O_2 to form N_2O_3 is extremely slow (159). While this may be true of the concentrations of NO formed from NO

synthase within human cells, at the concentrations of NO that have been found at the gastroesophageal junction this would be relevant chemistry.

1.9.2.2 *In vivo* effects of α-tocopherol

Administration of antioxidants including α-tocopherol has been found to prevent oxidative stress in the retina and heart of diabetic rats and to partially inhibit the development of retinopathy in these rats, where chronic elevation of NO is thought to be important (160). In erectile dysfunction there are changes in corpus cavernosal vascular smooth muscle cells cells that are induced by glucose changes in protein C and NO expression and activity within the cell. A-tocopherol has also been shown to prevent these glucose-induced changes in protein kinase C and NO (161). In pancreatic islet cells that were preincubated with α-tocopherol there was significant improvement in the cells' resistance to toxic doses of NO. This is significant as, *in vitro*, NO has been identified as the most potent islet toxic product of inflammatory macrophages (162). While it was suggested by Burkart et al (162), that the inhibition of toxic effects on the cells was likely to be due to direct effects of α-tocopherol on NO, it is more likely that the inhibitory effect was of α-tocopherol on nitrosating species. Mergens et al, was the first to show that α-tocopherol, in its reduced form, was protective against amine induced hepatotoxicity and in the same paper showed destruction of nitrite in an *in vitro* model of gastric juice (163). In Mergens et al studies, α-tocopherol was found to react with nitrosating agents both

in lipophilic and aqueous environments and they also described greater nitrosamine inhibition when α-tocopherol and ascorbic acid were used together (163).

1.9.2.3 The vitamin E/vitamin C interaction and possible recycling

Synergistic inhibition between vitamin C and vitamin E was first suggested in 1941 when it was found that although ascorbic acid alone was a very poor antioxidant for lard and oil, it enhanced the antioxidant activity of tocopherols (164). It was not until 1968 that Tappel suggested that vitamin C might reduce the tocopheroxyl radical formed from tocopherol during the scavenging of free radicals *in vivo*, permitting a single molecule of vitamin E to scavenge many radicals (165).

Further work has been performed to evaluate the synergy of ascorbate and tocopherols and to look at the possibility of recycling of tocopherol by ascorbate. In electron spin resonance studies regeneration of vitamin E from the chromanoxyl radical by GSH and vitamin C was detected (166,167). In experiments investigating the effect of vitamin E and vitamin C on methyl linoleate peroxidation, vitamin E was kept in the reduced state as long as there was vitamin C present (168). The synergistic effects of vitamin C and E were evaluated in preventing lipid peroxidation of NADPH/iron-ADP-supplemeneted rat microsomal fractions. In these experiments, it was found that any protective effect was due to ascorbic acid reducing vitamin E, which was then active and could act as a chain breaking antioxidant (169). It should be noted that these studies do not distinguish prevention

of α-tocopherol oxidation from regeneration of α-tocopherol. Therefore, they do not provide unambiguous evidence for tocopheroxyl radical repair. In similar studies using lipid peroxidation in liposomes, there has been the suggestion of recycling of vitamin E by ascorbic acid, since the ascorbate consumption in liposomes with α-tocopherol was much more rapid than would be expected from regeneration of tocopheroxyl radicals, based on the rate of tocopherol depletion in the absence of ascorbate (170,171). There was direct evidence of recycling in human LDL by Kagan et al. They created vitamin E radicals in LDL by ultraviolet rays and found reduction of these radicals by water soluble antioxidants such as ascorbic acid. It is thought that this increases the antioxidant effect of LDL (172). It was found that when α-tocopherol was oxidised to α-tocopheroxyl radical by NO in an anaerobic, *in vitro* experiment, ascorbic acid was depleted and α-tocopherol was regenerated. Gorbunov et al suggested that recycling of vitamin E may be necessary for effective protection against NO-induced damage (153). It has been proposed that effective hydrogen transfer between ascorbic acid and α-tocopherol can take place because the polar phenoxy head group of α-tocopherol is located near the polar lipid-water interface (173).

The first explicit, *in vivo*, test of ascorbate α-tocopherol recycling suggested conflicting results with the *in vitro* studies discussed (174). This study measured the turnover kinetics of α-tocopherol in several tissues from guinea pigs fed diets containing different levels of ascorbate. Because guinea pigs cannot synthesise ascorbate, rates of α-tocopherol turnover were expected to vary inversely with

ascorbate status. In fact, in these studies, the α-tocopherol turnover kinetics were unaffected by ascorbate status in all tissues examined and it was concluded that ascorbate-dependent recycling of α-tocopherol was of negligible importance, *in vivo*, in guinea pigs that were not oxidatively stressed. However subsequent studies have failed to confirm the absence of an *in vivo* recycling mechanism. Rat hepatocytes incubated with various doses of ascorbic acid were compared to cells that were incubated in the absence of ascorbic acid. Measurements of α-tocopherol, α-tocopherolquinone, ascorbic acid and dehydroascorbic acid were made. In the absence of ascorbic acid, α-tocopherol was undetected at the completion of the experiment, while levels of α-tocopherolquinone were high, whereas in those rat hepatocytes incubated with ascorbic acid there were varying concentrations of α-tocopherol and little α-tocopherolquinone (175). It has also been suggested that ascorbate within intact human erythrocytes protects α-tocopherol in the cell membrane by a direct recycling mechanism. This was the first study to evaluate recycling occurring across a cell membrane (176). A proposed mechanism for this recycling of α-tocopherol by ascorbate is shown in Figure 1.2.

There has been some controversy in this field regarding the synergy between the two vitamins and part of the function of our study was to investigate this phenonomen further, in a model of nitrosative stress at the gastroesophageal junction.

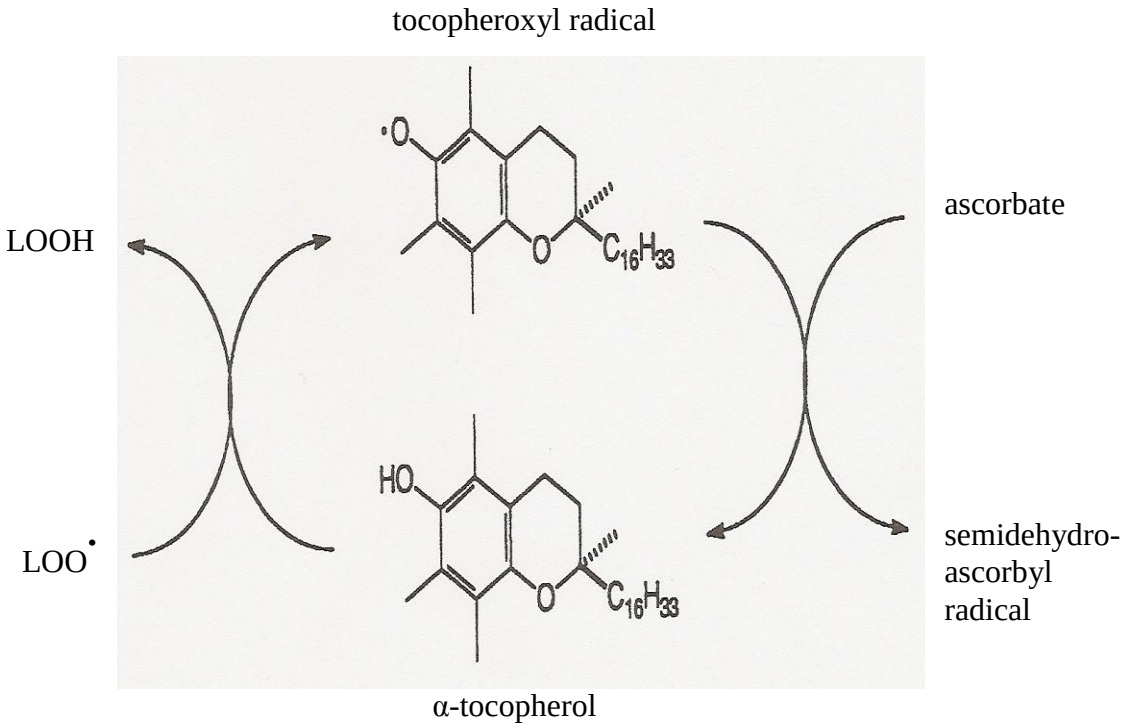


Figure 1.2: Representation of the proposed chemistry coccuring when α-tocopherol is regenerated from the tocopheroxyl radical by ascorbate. Alpha-tocopherol is shown to reduce the peroxyl radical (LOO·) to its antioxidant derived peroxyl radical (LOOH) and in so doing tocopheroxyl radical has been formed. The tocopheroxyl radical is then reduced by ascorbate back to the antioxidant α-tocopherol (adapted from Liebler (170)).

1.10 Duodenal refluxate, bile acids and carcinogenesis in the upper gastrointestinal tract

Many patients with Barrett's oesophagus have been found to have, not only gastric acid exposure, but also duodenal juice exposure of the oesophagus. The prevalence of increased bilirubin exposure significantly increases between the groups of patients with no mucosal injury (30%), oesophagitis (62%) and long segment Barrett's oesophagus (85%) (41,177-181). Several investigators have shown that duodeno-gastroesophageal reflux *per se* can induce Barrett's oesophagus to form oesophageal adenocarcinoma in rats (182-186).

There is information on the toxicity of bile acids on the oesophageal mucosa (187), gastric mucosa (188-190), colonic mucosa (191,192) and hepatocytes (193,194). However, the mechanism of action for bile refluxate causing oesophageal damage is not clear. It has previously been shown that bile, in itself, is not mutagenic, in the Ames test (195). There is the suggestion that at more alkaline pH, in their unionised form, bile acids can cross mucosal cell membranes and enter the cell, where they are noxious to the cell mitochondria. Also, due to its pKa, taurine is unionised at a low pH and will therefore be able to enter cells across their membranes. There have been reports of toxic synergism between taurine conjugates and acid in mucosal damage of the oesophagus (187). The accumulation of bile acids in the oesophageal mucosa has been a proposed method of toxicity for many years, after they were shown to accumulate in rat oesophageal mucosa (196). To elucidate the carcinogenic effects of duodenal contents, many researchers have

addressed the bile acids and pancreatic juice (197-199). It is known that conjugated bile acids, in the form of amides, are a target for nitrosative stress, leading to nitrosated compounds that are carcinogenic. The bile acids are the major source of nitrosatable amides in the body (200). Glycocholic acid, shown to make up 60% of the total bile acid composition of reflux into the oesophagus of patients with GORD (201), can be nitrosated under simulated gastric conditions to form *N*-nitrosoglycocholic acid, which is known to be highly mutagenic to bacteria and mammalian cells (200,202). *N*-nitroso bile salts such as *N*-nitrosoglycocholic acids and *N*-nitroso-taurocholic acids were also shown to be mutagenic and carcinogenic in rat stomachs as early as 1985 (197). Such bile salts have been implicated in gastric cancer as well as gastric stump carcinogenesis post gastrectomy (185). More recently, it has been demonstrated that bile acids can induce DNA breaks and oxidative damage in oesophageal cell lines and tissue (203-205) and importantly, duodenoesophageal reflux has been shown to be potentially mutagenic in rat models of Barrett's oesophagus (206).

In a rat model of oesophageal adenocarcinoma, induced by gastroduodenal reflux, it was found that the nitrite scavenger thioproline (207) was effective in inhibiting the development of oesophageal adenocarcinoma (208). In this rat model, there is reflux of duodenal contents but the stomach retains its normal function (209). This model simulates the situation of patients with duodeno-gastroesophageal reflux, including reflux of both duodenal contents and gastric juice into the oesophagus. In this study, thioproline inhibited the development of oesophageal

adenocarcinoma and iNOS protein was over expressed in Barrett's oesophagus of both the control and thioproline group. Thioproline could not suppress the overexpression of iNOS. The suggested mechanism of thioproline inhibiting oesophageal adenocarcinoma was summarised as follows by Kumagai et al: 'thioproline inhibits production of nitroso compounds by nitrite-reducing bacteria but also reactive nitrogen species such as NO, peroxynitrite and *N*-nitrosocompounds derived from reflux of duodenal contents'. This study therefore suggests that oesophageal adenocarcinoma is associated with reactive nitrogen species (such as NO, peroxynitrite and *N*-nitrosocompounds) derived from bile salts in the oesophagus (208).

It has been established that bile salts are implicated in colon cancer. Epidemiological studies have shown that high levels of secondary bile acids are found in the faeces of patients with colon carcinoma (210). It is also known that patients with Barrett's esophagus have a significantly higher incidence of colonic polyps and adenomas (211). It is therefore attractive to hypothesise that, as a high fat diet promotes the flow of bile, the excessive amount of bile acids in the alimentary tract may be a contributing factor which enhances carcinogenesis, not only in the colon, but also in the upper gastrointestinal tract, through reflux of gastro-duodenal content.

It is interesting to note that one of the few other cancers to have an increase in its incidence over the last couple of decades is cholangiocarcinoma. Studies have

shown that NO generated in response to inflammation may initiate malignant transformation of biliary epithelia and/or promote progression of established cholangicarcinoma (212). NO inactivation of DNA repair enzymes may provide a link between inflammation and the initiation, promotion, and/or progression of cholangiocarcinoma. This tumour also provides an insight into the potential mechanisms underlying NO induced malignancy, in a cancer also increasing in incidence. It is attractive to suggest that, perhaps, in this tumour there is also indirect carcinogenesis through nitrosation of bile acids.

It can be seen that the nitrosation of bile acids is an important carcinogenic mechanism and an environment rich in bile acid amides and nitrosative stress would potentially be a site of increased cancer risk. In this thesis we explore nitrosative stress in the upper gastrointestinal tract where there is the potential for bile acid refluxate.

1.11 Enterosalivary recirculation and NO generation at and around the GO junction

There are two mechanisms through which there can be nitrate circulating in the bloodstream. The first of these involves dietary nitrate. The increased nitrogen fertiliser use since the Second World War has resulted in an increase in nitrate concentrations in the diet (213). Nitrate is swallowed in the diet and then a large percentage of this is absorbed across the small intestine into the bloodstream (214). There is also a smaller contribution to the overall circulating nitrate concentration from endogenously produced nitrate, as the end product of enzymatically synthesised NO. Approximately 25% of the total nitrate in the bloodstream is actively taken up by the salivary glands and secreted into the mouth (214). The resulting concentration of nitrate in saliva is 10-20 times higher than that in the circulating blood (215). A significant proportion of salivary nitrate is then rapidly reduced to nitrite in the mouth by the action of nitrate reductase bacteria, colonised densely on the dorsum of the tongue (216,217) (Figure 1.3). Due to this enterosalivary recirculation of nitrate and subsequent conversion of nitrate to nitrite, human saliva contains very high concentrations of nitrite. Under fasting conditions the nitrite concentration of saliva is 10-1000μmol/l (143,215) and this rises 2-5 fold for several hours after the ingestion of nitrate containing foods (143). This is thought to be potentially beneficial to humans, as the nitrite formed on the tongue may then be acidified through encounter with the acid environment around the teeth and periodontal tissues, provided by the dental plaque microflora (218). The

acidification of nitrite produces nitrogen oxides as well as nitrous acid. The nitrous acid will spontaneously decompose to NO and nitrogen dioxide. NO has been shown to be produced here (219). This NO produced has an inhibitory effect on periodontal bacteria (220).

Previous studies, using validated microdialysis probes, have provided useful information regarding the localisation of chemistry into regions of the upper gastrointestinal tract (221,222). It has been shown that the most proximal cardia region of the stomach has the chemical conditions most favourable for luminal generation of *N*-nitrosocompounds (221). The concentrations of nitrite found in saliva are found in similarly high concentrations throughout the length of the oesophagus (45). It is known that approximately 1.5 litres of saliva is produced every 24 hours and all of this nitrite rich saliva passes down into the stomach. It has been observed that nitrite concentrations are highest in the cardia region of the stomach, consistent with where nitrite in swallowed saliva enters into the gastric cardia from the oesophagus (221). The more distal stomach has less nitrite present, in part due to dilution and also in part due to acidic gastric juice converting nitrite to nitrous acid and NO, with NO then able to pass into surrounding mucus and tissues. When this saliva reaches the proximal stomach, it is immediately converted to nitrous acid and nitrosating species including N_2O_3 and NO^+. As well as taking up and secreting nitrate, the salivary glands also actively take up and secrete thiocyanate and its concentration in saliva is approximately 1mmol, and even higher in smokers (223). This anion may also be secreted directly into gastric juice (223).

In determining localised regional chemistry, it was found that high concentrations of thiocyanate are present throughout the upper gastrointestinal tract but the concentrations in the proximal and distal stomach were 80% and 50% of those found in the saliva (221). Thiocyanate reacts with NO^+ to form NOSCN which is particularly potent as a nitrosating species (98,99). There is then the capability of nitrosating species to react with nitrosatable compounds such as amines and amides to generate potentially carcinogenic *N*-nitrosocompounds.

The main inhibitor of luminal acid-catalysed *N*-nitrosation is the aqueous antioxidant ascorbic acid, which is known to be present in gastric juice at high concentrations. Ascorbic acid is known to be actively secreted into gastric juice (96,134). In the study by Suzuki et al, it was shown that intragastric ascorbic acid and total vitamin C were lower in the cardia region of the stomach, as compared to the mid and distal stomach (221). A combination of the cardia of the stomach having the lowest ascorbic acid and highest nitrite means that the ratio of ascorbic acid to nitrite is significantly lower in the cardia. The ratio of ascorbic acid to nitrite is a critical determinant of acid nitrosation (139,224). When ascorbic acid is present in excess, acid nitrosation is prevented (139,224). Therefore, in this way the conditions for luminal acid nitrosation are maximal at the cardia region, as compared to the rest of the stomach. How much of a part this mechanism will play, depends, in part, upon the availability of ascorbic acid. When ascorbic acid is available to excess, significant NO will be generated.

Ascorbic acid is oxidised to dehydroascorbic acid in reducing nitrosating species to NO. Peak concentrations of NO generated in this way occur in the cardia of the stomach, as have been shown in previous studies by our group (43). There is a process of recycling of nitrite that will occur here, in the presence of initial excess ascorbic acid. It is through nitrosative stress generated by NO that our group proposes that dietary nitrate may have a role in carcinogenesis in the apparently healthy acid secreting stomach. This thesis explores such chemistry in the upper gastrointestinal tract.

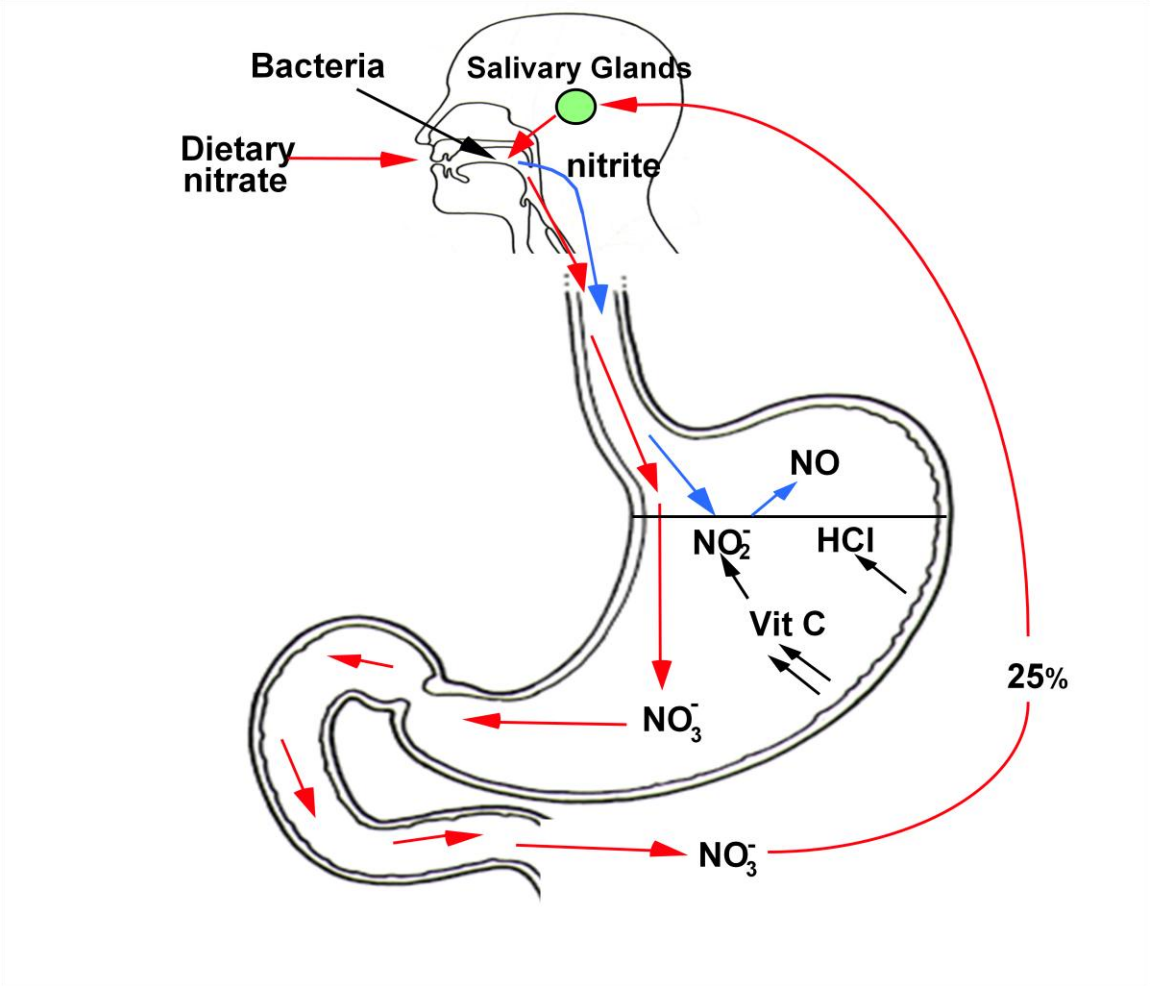


Figure 1.3**:** The process of enterosalivary recirculation of dietary nitrate and subsequent conversion to nitrite by bacteria on the dorsum of the tongue is illustrated in this figure. Once nitrite is swallowed in saliva it meets the acidic gastric juice and in the presence of vitamin C is converted to NO. The red arrows represent the passage of nitrate while the blue arrow represents the pathway for nitrite.

1.12 Overall

It has been shown that there are potentially three mechanisms of nitrosative stress, with two of these occurring maximally at the cardia region of the stomach and the gastroesophageal junction. Two of the three mechanisms, luminal acid nitrosation and nitrosation from denitrifying bacteria are well established in the literature. However, the third mechanism of nitrosative stress in the healthy acid secreting stomach, from swallowed nitrite, with NO formation and the possibility of subsequent diffusion of NO into tissues, is a relatively recent addition to our understanding of nitrosative stress in the gastrointestinal tract. The purpose of this study was to look further at NO related nitrosative stress in models of the upper gastrointestinal tract and to take this into an *in vivo* setting, to assess nitrosative stress.

Chapter 2: Materials and Methods

Throughout the studies, there were several techniques and analyses that were regularly undertaken and these will be described in this chapter. For each series of experiments, there were more specific materials, methods and experimental design used and these will be described within the respective chapters.

2.1 Methods

2.1.1 NO Determination by Amperometry

NO was measured using a World Precision Instruments (WPI) ISO-NO^{TM} Mark II NO sensor (World Precision Instrument, Sarasota, Florida). This type of biosensor has been used in experiments to measure NO since 1993 (225) and has been used in many different experimental situations since then (43,226,227,228,229). This was a 2mm diameter stainless steel shielded sensor as shown in figure 2.1.

This probe was amperometric. NO diffused through a selective gas permeable membrane and was oxidised at an electrode, resulting in an electrical current. This redox current was proportional to the NO concentration of the sample. The sensors were connected to a WPI ISO-NO NO meter (World Precision Instrument, Sarasota, Florida) which showed both the NO concentration and the redox current. The sensor

could measure NO concentrations down to 1nM. The meter was connected to a computer which converted the electrical signal (pA) to NO concentrations in µM.

The probe was calibrated to ensure accurate NO measurements. Calibration was achieved using the chemical generation of NO. A set of calibration sodium nitrite ($NaNO_2$) standards were created from a 100mM $NaNO_2$ stock solution. The method of calibration is based on the following reaction:

$$2NaNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2NaSO_4$$

where a known amount of $NaNO_2$ was added to produce a known amount of NO. KI and H_2SO_4 were added to excess, making KNO_2 the limiting reagent. Since the reaction goes to completion, the equation above states that the ratio between $NaNO_2$ and NO is 1:1. Therefore the amount of NO generated in the solution will be equal to the amount of $NaNO_2$ added. The final concentration of NO will be equal to the diluted concentration of $NaNO_2$ in the solution. As the ISO-NO sensor responds to partial pressure of NO, as opposed to the concentration, the temperature of calibration had to be the same as the temperature of the experimental setting. H_2SO_4 (0.1M) and KI (0.1M) were added to a glass vial and a small magnetic stirrer was added to the bottom of the vial. This was then placed in a water tank containing deionised H_2O, maintained at 37^oC on a heater magnetic plate in a fume cupboard. The NO probe was suspended in the solution and allowed to settle until a steady baseline was achieved. A known $NaNO_2$ calibration standard was then added and

the highest pA reading achieved was recorded. The baseline recording is subtracted from this reading to ensure that all the change in pA is due to NO and not just from background noise. This was repeated for different $NaNO_2$ concentrations, allowing the sensor recording to drop to the baseline in dH_2O before adding the probe to the next standard. The response was linear for the range of concentrations measured. From these readings a calibration curve was created each time the sensor was used (figure 2.2).

The standard curve enabled us to determine the unknown NO concentration of a sample. For any sample that had been diluted a conversion factor was allowed based on the degree of dilution. The concentrations of NO determined were given in µM.

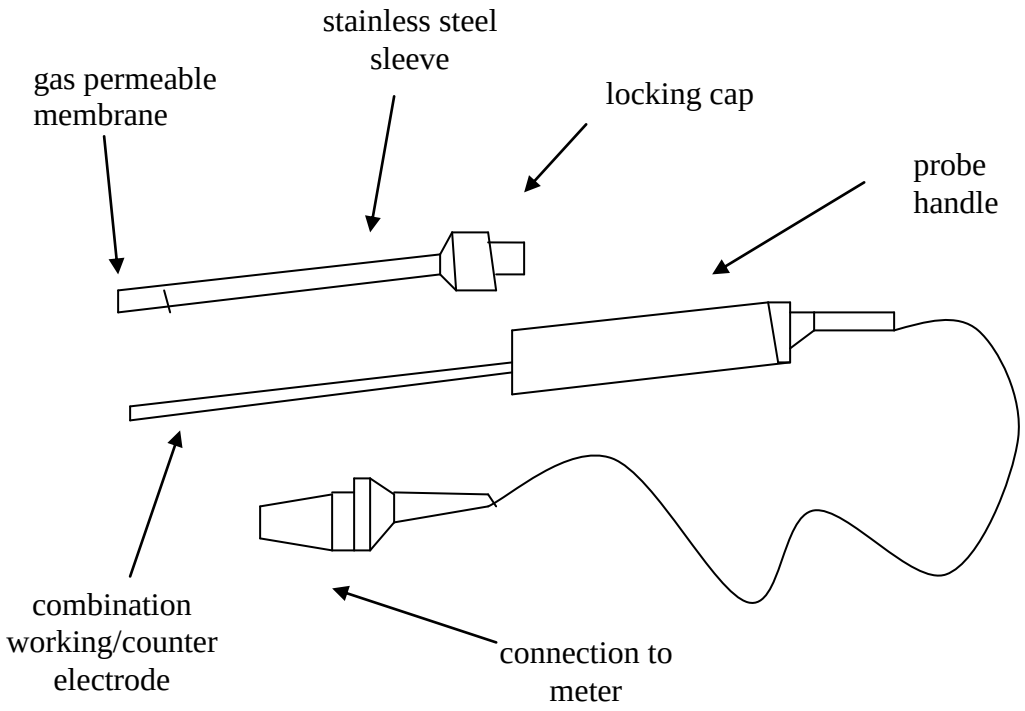


Figure 2.1: Schematic diagram representing the World Precision Instruments ISO NO™ Mark II NO probe. The probe is composed of a stainless steel sleeve, with a gas permeable membrane tip, filled with an electrolyte conducting solution. The electrode was linked to a computer recording the data as a pA output.

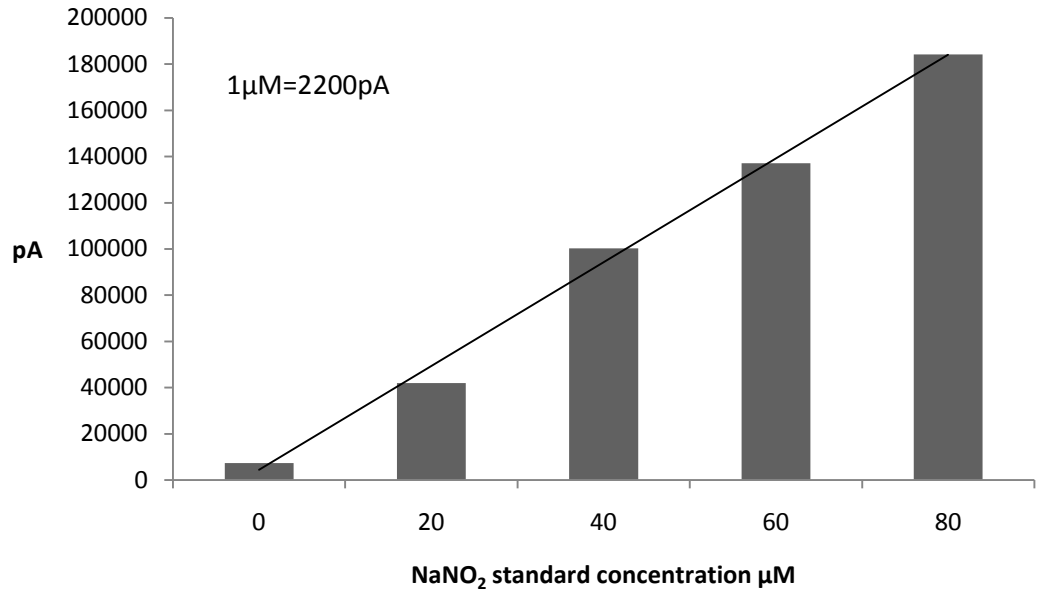


Figure 2.2: Typical NO calibration curve determined by a set of known sodium nitrite standards that generate 1µM NO per 1µM of sodium nitrite. The electrical current generated by the gaseous NO diffusing across the gas permeable membrane of the NO senor is recorded in pAs. NO is generated by using set concentrations of sodium nitrite standards. The pA generated is plotted against the given nitrite concentration. By fitting the best fit straight line through the peak pA reached per standard, a slope is created that can determine the pA per 1µM NO. Slope = pA/µM

2.1.2 Oxygen determination by amperometry

A World Precision Instrument ISO_2 meter and its associated OXEL-1 probe (World Precision Instrument, Sarasota, Florida) were used to measure O_2 levels within the experimental design. The sensor amperometrically measured the concentration of O_2 in an aqueous mixture. The O_2 probe housed a platinum working electrode and a silver reference electrode inside a stainless steel sleeve of 66mm length. The tip diameter was only 2mm, allowing detection in small vials. At the tip end of the sleeve there was a gas permeable polymer membrane which allowed O_2 to pass. O_2 was then reduced at the platinum cathode, which was held at -0.7V when the instrument was on, thus resulting in an electrical current. The magnitude of the electrical current was determined by the rate of diffusion to the electrode, which was proportional to the partial pressure of O_2 outside the membrane. Measurement modes were percent O_2, parts per million, and O_2 reduction current in nanoamperes. This could then be converted to µM concentration. The lower detection limit for ISO_2 (in liquid phase) was 0.1 ppm or 0.1%. O_2 concentration, around 0.5 ppm or 0.5% and upward, could be routinely measured in the gaseous or in the liquid phases. The accuracy of the sensor was $\pm$1.5% with a 0.1ppm resolution.

The ISO_2 sensor probe was calibrated at the same temperature (usually 37^{o}C) as was used in the experiment. Calibration took place in water saturated with nitrogen as per the manufacturers instructions.

2.1.3 Protein measurement

2.1.3.1 Introduction to protein measurement

Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd, Hertfordshire, UK). This is based on the absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifting from 465nm to 595nm when binding to protein occurs (230,231). Protein based samples were added to dye reagent concentrate and measured against bovine serum albumin standards on a colorimetric spectrophotometer (Dynatech MR 500, Dynatech Laboratories, Billingshurst, W. Sussex, UK). The Bio-Rad kit came complete with dye-reagent concentrate, which was a solution of dye, phosphoric acid and methanol, and bovine serum albumin standardised plasma protein. The dye reagent required a dilution of 1 volume dye reagent concentrate with 4 volumes dH_2O, with subsequent filtering through Whatman No. 1 paper (RA Lamb Ltd, East Sussex, UK). The Bio-Rad method was used because it is sensitive down to 1 µM of protein, is easy and rapid to use and has less in the way of chemical interference compared to the more basic Lowry and Biuret methods.

2.1.3.2 Protein Standards and use of assay

Bovine serum albumin was used as the protein standard. The stock standard was 20 mg/100mL water. To make up a working standard, the stock was diluted 1:5

with dH_2O (4mg/100mL). Standards were made of 0.25 – 2.5 mg/100mL by diluting the original working standard with deionised water. A standard curve was prepared each time the assay was performed. The standard (0.2 mL) and its dilutions were placed into a test tube. A 0.2 mL sample buffer was in a 'blank' test tube. Diluted dye reagent (0.1 mL) was added, and the samples inverted several times to allow mixing. After 15 minutes the OD_{595} was measured against the reagent blank using the colorimetric plate reader at wavelength 595nm. The OD_{595} was then plotted for the standards and the unknowns were read from the standard curve. When this assay was used for tissue samples, random, human and rat, biopsy samples were obtained from different regions of the upper gastrointestinal tract to show that there was no difference in the linearity of protein from within this heterogeneous group of samples.

2.1.4 Nitrite analysis using the modified Greiss reaction

There are many different ways to measure nitrite. These can be divided into four main categories: spectroscopic detection, electrochemical detection, chromatography and capillary electrophoresis. Spectroscopic methods are by far the most widely used and within this category the Greiss Assay is the most extensively assessed (232). This assay was first developed in 1879.

Samples in this study were analysed against known concentrations of nitrite ($NaNO_2$) using the modified Greiss reaction (233). The Greiss reagent method is based on the reaction shown in figure 2.3, where the reaction of nitrite and sulfanilic acid in an acidic environment produces an azo dye detected spectrophotometrically at 540 nm (232).

The Greiss reagent was made up with sulphanilamide, concentrated HCl, dH_2O and NEDD (N-1-Napthylethylene diamine dihydrochlorlide). In this arrangement of the Greiss reaction the NEDD is an aromatic amine acting as the coupler, while the sulphanilamide is the target aromatic amine. The detection limit was 0.1 µM.

$NaNO_2$ standards were made up in PBS at different concentrations. The general standards that were made up were 10 µM, 25 µM, 50 µM, 75 µM and 100 µM.

Using a 96 well plate (Labtech International Ltd, East Sussex, UK), 60 µL of standard solutions and samples were added to the wells before, immediately, adding 60 µL of Greiss reagent and 60 µL of dH_2O. Three 'blank' wells were created, by adding only dH_2O. The reaction was left at room temperature for thirty minutes before the optical density at 540 nm was measured on the Dynatech MR 500 colorimetric plate reader. All samples and standards were analysed in triplicate.

Figure 2.3: The chemical reaction of the Greiss reaction

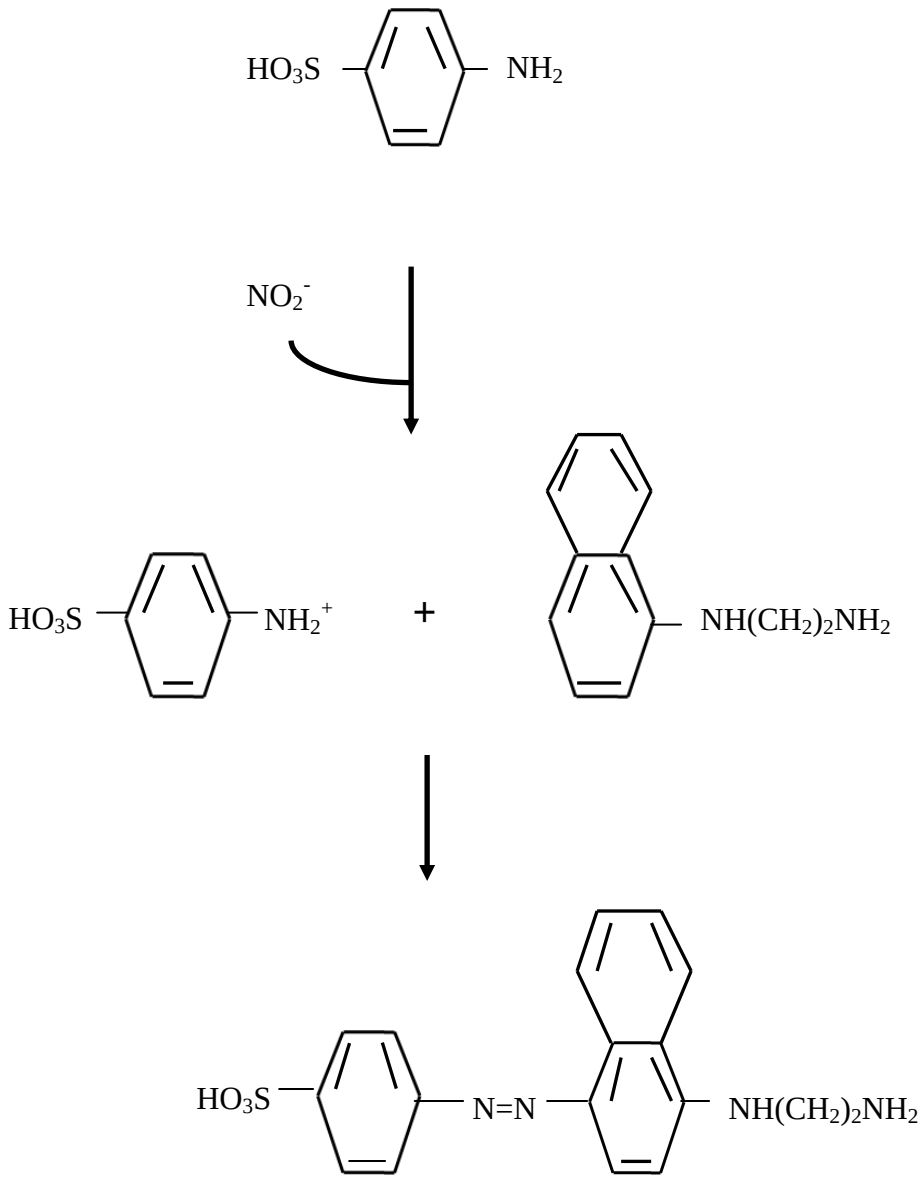


Figure 2.3: The Greiss reaction involves the reaction of nitrite (NO_2^-) under acidic conditions, with aromatic amines, such as sulphanilic acid, to form a diazonium cation; in a second step, this ion reacts with a further aromatic amine, such as NEDD, as a target coupling amine to form a purple azo dye.

2.1.5 Nitrate analysis

Nitrate was measured indirectly by the reduction of nitrate to nitrite by the bacterial enzyme nitrate reductase. The nitrate reductase was made up into a reaction buffer in the presence of β-Nicotinamide Dinucleotide Phosphate (NADPH) and Flavin Adenine Dinucleotide (FAD), Phosphate Buffer Solution (PBS) and dH_2O. PBS is present as the reaction will only take place at pH 7.2-7.4. One unit of nitrate reductase will reduce 1.0 µM of nitrate per minute in the presence of NADPH at 25^{o}C. Nitrate reductase (10 units) was added to 4 mg of NADPH and 33.2 mg of FAD to make up the reaction buffer. The total volume of reaction buffer that was used was 4000 µL which was made up of the following: NADPH 4 mg in 800 µL, FAD 33.2 mg in 800 µL, PBS (pH 7.4) 800 µL, dH_2O 1520 µL, nitrate reductase 10 U/450 µL 80 µL. The reaction of nitrate reduction with nitrate reductase converts the nitrate to nitrite, and the nitrite is subsequently measured by the Greiss reagent method as described above. As the nitrate method does not take into account any nitrite within the sample to start with, we also plated nitrite standards and samples on a separate well plate. This nitrite concentration was subtracted from the concentration from the nitrate well samples. The reaction of nitrate reductase relies on the pH of the sample being between pH 7.2 – 7.4 and therefore for alkali samples, from rat studies, adjustment using a 'superbuffer' was performed. The superbuffer consisted of 0.1 M NaOH and a half volume 0.3 M HCl.

Well plates for nitrate (Plate I)/nitrite (Plate II) analysis were set up as follows. In both plates there were three 60 µL water 'blanks', then three 60 µL standards for

each concentration of nitrite. In the nitrate plate (Plate I), there were also three wells for each 60 µL nitrate concentration. Sample (60 µL) was added to each of three sample wells. To plate I, 30 µL of reaction buffer was added, while 30 µL of water was added to each well of plate I. Both plates were incubated at 25^{o}C, in the dark, for 2 hours, to allow the reaction buffer to convert nitrate to nitrite. At the end of 2 hours, 60 µL Greiss reagent and 30 µL distilled water was added to each well of both plates. The plates were then left at room temperature, for 20 minutes, before being analysed on the colorimetric plate reader (Dynatech MR 500). The original nitrate in samples was calculated from the nitrate standard curve.

The reaction principle of nitrate reductase is

$$\beta\text{-NADPH} + \text{Nitrate} \xrightarrow[\text{Nitrate reductase}]{} \beta\text{-NADP} + \text{Nitrite} + H_2O$$

2.1.6 ^{15}N analysis

Analysis for ^{15}N was undertaken in studies to examine the effectiveness of cleaning samples deliberately exposed to $K^{15}NO_2$ and $K^{15}NO_3$. The analysis for total ^{15}N content (ng) was undertaken in collaboration with Dr T Preston who kindly performed the analysis on a tandem mass spectrometer (ThermoFinnigan, Hemel Hempstead, UK).

2.1.7 S-Nitrosothiols

Nitrosothiols were measured at pH 7.4, using the release of NO from nitrosothiols by copper in an acidic environment, in the presence of glutathione acting as an excess reducing agent (234,235). The NO was measured using the WPI NO probe, as described above in Chapter 2.1.1. The copper used was in the form of copper nitrate. To calibrate the NO probe, nitrite standards (of concentration 20, 40, 60 and 80 μM) were used, as in the presence of excess glutathione all the nitrite would be converted to nitrosothiols before the NO is then released by the copper.

The following reagents were used

a. pH 7.4 50 mM H_3PO_4/K_2HPO_4 Buffer. This acts to keep the reaction at pH 7.4. H_3PO_4 of 50 mM is made up by adding 0.5 mL H_3PO_4 (concentrate) in 100 mL dH_2O to give a pH of 1.7. K_2HPO_4 of 50 mM is made up by dissolving 0.871g

of K_2HPO_4 in 100 mL of dH_2O to give a pH of 8.9. K_2HPO_4 solution was put into a beaker and the pH adjusted by adding H_3PO_4 solution to give a pH of 7.4 (roughly H_3PO_4:K_2HPO_4 = 6:100)

b. 500 mM $Cu(NO_3^-)_2$. This provides copper, which, when added to a nitrosothiol, displaces the NO from the GSNO thiol group, liberating NO. To make up this solution, 0.938 g of $Cu(NO_3^-)_2$ was dissolved in 10 mL of pH 2.5 HCl. $Cu(NO_3^-)_2$ was used at 5mM final concentration.

c. 100mM Glutathione (GSH). This acts as a reducing agent and also a thiol donor for the production of nitrosothiols. 0.307 g of GSH is dissolved in 10 mL of dH_2O. GSH was used as a final concentration of 1 mM

From these standards a graph can be plotted and, from the graph, we can determine the NO release from the unknown samples (Figure 2.4).

Human biopsy samples were acidified to pH 2.5 by adding 19 µL of 0.1 M HCl. GSH (1 mM) was added to act as a thiol donor group and reducing agent, thus allowing any nitrite present to be converted to nitrosothiols. In a separate 1 ml plastic Eppendorf test tube 99 µL of buffer solution and 1 µL of 100 mM GSH were added together. The 80 µL sample was then added to 100 µL of buffer based solution. A calibrated WPI NO probe was placed into the Eppendorf tube before the NO was liberated. To the 200 µL solution, 2 µL of 500 mM of $Cu(NO_3^-)_2$ (5mM) was added to liberate NO. NO production was recorded and the peak measurement obtained.

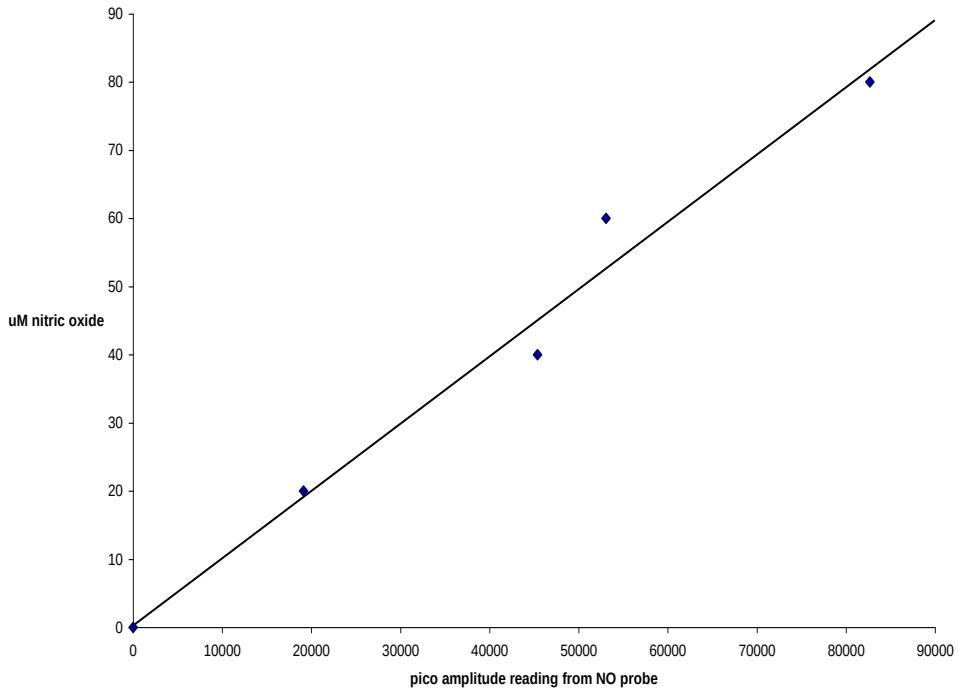


Figure 2.4: Standard calibration curve for NO release from nitrosothiols. Nitrite samples of known concentration were mixed with glutathione as standards. In the presence of excess glutathione all the nitrite is converted to nitrosothiols, which can then be measured through the release of NO. NO is released by the reduction of nitrosothiols by copper in an acidic environment. Since there is a linear relationship between pA and µM from known concentrations of nitrite standards, in the sample, unknown nitrosothiol concentrations can be calculated.

2.1.8 Ascorbic acid/total vitamin C analysis

2.1.8.1 Collection and preparation of samples

The aqueous phase of bench top model studies was sampled at times 0, 1, 2, 5, 10 and 15 minutes through a sampling port. The sample (100 µL) was added to 900 µL of dH_2O and split into two test tubes. Both test tubes contained 0.5 mL of 2% metaphosphoric acid/0.5% sulphamic acid and one of the test tubes also contained 6mg/ml dithiothreitol (DTT). The DTT was used to regenerate ascorbic acid from any dehydroascorbic acid in the sample, allowing quantification of total vitamin C levels. The sulphamic acid was added to remove any remaining nitrite.

Ascorbic acid (ASC) and total vitamin C (TVC) samples were immediately frozen in a -70 ^{o}C freezer after being obtained. Stored samples were analysed within 4 weeks. Prior to analysis, samples were thawed and centrifuged briefly at 1000 g, using a microcentrifuge (DJB Labcare Ltd, Buckinghamshire, UK).

2.1.8.2 Analysis of sample by HPLC

ASC and TVC levels were measured by high performance liquid chromatography (HPLC), employing an electrochemical detector. This method is based on a previously validated method for ascorbic acid measurement in gastric juice (43,143,221,222,236). The instruments comprised of an automated sample injector (Shimadzu SIL-10AD VP) 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 model C-R3A Chromatopac). ASC was separated using the reverse phase, ion pair chromatography on a Phenomenex 5µM C18 Luna 150 x 4.6 mm analytical column (Phenomenex, Macclesfield, UK) protected by a guard column (Anachem, UK, 20 x 3 mm) hand packed with Lichroprep RP-18 (25-40 µm, BDH-Merck Ltd, Poole, UK). A precolumn (Phenomenex Security guard C18 ODS Octadecyl filters 4 x 3 mm id) was also used. The mobile phase consisted of 0.1 M sodium acetate containing 0.1 M ocytlamine and 15% acetonitrile with a final pH of 4.3, adjusted with glacial acetic acid. The flow rate was 0.8mL/min and the retention time of ASC was approximately three minutes. An aqueous stock standard solution (1 mg/mL) was prepared by adding 5 mL of ASC stock solution to 5ml of DTT (3.5 mg/mL). Working standards 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 DTT solution to remove oxidising sites on the column. A blank sample of 1:1 metaphosphoric acid : sulphamic acid (MS) diluted 1 in 2 using dH_2O was processed for each run and subtracted from the measured ascorbic acid of the samples, as the MS solution had a positive ASC reading when analysed.

After obtaining a value for both total vitamin C and ASC, the dehydroascorbic acid could be calculated. All measurements were expressed in µg/mL.

2.1.9 Vitamin E analysis

The analysis of α-tocopherol which was used was based on a method previously described for non biological samples, using high performance liquid chromatography (HPLC) employing an electrochemical detector. The analysis with HPLC and electrochemical detection offers more sensitivity and selectivity compared with HPLC and UV-visible or fluorescent detection (237). The instruments comprised of an automated sample injector (Shimadzu SIL-10ADvp) with a 50µL loop, a pump (Shimadzu LC-10AT) and an electrochemical detector (Applied Biosystems 980 Programmable Fluorescent detector, Applied Biosystems, Warrington, UK) with excitation 295 nm and emission 340 nm.

A-tocopherol was separated from the rest of the sample using a Jones Spherisorb 5µ silica analytical column (25 x 4 mm) with a C18 guard. The mobile phase consisted of HPLC grade methanol. The flow rate was 0.8 mL/min, giving a column pressure of approximately 600psi and the retention time of α-tocopherol was approximately 4 minutes.

Stock solutions of α-tocopherol were used to generate a standard curve. 92mg of α-tocopherol was weighed out and dissolved in 100 mL methanol, giving a standard stock of 920 µg/m or 2.14mM. Before preparing the standard curve, the stock solution was diluted in methanol to final concentrations of 2140µM, 1070µM, 535µM, 268µM, 134µM, 67µM and 33µM. The autosampler was programmed to

inject 10µL of standards, quality control and samples. The autosampler dilutor solution used was filtered and degassed 50% methanol and distilled water.

Data collection and processing was via Viglen p5/166 Genie ATX and Jones Chromatography software (Jones Chromatogarphy Ltd, Mid Glamorgan, UK). Linear regression was carried out within the program. The quality control and unknown sample results were calculated from parameter values generated.

Using this method allowed separation of α and β-tocopherol individually but under the chromatographic conditions γ and δ-tocopherol could not be separated. In the experiments performed throughout this thesis, we were primarily interested in α-tocopherol and measurement of γ and δ tocopherol was not relevant.

2.1.10 *N*-nitrosamine measurement

Nitrosamine analysis has improved greatly over the past twenty years but nitrosamines remain technically difficult to analyse, due the range of chemical interactions that they can undergo. It is considered best for volatile nitrosamines to be measured by gas chromatography (GC) mass spectrometry (MS) and ideally by tandem mass spectrometry. Nitrosamines present several problems in terms of their analysis. In principle, any secondary nitrogen atom can be nitrosated, with the resulting formation of the corresponding *N*- nitrosocompound. The potential range of analytes is further increased by differences in the polarity, volatility, size and solubility of the molecule containing the *N*-nitroso moiety. The nitrosamines that we are interested in are the volatile nitrosamines, as they are more widely implicated in biological tissues.

Another problem is artefactual formation of the nitrosamine. If the sample contains artefactual nitrite, formation of the *N*-nitrosocompounds may occur during the analysis, as a result of secondary nitrogen compounds in the sample. It is therefore necessary, as a first step, to destroy the nitrite with a suitable agent. Sulphamic acid is suitable for this and forms a nitrogen gas on reacting with the nitrite. The sulphamic acid more rapidly destroys nitrite in the presence of an acidic pH and it is therefore important to make a solution of sulphamic acid that is acidic for this purpose. In our experiments we use a solution of 0.08 M HCl in combination with sulphamic acid and saturated NaCl solution.

2.1.10.2 *N*-nitrosamine extraction from aqueous phase

A 1 mL aqueous phase aliquot was added to 0.5 mL 0.08 M HCl and 5% sulphamic acid solution in saturated 6 M NaCl, in a 2 mL glass tube. This sample was then well mixed by inversion and 10 µL of the internal standard 0.01% NDBA was added. This sample was again mixed well before adding 0.5 mL of an extraction solvent mix (45: 55 v/v dichloromethane: diethylether). This sample was mixed and allowed to separate into two distinct layers. The density of the extraction solvent mix was 1.03. The density of dichloromethane on its own is such that separation of the aqueous phase from the solvent phase is difficult, therefore, diethylether is added. This meant that the solvent formed the upper layer, with the nitrosamine extracted into the solvent. The upper layer from this sample was removed by glass pipette and transferred to a tapered glass vial. The extraction was carried out twice. The sample was concentrated under a gentle stream of nitrogen, to concentrate the sample down to 100 µL. The glass vial was crimp capped and stored at -20^{o}C until further analysis.

2.1.10.3 Extraction of *N*-nitrosamine from lipid phase

A 500 µL sample was taken with a glass pipette from the lipid phase and 50 µL 0.01% NDBA was added as an internal standard. The lipid was then subjected to solid phase extraction, to remove the nitrosamines from the lipid tributyrin phase. The solid phase extraction process involved adding 1000 µL of dichloromethane to

the lipid phase. This was mixed and 500 µL of the lipid/dichloromethane mix was added to 1.5 mL of hexane before further mixing. A DSC-Diol solid-phase extraction cartridge (500 mg, 3 mL) (Phenomenex, Macclesfield, UK) was then conditioned with solvent before the lipid mix was eluted drop-wise into the column. The column was washed with 1 mL hexane. The nitrosamines were eluted into a glass vial using 2.5 mL methanol. The methanol nitrosamine mix was concentrated to 500 µL using 35^{o}C Gyro-vap (Howe, Banbury, UK) for 10 minutes. The glass vial was crimp capped and stored at -70^{o}C before analysis by tandem mass spectrometry. Only NPIP and NMOR were measured within the lipid phase due to difficulty with our solid phase extraction method for the more volatile nitrosamine NDMA.

2.1.10.4 *N*-nitrosamine analysis by gas chromatography tandem mass spectrometry

To measure *N*-nitrosamine concentrations, a gas chromatography ion trap tandem mass spectrometer, on a TRACE GC 2000 Series gas chromatograph (ThermoFinnigan, Hemel Hempstead, UK) interfaced to a Polaris Q mass spectrometer (ThermoFinnigan, Hemel Hempstead, UK), was used. On the mass spectrometer there was an AS2000 autosampler and programmable temperature vaporising (PTV) injector.

The *N*-nitrosamines within a sample were separated by chromatography on a ZB-5MS'fused silica capillary column, internal diameter 30m x 0.25mm, 0.5µm fill thickness (Phenomenex, Macclesfield, UK)). The oven temperature programme was as follows: 37^{o}C (held for 2 minutes) to 160^{o}C at 10^{o}C/min, to 250^{o}C at 30^{o}C/min (held for 2 minutes). Helium was used as a carrier gas, at a flow rate of 1ml/min. The PTV injector was programmed from 36^{o}C to 115^{o}C (held for 2 minutes) at 10^{o}C/min and a pressure of 22psi, prior to a cleaning phase at 325^{o}C (held for 12 minutes).

Acquisition and treatment of data obtained was performed on Xcalibur version 2 (ThermoFinnigan, Hemel Hempstead, UK). All samples were spiked with 10µL internal standard (0.01 NDMA), and tandem mass spectrometer spectra were calibrated with spiked standard *N*-nitrosamines solutions, covering concentrations from 0-70 µM. The response was linear for the concentrations studied.

A standard trace obtained for NDMA, NDEA, NDBA, NPIP and NMOR standards is shown in figure 2.5. The amount of *N*-nitrosamine present is shown by the area under the curve. It is from the area under the curve that calculations can be made of how much actual sample is present, adjusting for differences between individual nitrosamines. Calculations are also based on a correction factor for the internal standard. There is therefore both an internal and external standard.

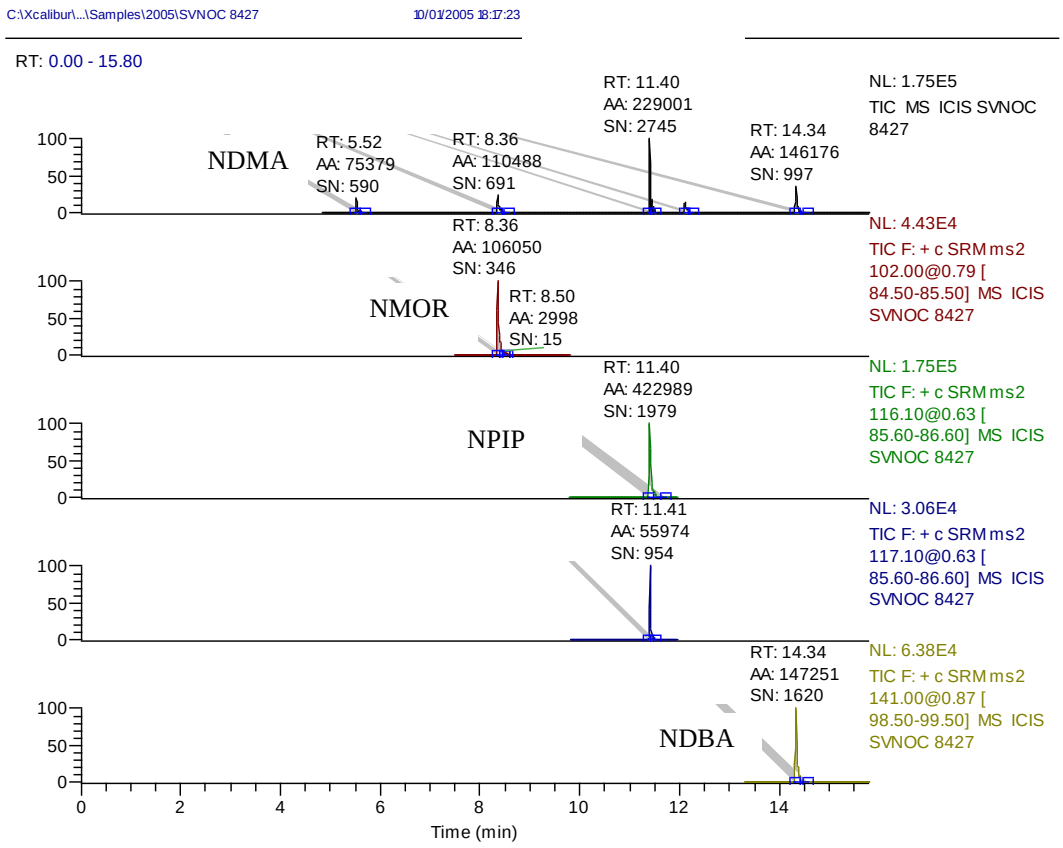


Figure 2.5: Chromatogram of GC-MS/MS for the *N*-nitrosamine standards. Due to different volatilities and mass of daughter compounds, the various nitrosamines have different retention times within the mass spectrometer and are detected at different times. Their abundance is shown on the y axis representing the signal intensity. (RT=retention time, AA=area under curve, SN=signal intensity).

2.2 Materials - Chemical Reagents Used

Sigma-Aldrich (Poole, UK)

Acetonitrile HPLC grade (15%), Ascorbic acid, Butylated hydroxytoluene (BHT), Copper nitrate ($Cu(NO3^-)_2$), Dichloromethane, Diethylether Flavin adenine dinucleotide (FAD), Glutathione Hexane, Hydrochloric acid, Dibasic potassium phosphate (K_2HPO_4), Metaphosphoric acid (2%), N 1-Naphthylethylenediamine dihydrochloride (NEDD), N-acetyl cysteine (NAC), Nitrate reductase, Octylamine (0.1M), Phosphate buffer solution (PBS), Phosphoric acid (H_3PO_4), Potassium iodide (KI), Pronase E from *Streptomyces griseus*, Sodium acetate, Sodium nitrate ($NaNO_3^-$), Sodium nitrite ($NaNO_2^-$), Sodium thiocyanate (NaSCN), Sulphamic acid (0.5%), Sulphanilamide, Sulphuric acid (H_2SO_4), Triacetin (Glyceryl triacetate), Tributyrin (Glyceryl tributyrate), α-tocopherol,β Nicotinamide dinucleotide phosphate (NADPH), β-carotene

Qmx Laboratories (Thaxted, UK)

N-nitrosodibutylamine, *N*-nitrosodiethylamine, *N*-nitrosodimethylamine, *N*-nitrosomorpholine, *N*-nitrosopiperidine

Bio-Rad (UK)

Bio-Rad Protein Assay Kit

BDH Ltd (Liverpool, UK)

EDTA

Rathburn Chemicals (UK)

Methanol (HPLC grade)

RA Lamb Ltd (Eastbourne, UK)

Whatman No1 paper

2.3 Statistical Methods

Using online statistical software, Smiths Statistical Package, median values with 1^{st} and 3^{rd} quartile values were obtained. Where appropriate, the data was analysed in this way to allow for inter individual variation to be shown without altering the statistical pattern of results. Statistical significance was determined using a 1-sample Wilcoxon test for paired data and Mann-Whitney *U* test for unpaired data.

Smith Statistical Package was also used to obtain mean values, standard deviation and analysis of variance (F Statistics for p value analysis).

Correlation analysis was performed using Mini Tabs statistical analysis software to determine Spearmans' rank correlation coefficient (p value) for non parametric rank testing and Pearson coefficient of correlation (r^2) for parametric testing.

Chapter 3 Nitrosamine formation in a single and two-phase model representing the healthy gastroesophageal junction

3.1 Introduction

The relationship between dietary nitrate and ascorbic acid induced nitrosative stress, at the gastroesophageal junction and cardia of the stomach, has been the subject of increasing research (43,44,144,221,238,239). It had previously been thought that nitrosative stress was inhibited within the upper gastrointestinal tract by ascorbic acid (240-242). This is explained by the antioxidant effect of ascorbic acid on nitrite, forming NO within the lumen. However, in the upper gastrointestinal tract there is both a luminal compartment and a lipid rich environment. There are two major sources of lipids: the first is the mucosa where there is both a lipid bilayer formed by epithelial cells, which also creates intraepithelial compartments, and there is also lipid within the cells. A high intracellular fat content is found in intestinal metaplastic epithelium (243), the same epithelium in which there is development of gastroesophageal junction cancers. Unlike normal gastric or oesophageal epithelium, intestinal metaplasic epithelium absorbs luminal fat, a process facilitated by bile salts (243). As well as lipids from the mucosa, it has been shown that lipids sit within the more proximal region of the stomach in the postprandial state (244). There are, therefore, two potential areas where lipid will alter the chemistry within the upper gastrointestinal tract.

The aim of this study was to generate a model to simulate the chemistry at the gastroesophageal junction and cardia of the stomach, that allowed the addition of a lipid phase, to examine the effect this had on the nitrosative chemistry. The model prepared would also allow chemical manipulation of the lipid phase.

3.2 Experimental design

3.2.1 Bench Top Model

A bench top model was designed to enable us to study the influence of lipid on the nitrite chemistry occurring when nitrite swallowed in saliva encounters gastric acid (figure 3.1). The model consisted of a 250 mL specially designed glass beaker containing 50 mL HCl pH 1.5 representing the stomach. The pH of 1.5 was chosen as it has previously been shown that this is the pH in the post prandial situation in the proximal region of the stomach (245). NaSCN (1mM) was also added to the gastric compartment, representing its physiological concentration in gastric juice. EDTA (1mM) was added to chelate metals known to be present in chemical reagents. Secondary amines were added to the aqueous solution to allow us to monitor *N*-nitrosation, by measuring the generation of *N*-nitrosamines. The secondary amines used were one of morpholine (5mM), piperidine (5mM) or dimethylamine (5mM). Experiments were performed with and without ascorbic acid (1mM).

To investigate the influence of lipid, studies were undertaken with and without lipid added to the acidic aqueous solution. Two different lipids were used, tributyrin (glyceryl tributyrate) and triacetin (glyceryl triacetate). These two lipids both have a higher density than water and sink, forming a layer in the case of triacetin, and a globule in the case of tributyrin, in the bottom of the beaker. The lipid was added by slowly injecting the lipid out of a syringe with a 21 gauge needle

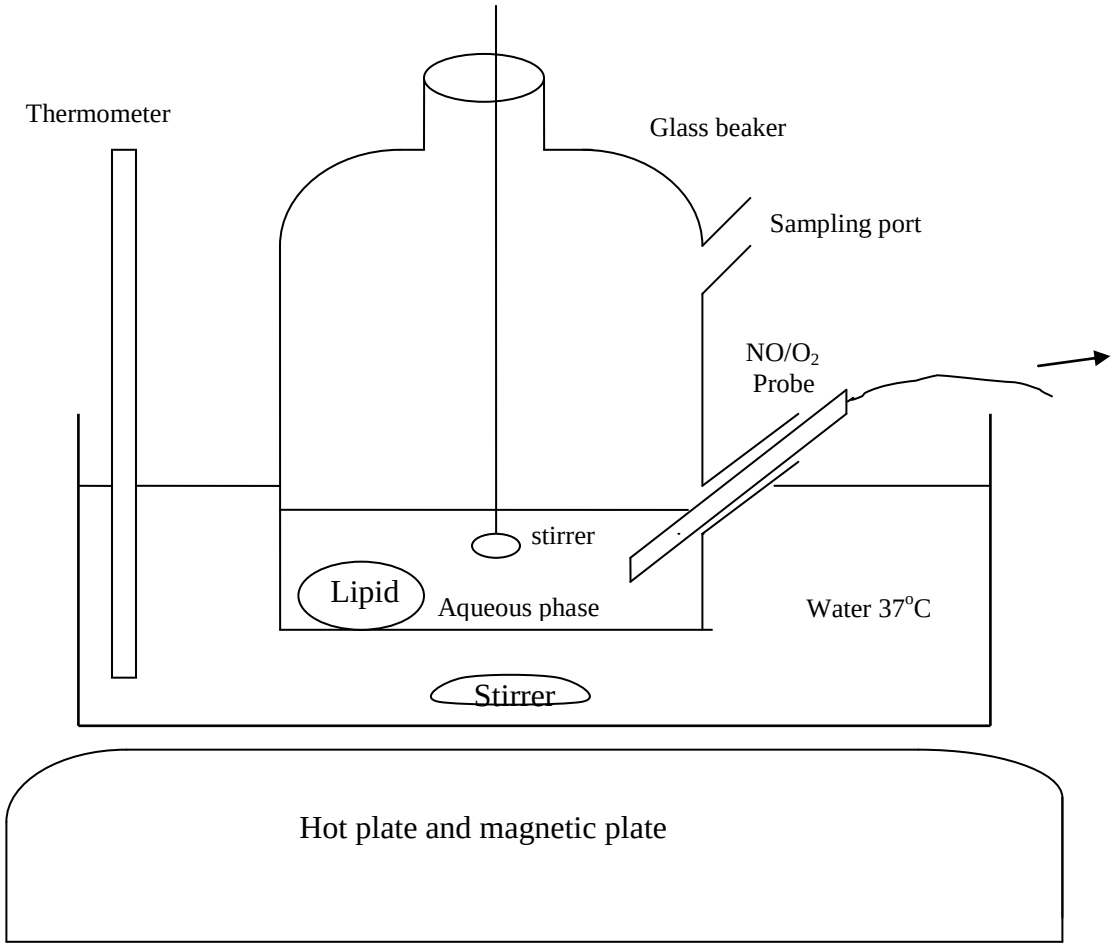


Figure 3.1: Bench top model used to represent the gastroesophageal junction in single and two-phase models. The aqueous phase total volume was 50 mL, consisting of HCl (pH 1.5), NaSCN (1 mM), EDTA (1 mM), secondary amine (5 mM) and $NaNO_2$ (range of concentrations) ± ascorbic acid (1 mM). To create a two-phase model a lipid (tributyrin or triacetin) was added.

(outer diameter 0.813mm, internal diameter 0.495mm) into the bottom of the aqueous phase. Varying volumes of lipid were added, giving ratios of aqueous to lipid ranging from 50:1 to 5:1. The majority of the studies were performed with a ratio of 10:1 aqueous to lipid, as this is most likely to represent the luminal conditions of the upper part of the stomach. It is known that 20-30% of solid ingested food is present as a form of fat. This will also be diluted by gastric juices, hence the reason we chose 10% as a percentage of fat to other contents in the model stomach. Secondary amines were also added to the lipid phase in some of the experiments. The three secondary amines (morpholine, piperidine or dimethylamine), were added to the lipid phase, at a concentration of 5mM. The experiments were all performed at 37^{o}C, for a time period of 15 or 60 minutes. The contents of the beaker were constantly stirred using a magnetic stirrer suspended in the aqueous phase. The experiments were initiated by adding $NaNO_2$ (100 µM).

Throughout the study dissolved O_2 and NO concentrations were monitored in the aqueous phase, using the probes and sensors as described in Chapter 2.1.1 and 2.1.2. Samples (100 µL) were obtained at 0, 1, 2, 5, 10, and 15 minutes after adding nitrite, for determination of ascorbic acid and total vitamin C. At the end of the 15 minute experiment, a 1 mL aqueous sample was removed for *N*-nitrosamine measurement. A 500 µL sample of the lipid phase with ascorbic acid present, from experiments involving piperidine and morpholine, was taken at 15 minutes for analysis of the nitrosamine content in the lipid phase. In experiments lasting 60 minutes a 500 µL aqueous sample was taken at times 15 and 60 minutes.

3.2.2 Increasing the surface area of the lipid phase

As well as having the tributyrin lipid phase present as only one single globule at the bottom of the beaker, a model was also created where several globules of tributyrin were present. Tributyrin was injected into the aqueous phase using a needle and syringe. In order to create different globules the injection had to be started and stopped such that the 5 mL volume was separated into four equal parts of 1.25 mL. These four globules then had to remain distinct and not coalesce during the 15 minutes over which the experiment was run for the results to be recorded. NO and O_2 concentrations were monitored and samples were taken for ascorbic acid and *N*-nitrosamine, as previously described.

3.2.3 Triacetin as the lipid phase

Experiments were performed using the lipid triacetin as a substitute for tributyrin. Like tributyrin, triacetin has a higher density (d=1.16 g/mL at 25^{o}C)) than water and therefore sinks into the aqueous phase. A difference with triacetin is, that rather than forming a globule, it sinks to the bottom of the beaker model to form a bottom layer. Experiments were then performed as for tributyrin, although we were unable to form globules with this lipid.

3.3 Results

3.3.1 Studies in aqueous solution with no lipid (single phase model)

3.3.1.1 In the absence of ascorbic acid

When nitrite was added to HCl pH 1.5 containing thiocyanate (1mM) but without ascorbic acid, nitrosation of the secondary amines was observed. After 15 minutes 6.71 (±0.82) µM NMOR, 0.014 (±0.005) µM NPIP and 0.58 (±0.21) µM NDMA were present (figure 3.2). In these experiments, the addition of nitrite produced only a very small increase in the concentration of NO. This increase was small and steady throughout the 15 minutes of the experiment, to a maximal concentration of 2 µM (figure 3.3). There was only a small decline over time of the O_2 concentration from 180 µM to 175 µM (figure 3.3).

3.3.1.2 In the presence of ascorbic acid

The presence of ascorbic acid markedly inhibited the nitrosation of each of the secondary amines. The concentration of each *N*-nitrosamine formed was significantly less for each amine (figure 3.2). NMOR and NDMA concentrations were just over 5% of the total found in the absence of ascorbic acid, with mean values of 0.37 (±0.08) µM and 0.03 (±0.01) µM, while the concentration for NPIP was only 0.01 (±0.005) µM compared to 0.014 (±0.005) µM.

In the presence of ascorbic acid (1mM), the addition of nitrite produced a rapid increase, followed by slow decline in NO concentrations. Within 60 seconds of adding nitrite (100µM) the dissolved NO concentration was approximately 80µM. This rapid rise in NO was followed by a rapid fall in the dissolved O_2 concentration. Within 60 seconds of adding the nitrite, no dissolved O_2 was detected until 10 minutes later, when the concentration slowly increased to reach 5–10% of its maximal concentration (figure 3.4).

The addition of nitrite also produced a fall in the ascorbic acid concentration. It rapidly fell from its original concentration of 970 (± 75) µM to 580 (±40) µM at 2 minutes and then slowly fell to 480 (± 40) µM at 15 minutes (Figure 3.5).

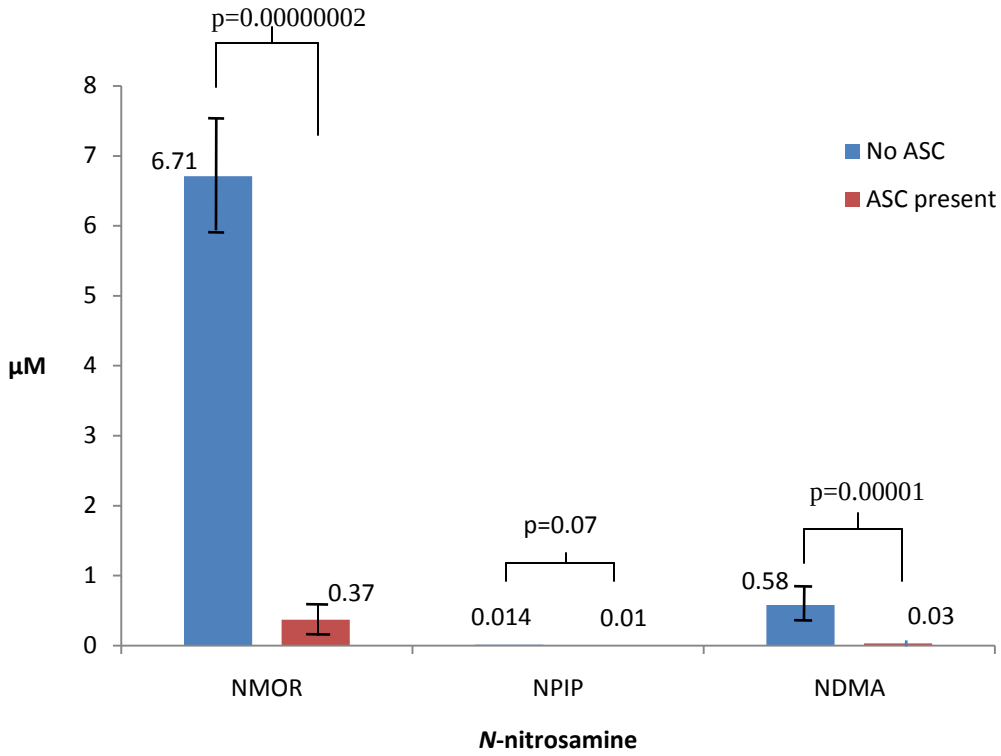


Figure 3.2: *N*-nitrosamine (µM) produced in a single phase system, both in the presence (red column) and absence (blue column) of ascorbic acid (1 mM), for the three secondary amines morpholine, piperidine and dimethylamine, after the addition of nitrite (100 µM). Data labels show mean nitrosamine concentration. Error bars represent 1 standard deviation. (n=18)

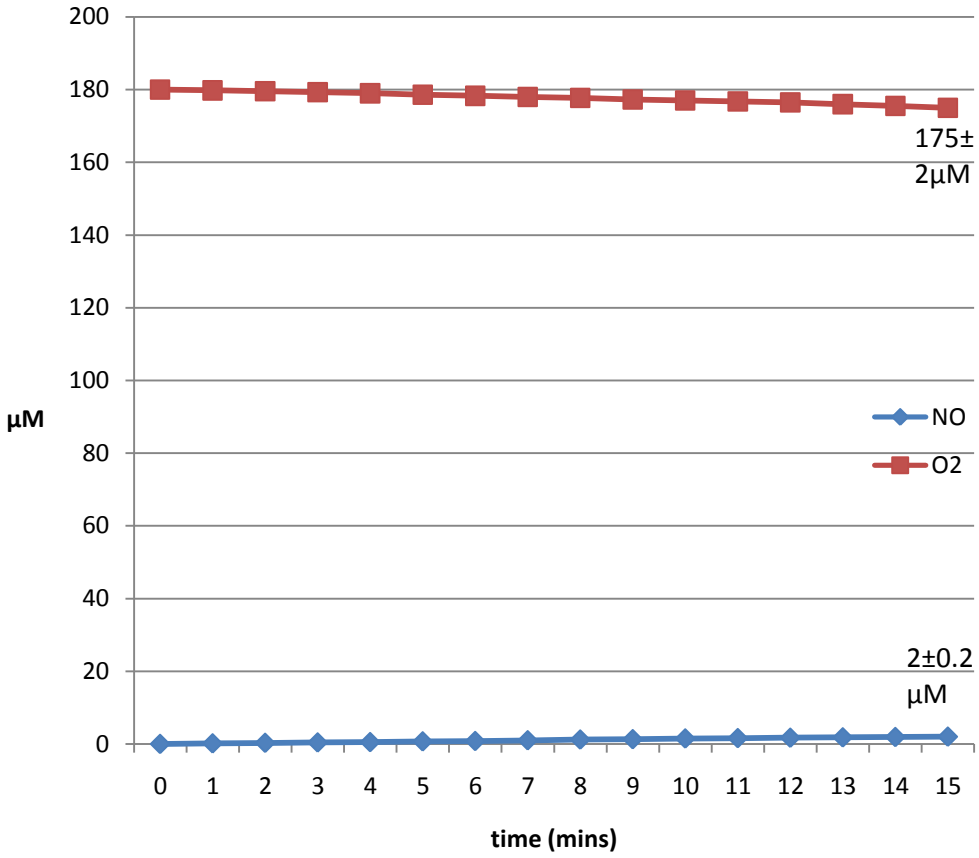


Figure 3.3: Mean NO (blue line with diamond markers) and O_2 concentrations (red line with square markers) in a single phase system, after the addition of nitrite (100µM) over 15 minutes (n=18)

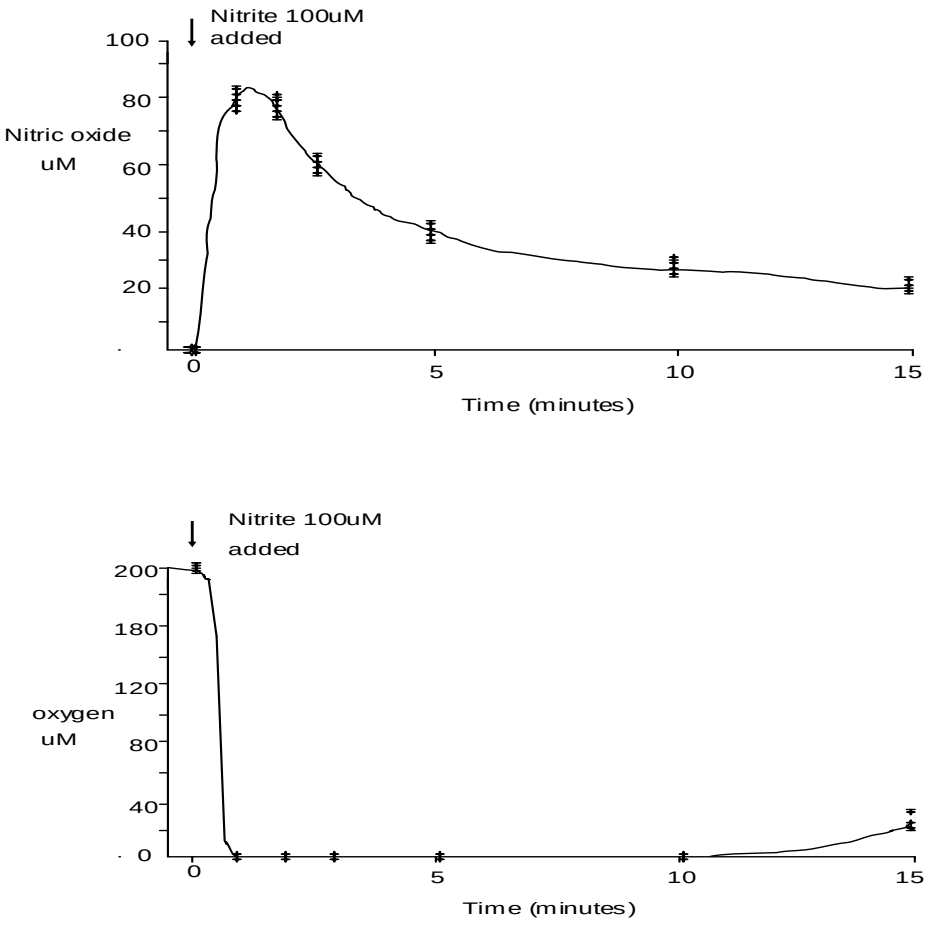


Figure 3.4: Aqueous NO and aqueous O_2 concentrations in a two-phase model representing the chemistry occurring at the gastroesophageal junction, after the addition of sodium nitrite (100 µM). The acidic aqueous phase contained the aqueous antioxidant ascorbic acid to an excess concentration of 1000 µM. The experiment was performed over 15 minutes. (n=18)

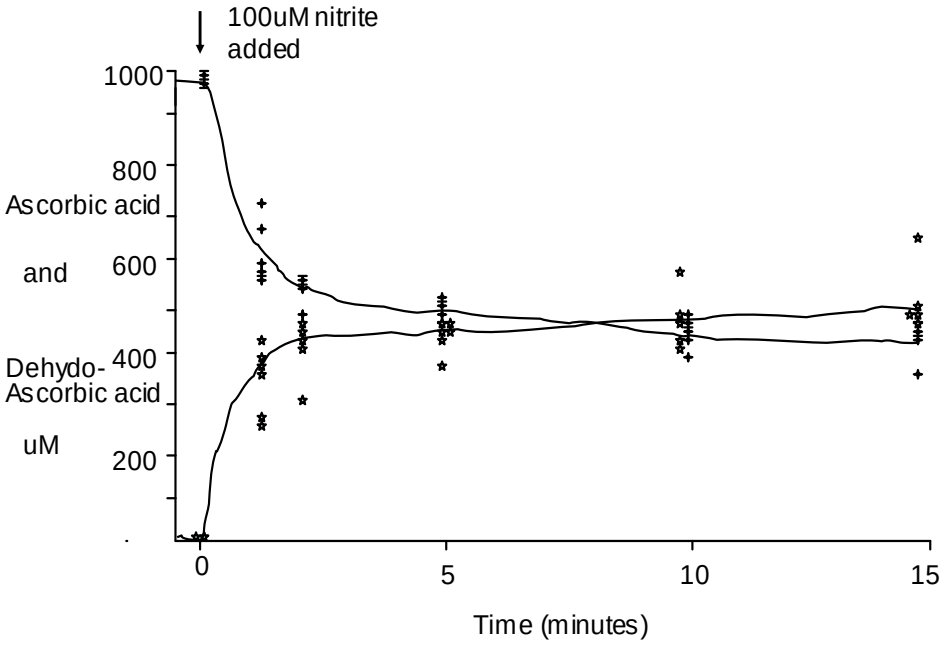


Figure 3.5: Ascorbic acid and dehydroascorbic acid concentrations in a two-phase model representing the chemistry occurring at the gastroesophageal junction after the addition of sodium nitrite (100 µM). The acidic aqueous phase contained the aqueous antioxidant ascorbic acid, to an excess concentration of 1000 µM, at initiation of the experiment. The experiment was performed over 15 minutes. (n=18)

3.3.2 Studies in aqueous solution containing 10% tributyrin globule (two-phase model)

3.3.2.1 Aqueous phase in absence of ascorbic acid with amine in both phases (two-phase model)

When nitrite was added to HCl pH 1.5 containing thiocyanate (1 mM), no ascorbic acid and a 10% globule of tributyrin, nitrosation of the secondary amines was observed. The concentrations generated were different from those found in the single phase study in the absence of ascorbic acid ($p<0.001$) although the same pattern was seen. Aqueous phase mean concentrations observed were 4.33 (±0.8) µM NMOR, 0.19 (±0.07) µM NPIP and 0.64 (±0.29) µM NDMA (figure 3.6).

3.3.2.2 Aqueous phase in presence of ascorbic acid with amine in both phases (two-phase model)

In marked contrast to the observations in the absence of the lipid globule, the addition of ascorbic acid did not inhibit the generation of *N*-nitrosamines in the presence of the lipid globule. Indeed, in the presence of the lipid globule, the ascorbic acid actually increased the concentration of *N*-nitrosamine formed. The degree to which this happened varied with the different secondary amines. The concentration of NMOR formed in the absence of ascorbic acid (4.82 µM ± 0.60 µM) was only slightly, and not significantly, higher than that in the absence of

ascorbic acid. However, the concentration of NDMA formed in the presence of ascorbic acid (2.18 µM ± 0.31 µM) was approximately four fold higher than in the absence of ascorbic acid. The most dramatic effect occurred with piperidine, where the concentration of NPIP formed in the presence of ascorbic acid (6.15 µM ± 0.54 µM) was thirty-two fold higher than in the absence of ascorbic acid (figure 3.6).

In the presence of lipid, the addition of nitrite produced similar changes in the concentrations of NO, O_2 and ascorbic acid to those seen in the absence of lipid.

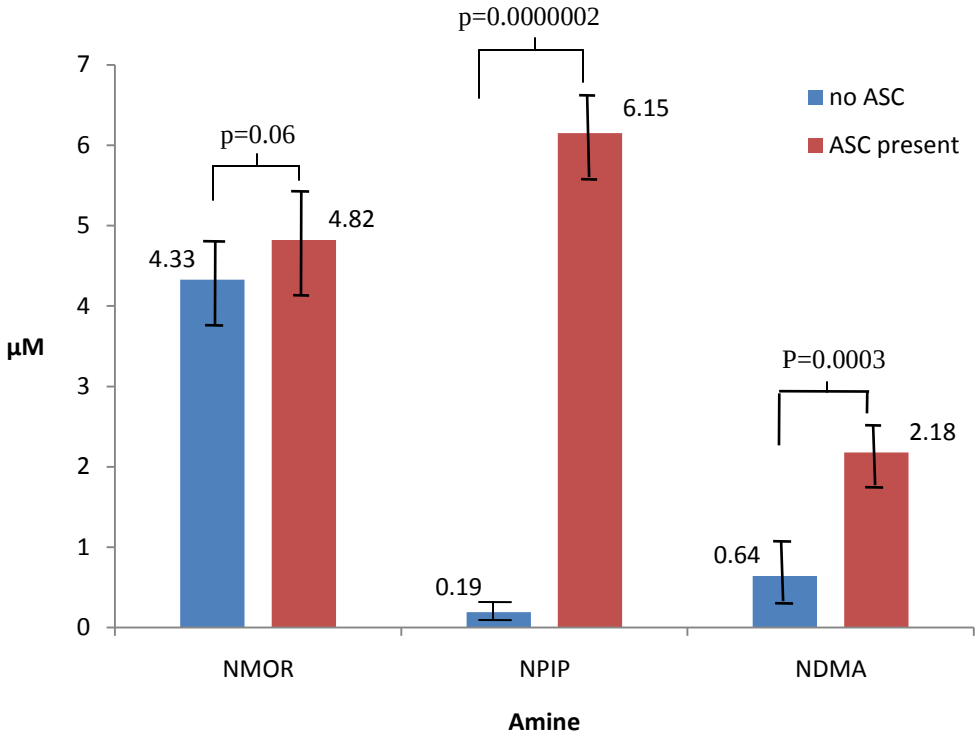


Figure 3.6: Aqueous *N*-nitrosamine (µM) produced in a two-phase system, both in the presence (red column) and absence (blue column) of ascorbic acid, for the three secondary amines morpholine (MOR), piperidine (PIP) and dimethylamine (DMA), after the addition of 100µM nitrite. The second phase is formed by the lipid tributyrin in a ratio of 1:10 with the aqueous phase volume. Error bars represent 1 standard deviation. (n=18)

3.3.2.3 Aqueous phase *N*-nitrosamine in absence of ascorbic acid with amine (5mM) in aqueous phase only (two-phase model)

The concentrations of *N*-nitrosamine generated in the aqueous phase were not significantly lower for NMOR and NDMA, than when the amine was present in both phases in the absence of ascorbic acid. The mean concentrations formed for the individual nitrosamines were 3.90 (±0.42) µM NMOR and 0.41 (±0.15) µM NDMA. This compares to 4.33 (±0.8) µM for NMOR and 0.64 (±0.29) µM for NDMA when amine was present in both phases. However, there was significantly less NPIP when the amine was present only in the aqueous phase as compared to when it was in both phases. The mean NPIP concentration when piperdine was present ony in the aqueous phase was 0.013 (±0.02) µM compared to 0.19 (±0.07) µM when piperidine was present in both phases ($p=0.0003$).

3.3.2.4 Aqueous phase *N*-nitrosamine in absence of ascorbic acid with amine in lipid phase only (two-phase model)

The concentrations of NMOR and NDMA were nearly half those seen when amine was present in both phases in the absence of ascorbic acid. The mean concentrations of NMOR and NDMA generated were 2.64 (±0.37) µM and 0.31 (±0.15) µM respectively. The mean concentration of NPIP was not statistically different from those seen when amine was in both phases in the absence of ascorbic acid, with a mean concentration of 0.24 (±0.09) µM.

3.3.2.5 Aqueous phase *N*-nitrosamine in presence of ascorbic acid with amine in aqueous phase only (two-phase model)

There was a dramatic reduction in the nitrosamines formed in this system with amine present in the aqueous phase only, as compared to the same model but with amine present in both phases. The mean NMOR concentration in the aqueous phase at 15 minutes was only 0.08 (±0.01) µM, the lowest concentration of NMOR found in any of the experiments. Mean NPIP and NDMA concentrations were also very low, with mean values of only 0.01 (±0.01) µM and 0.02 (±0.01) µM generated within this system, suggesting little partitioning of these amines in that time period.

3.3.2.6 Aqueous phase *N*-nitrosamine in presence of ascorbic acid with amine in lipid phase only (two-phase model)

The concentrations in these experiments were the closest of any where the amine was omitted from one phase compared to when amine was present in both. The mean concentrations of NMOR, NPIP and DMA in this model were not significantly different when compared to concentrations in a two-phase model in the presence of ascorbic acid with amine present in both phases. The mean concentrations were 4.20 (±0.66) µM, 6.43 (±0.85) µM and 2.12 (±0.58) µM for NMOR, NPIP and NDMA respectively.

3.3.2.7 Lipid phase in the presence of ascorbic acid (1mM) with amine in both phases (two-phase model)

Measurement of *N*-nitrosamine concentration was undertaken in two-phase 15 minute experiments, where ascorbic acid (1mM) was present, fifteen minutes after the addition of sodium nitrite (100µM). *N*-nitrosamines measured in the lipid phase were present in higher concentrations than in the corresponding aqueous phase. The mean NMOR present in lipid was 11.5 fold higher (55.3 ± 6.7µM) and NPIP 17.8 fold higher (109.7 ± 17.5µM) than that of the aqueous phase with amine present in both phases in the presence of ascorbic acid (1mM).

3.3.3 Aqueous phase *N*-nitrosamine in two-phase studies wth ascorbic acid present with prolonged time period

When experiments were allowed to run for 60 minutes rather than just 15 minutes there was a significant increase in *N*-nitrosamine production. The concentration increase however, was not proportional to the increase in time. There was just over double the concentration of NMOR and NPIP when the time interval was increased four fold. The mean NMOR concentration at 60 minutes was 10.14 (±1.12) µM compared to 4.82 (±0.6) µM after 15 minutes. The mean NPIP concentration increased from 6.15 (±0.5) µM at 15 minutes to 14.02 (±1.84) µM at 60 minutes. During this time NO and O_2 were both still present, as was ascorbic acid. The NO concentration gradually fell to approximately 15µM, while O_2 concentrations were 100 µM at the end of the 60 minute experiment. Ascorbic acid continued to be gradually consumed but there was still 220 (±36) µM present at the end of the 60 minute experiment.

3.3.4 Two-phase studies with different volumes of tributyrin in the presence of ascorbic acid with amine in both phases

Increasing the ratio of the lipid phase tributyrin to the acidic aqueous phase increased the concentration of *N*-nitrosamine produced in the aqueous phase at 15 minutes. For all three of the *N*-nitrosamines examined, there was a non linear relationship between the volume increase in tributyrin and concentration of *N*-nitrosamine produced. For NMOR, the mean concentration produced increased from 2.03 (±0.5) µM with a ratio of 1:50 to 4.82 (±0.6) µM with a ratio of 1:10. The concentration of NMOR produced was greater again when a ratio of 1:5 was used with 8.74 (±0.72) µM produced giving over a four fold increase in NMOR production when increasing the lipid volume ten fold. There were similar percentage increases in NPIP and NDMA concentrations with increasing tributyrin to aqueous ratios. NPIP concentrations increased from 2.75 (±0.30) µM to 6.15 (±0.51) µM when the tributyrin ratio was increased from 1:50 to 1:10 and to 10.26 (±0.52) µM when the ratio was increased further to 1:5. The rise in concentration of NDMA with increasing volumes of tributyrin followed a similar pattern, with concentrations 0.80 (±0.29) µM, 2.18 (±0.0.31) µM and 3.82 (±0.57) µM NDMA in experiments with tributyrin to aqueous ratios of 1:50, 1:10 and 1:5 respectively (figure 3.7).

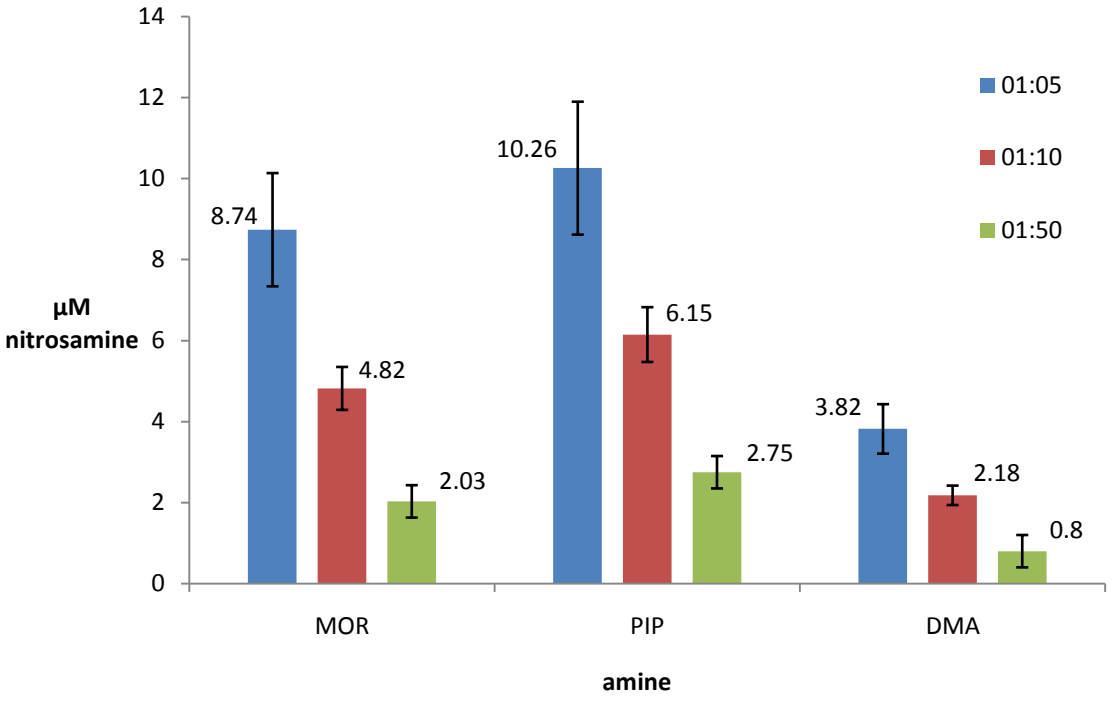


Figure 3.7: *N*-nitrosamine production with ascorbic acid present in a two-phase system with different ratios of lipid to aqueous phase. The ratio was altered by adjusting the volume of lipid placed in the aqueous solution. The three ratios assessed were 1:5 (blue column), 1:10 (red column) and 1:50 (green column). The aqueous solution remained at a fixed 50mls. One of three secondary amines (5mM) was present in both aqueous and lipid phases: morpholine (MOR), piperidine (PIP) or dimethylamine (DMA). The corresponding *N*-nitrosamine concentration was measured from the aqueous phase. The mean values are annotated and the error bars represent 1 standard deviation. (n=12)

3.3.5 Two-phase studies with varying surface area of lipid in the presence of ascorbic acid with amine in both phases

With the greater surface area provided by the division of the volume of tributyrin into smaller globules, there was a significant increase in the aqueous concentration of *N*-nitrosamine produced. For both NMOR and NPIP there was a more than two fold increase in the production of the nitrosamine. The mean NMOR concentration was increased from 4.82 (±0.60) μM when a single lipid globule was present to 9.93 (±0.72) μM when the same total volume of tributyrin was divided into four globules, in a 15 minute experiment. A similarly significant increase in mean NPIP concentration was observed with 13.25 (±1.11) μM NPIP formed when 4 globules were present compared to 6.15 (±0.54) μM with a single globule (figure 3.8).

There was no measurable statistically significant difference in NO and O_2 concentration within the aqueous phase in the presence of four globules of tributyrin compared to a single globule (data not shown).

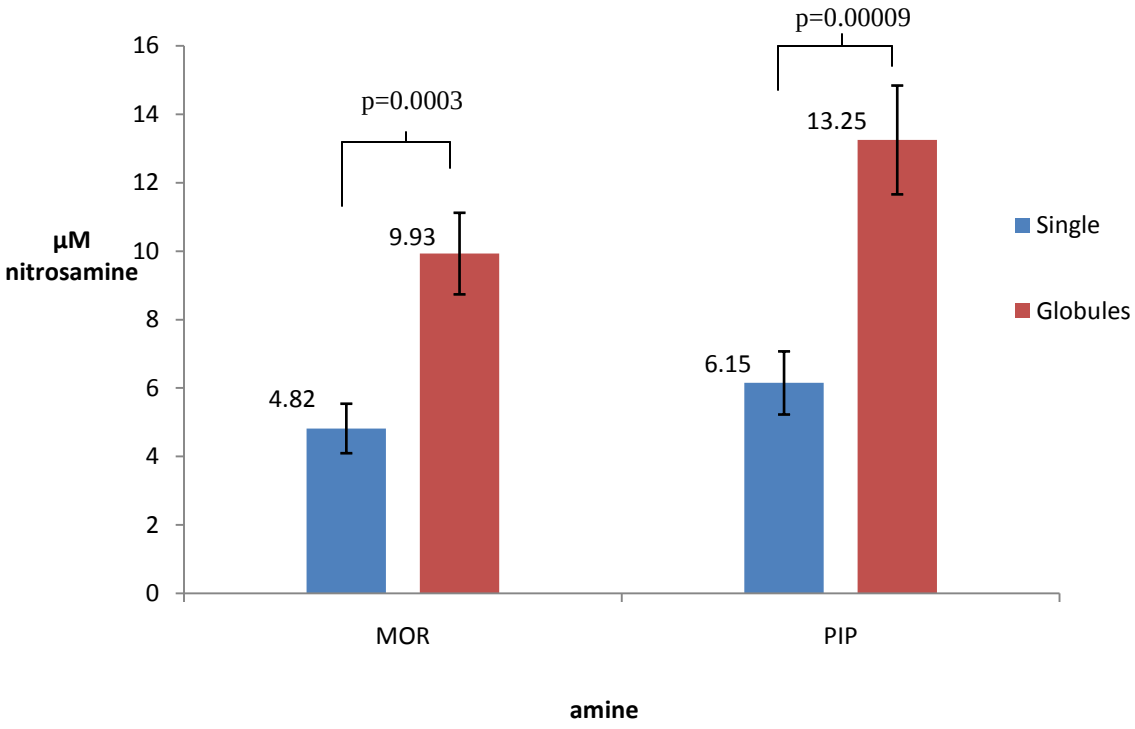


Figure 3.8: Mean *N*-nitrosamine concentrations (µM) from two-phase model experiments where the lipid phase is either a single globule (blue column), or the tributyrin is divided into four globules (red column). These experiments were performed for two secondary amines, morpholine (MOR) and piperidine (PIP), with the corresponding nitrosamine measured from the aqueous phase. Experiments were run over 15 minutes after the addition of 100µM nitrite to an acidic aqueous phase containing 1mM ascorbic acid. Error bars represent 1 standard deviation. (n=12)

3.3.6 Triacetin as the lipid phase

In two-phase studies where the lipid triacetin was used in place of tributyrin as the lipid phase, very similar results were observed. In the absence of ascorbic acid, mean concentrations of NMOR, NPIP and NDMA in the aqueous phase were 4.13 (0.30) µM, 0.11 (0.02) µM and 0.42 (0.17) µM. In the presence of excess ascorbic acid there was a mean NMOR concentration of 4.21 (0.44) µM, mean NPIP concentration of 5.28 (0.63) µM and a mean NDMA concentration of 1.84 (0.28) µM, 15 minutes after the addition of 100 µM nitrite. These results are not statistically different from the results from experiments using tributyrin as the lipid phase.

NO, O_2 and ascorbic concentrations were all comparable in experiments where triacetin was used in place of tributyrin (data not shown).

3.4 Discussion

These studies indicate that the presence of lipid profoundly alters the nitrosative chemistry in the bench top model. The lipid is able to convert the influence of ascorbic acid from one that protects against nitrosation to one that promotes it. This effect is due to the ability of the NO formed by the ascorbic acid within the aqueous phase to regenerate nitrosative species within the lipid.

In the studies performed without lipid, and without ascorbic acid, the addition of nitrite to HCl pH 1.5 containing thiocyanate resulted in nitrosation of the three secondary amines. The main nitrosating species formed under these conditions is NOSCN (137,223,246). It reacts with the secondary amine, in its unprotonated, uncharged state (247). The amount of the secondary amine, in its nitrosatable form, depends upon its –log dissociation constant (pK_a). The pK_a of the amines studied is 8.33 for morpholine, 10.68 for dimethylamine and 11.123 for piperidine. The amount of the secondary amine in a form available for nitrosation by NOSCN will, therefore, be greatest for morpholine and least for piperidine. The differences in pK_a can partly explain why different concentrations of the three *N*-nitrosamines were generated.

In the above experiments performed in the absence of lipid, the addition of ascorbic acid effectively prevented the nitrosation of the three amines. This can be explained by the ascorbic acid competing with the secondary amines for the NOSCN

(239,240). In the reaction between ascorbic acid and NOSCN, the latter is reduced to NO and the former oxidised to dehydroascorbic acid. Consistent with this, we observed a rapid rise in the concentration of NO and a fall in the ascorbic acid concentration. There was also a rapid fall in the O_2 concentration. This can be explained by the NO reacting with dissolved O_2 to form N_2O_3 (248,249). The N_2O_3 formed in this way is a nitrosating species and again preferentially reacts with ascorbic acid and is reduced back to NO (243). This recycling continues until either the O_2 or the ascorbic acid is consumed. Under our experimental conditions the O_2 was the first to be depleted. Stoichemically one mole of ascorbic acid can reduce two moles of nitrite to NO. The greater consumption of ascorbic acid observed is due to this recycling of NO.

In the presence of the lipid, nitrosamines were formed despite the presence of ascorbic acid. Indeed, the concentrations of the nitrosamines formed were higher in the experiments with lipid and ascorbic acid than experiments without lipid and without ascorbic acid. This was particularly the case for NPIP, where the concentrations formed in the experiments with ascorbic acid and lipid were 440 (6.15 μM in the presence of lipid versus 0.014μM in the absence of lipid) times higher than those without lipid and without ascorbic acid. Though the presence of lipid markedly altered the amount of the nitrosamine formed in the experiments with ascorbic acid, it did not alter the changes in the concentrations of NO, O_2 or ascorbic acid.

What is the explanation for the ability of the lipid to convert the effect of the ascorbic acid from being an inhibitor to a promoter of nitrosation? The effect observed is likely to be mediated by the NO produced by the reaction between the ascorbic acid and the nitrosating species. In the absence of the lipid this NO can react with O_2 to form the nitrosating species N_2O_3 (248,249). However, in the aqueous phase the N_2O_3 formed in this way does not nitrosate the secondary amines as it preferentially reacts with the ascorbic acid which reduces it back to NO (243). The ascorbic acid, in the aqueous phase, thus protects against nitrosation by both the nitrosating species NOSCN, formed by the acidification of nitrite in the presence of the thiocyanate, and by N_2O_3 formed by the NO (produced by the reaction between NOSCN and ascorbic acid) and O_2 (250). However, when a globule of lipid is present, the NO, formed by the reaction between the nitrosating species and ascorbic acid, diffuses into the lipid and within it reacts with O_2 to form N_2O_3. As ascorbic acid is not lipid soluble, it will be unable to enter the lipid and, thus, the N_2O_3 generated within the lipid will be able to nitrosate the secondary amines within the lipid. The *N*-nitrosamines generated within the lipid in this way will then diffuse out of the lipid and produce the rise in their concentration observed in the aqueous compartment.

The potential of NO to generate nitrosating species by reacting with O_2 was recognised many years ago. More recently the potential for this reaction to occur preferentially within lipids was recognised. Liu et al (251) demonstrated that the

reaction between NO and O_2 was 300 times faster in lipid than in aqueous solutions and that this was due to the increased solubility of both gases in lipids.

These studies also indicated that the relative extent to which the lipid promoted nitrosation varied between the different secondary amines studied. In the absence of lipid and absence of ascorbic acid, morpholine was nitrosated to a greater extent than dimethylamine and the latter more than piperidine. As discussed above, this can be explained by their different pK_a and, consequently, the proportion present in protonated form, with the resulting susceptibility to nitrosation by NOSCN and N_2O_3. However, in the studies with lipid and with ascorbic acid, piperidine was nitrosated more than morpholine and the latter more than dimethylamine. Previous studies have shown that this nitrosation of secondary amines in organic solvents is unrelated to their pK_a (253). The relative lipid solubility of both the amine and N-nitrosamine may explain the results observed. Piperidine is the most hydrophobic and therefore more of the secondary amine is likely to accumulate in the lipid phase, compared to morpholine and dimethylamine (252).

The importance of the nitrosation occurring within the lipid can be seen in the two-phase experiments, where the availability of the secondary amine was changed from being in both phases to being present in one or other of the lipid or aqueous phase. When the amine was present in the lipid phase only, in the presence of ascorbic acid, the concentrations of the three *N*-nitrosamines produced was not significantly different from those observed when amine was in both phases of the

model. When the amine was present only in aqueous phase in the presence of ascorbic acid the concentrations of the *N*-nitrosamines were only just detectable. This also shows that the partitioning of these amines into the lipid phase does not occur, as these experiments took place over 15 minutes. If there had been more rapid partitioning of amine into the lipid phase, then it could be expected that more amine would have been nitrosated and hence detectable after 15 minutes.

These studies also show that changing the surface area and therefore the interface between the aqueous and lipid phase, has an effect on the concentration of *N*-nitrosamine generated. By simply increasing the surface area, by splitting one lipid globule into four, around double the concentration of N-nitrosamine was generated. This suggests that the increased surface area allows more NO to diffuse over and also increases the diffusion back into the aqueous phase of generated *N*-nitrosamine, dependent on its partition co-efficient. This has relevance for the situation we would expect to find *in vivo*. There are two potential situations in the upper gastrointestinal tract where this lipid chemistry is important. Firstly, with regard to ingested fat in foodstuffs (244), and secondly in relation to lipid within the surrounding epithelial cells (243). As regards ingested fat, it is unlikely that all of this will sit as one globule within the proximal stomach but rather would be expected to form a larger surface area by being split up. The other area of lipid is within the epithelial layer of cells in the gastric cardia and the gastroesophageal junction. The surface area here is potentially very large, giving more in the way of an interface between the aqueous and lipid phases, thus allowing increased NO diffusion into the

epithelial cells. The ratio of lipid to aqueous phase also makes a difference to the generation of *N*-nitrosamine. This may simply be due to the larger surface area of the globule resulting from the increased volume since the increase in total volume is not matched by a proportionate increase in the *N*-nitrosamine generated. These studies highlight the importance of the interface between the lipid and aqueous phase for the nitrosative chemistry occurring.

The explanation of why the presence of lipid lowers the concentration of NMOR found at 15 minutes is not entirely clear. In these two-phase model studies in the presence of ascorbic acid, where morpholine is replaced by piperidine, the concentration of piperidine is slightly higher than in a single phase model, while when dimethylamine is substituted as the amine there is no statistical difference. It would be attractive to suggest from these studies that NMOR has a higher affinity for lipid than the other two *N*-nitrosamines. However, NMOR has a low lipid solubility while the the lipid solubility of NPIP is the highest of the three N-nitrosamines used in this study (ClogP 0.73 compared with ClogP of -.033 for NMOR and 0.01 for NDMA). It is therefore not clear why the NMOR present in the aqueous phase is significantly lower in the absence of ascorbic acid in a two-phase model compared to a single phase model. Differences in partitioning of the amines and *N*-nitrosamines between the lipid and aqueous phase may contribute to the differences. It is important to note that partition coefficients into a lipid may not fully represent what will happen *in vivo*. This is because, for small nonelectrolytes, Overton's rule of permeability coefficients correlating well with oil/water

coefficients cannot be applied to lipid bilayer membranes. The membrane behaves more like a polymer than a liquid hydrocarbon (254).

Within the lipid phase of two-phase studies, the measured NMOR and NPIP were significantly higher than the concentrations found in the aqueous phase, in the presence of ascorbic acid. The mean concentration of lipid NMOR was 11.5 times higher than aqueous concentrations and mean NPIP lipid concentration was 17.8 times higher than aqueous There were technical difficulties measuring NDMA using the methods described above. However, further published work shows that NDMA concentrations are also higher in the lipid phase as compared to the aqueous phase (255) when a suitable method of measuring NDMA within the lipid was used. This once again suggests that the generation of *N*-nitrosamines is occurring within the lipid layer. The proposed mechanism for this is shown in figure 3.9. The higher percentage increase in NPIP formation over NMOR formation can be explained by the lipophilicity of both piperidine and NPIP being higher than the lipopholicity of morpholine and NMOR (253). This means that there will be more amine present within the lipid to be nitrosated by NO and its nitrosating products, and also that the *N*-nitrosamine generated within the lipid is more likely to stay within the lipid layer than diffuse into the aqueous phase.

The shift in nitrosative chemistry from the aqueous to the lipid phase in the presence of ascorbic acid is not confined to one form of lipid. The results discussed so far have related to tributyrin. However, when triacetin replaced tributyrin in two-

phase models, nitrosative stress was again shown when NO was formed from acidified nitrite in the presence of ascorbic acid. The concentrations of NMOR, NPIP and NDMA were similar when comparing the lipid triacetin and tributryin for the different experimental conditions measured.

The above studies highlight the way in which lipid can profoundly alter acid induced nitrosative chemistry. Our studies were stimulated by the chemistry occurring when salivary nitrite enters the acidic stomach. Traditionally, the ascorbic acid secreted in gastric juice has been considered to be beneficial, by converting the nitrosating species to NO and thus inhibiting generation of potentially carcinogenic *N*-nitrosocompounds, within the gastric lumen. However, the NO produced in this way may itself induce nitrosation as it diffuses into lipid in the lumen or in the lipid bilayer membrane of the epithelial cells. The ascorbic acid therefore appears to have the potential to transfer the nitrosative stress from the aqueous phase into the adjacent lipid compartments.

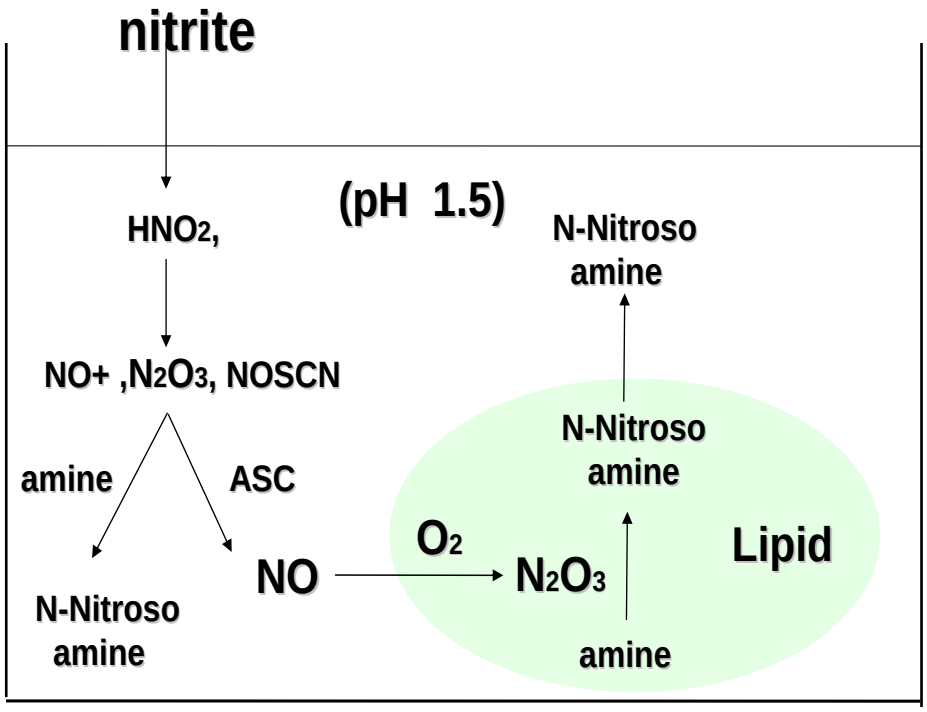


Figure 3.9: The proposed mechanism of *N*-nitrosamine formation in the lipid layer of a two-phase model of the gastroesophageal junction. The aqueous antioxidant ascorbic acid (ASC) shifts nitrosative stress from the aqueous to lipid phase.

Chapter 4 Lipid antioxidant effect on nitrosative chemistry in a model of the gastroesophageal junction

4.1 Introduction and Aims

The generation of *N*-nitrosocompounds within the upper gastrointestinal tract has been the subject of much research. Studies in Chapter 3 showed that, as well as acid-catalysed nitrosative stress in the absence of the aqueous ascorbic acid, there is a potential for NO induced nitrosative stress in the presence of ascorbic acid when lipid is present. These studies have shown that fat counteracts the ability of ascorbic acid to prevent nitrosation.

In biological systems there are lipid antioxidants as well as aqueous antioxidants. The interaction between lipid and aqueous antioxidants is thought to be important within these biological systems. Three major chain breaking lipid antioxidants are α-tocopherol, β-carotene and butylated hydroxytoluene (BHT). A-tocopherol is the major vitamin E compound found in the lipid fraction of living organisms (256-258). It is thought to be one of the most powerful naturally occurring lipid antioxidants known (145,259) and has previously been shown to inhibit the formation of nitrosamines in an *in vitro* gastrointestinal model system (260). B-carotene is another naturally occurring antioxidant that has been found in many biological systems (261). BHT is a synthetic phenolic lipid antioxidant that is

used in food preservatives (147,262). These, and other, lipid antioxidants may be important in preventing nitrosative stress occurring at the gastroesophageal junction.

The aim of the studies described in this chapter was to assess the effect on nitrosative stress, as determined by nitrosamine formation, of the lipid antioxidants α-tocopherol, β-carotene and butylated hydroxytoluene in a model simulating the chemistry occurring at the gastroesophageal junction.

4.2 Experimental design

A two-phase bench top model was created to assess the chemistry occurring at the gastroesophageal junction as described in Chapter 3. This model was modified such that a given concentration of one of three antioxidants, α-tocopherol, β-carotene or BHT was present within the lipid phase tributyrin (figure 4.1). This model consisted of a 250 mL glass beaker. Into this beaker an aqueous phase consisting of 50 mL HCl (pH 1.5) was added together with NaSCN (1mM) and EDTA (1mM). Ascorbic acid (1mM) was added to the aqueous phase in certain experiments. This provided an excess of ascorbic as compared to the later added nitrite. In other experiments a smaller dose of ascorbic acid was added such that all available ascorbic acid would be consumed. The aqueous phase represents the lumen of the stomach, with solutions present in physiological concentrations. Into the beaker was added 5 mL of a lipid, to generate a second phase. The two lipids used were tributyrin and triacetin. A secondary amine was added to both the lipid and aqueous phases to allow monitoring of the nitrosative stress, by measuring the generation of *N*-nitrosamines. The three secondary amines used were morpholine, piperidine and dimethylamine at concentrations of 5 mM. To determine the effect of various antioxidants on nitrosative stress, one of the lipid antioxidants α-tocopherol, β-carotene or BHT was added in various concentrations to the lipid. The concentrations used were 1 µM, 10 µM, 100 µM, 1 mM, 10 mM and 100 mM.

The contents of the beaker were stirred with a magnetic stirrer during the course of the experiment. Experiments were performed at 37^{o}C, the temperature being kept constant by having the beaker set up in a heated water bath. The experiments were initiated by adding $NaNO_2$ to the aqueous phase. Nitrite concentrations of 25 μM and 100 μM, after addition to the aqueous phase, were used in different experimental conditions.

Dissolved O_2 and NO concentrations were monitored in the aqueous phase throughout the study. At the end of the experiment, or at a given time point, a 1 mL aqueous sample was removed through a sampling device in the upper port of the beaker model for measurement of the relevant *N*-nitrosamine. Experiments were performed over 15 minutes, except in those studies where the time interval was being evaluated and in these experiments the time interval for sampling was 30 and 60 minutes.

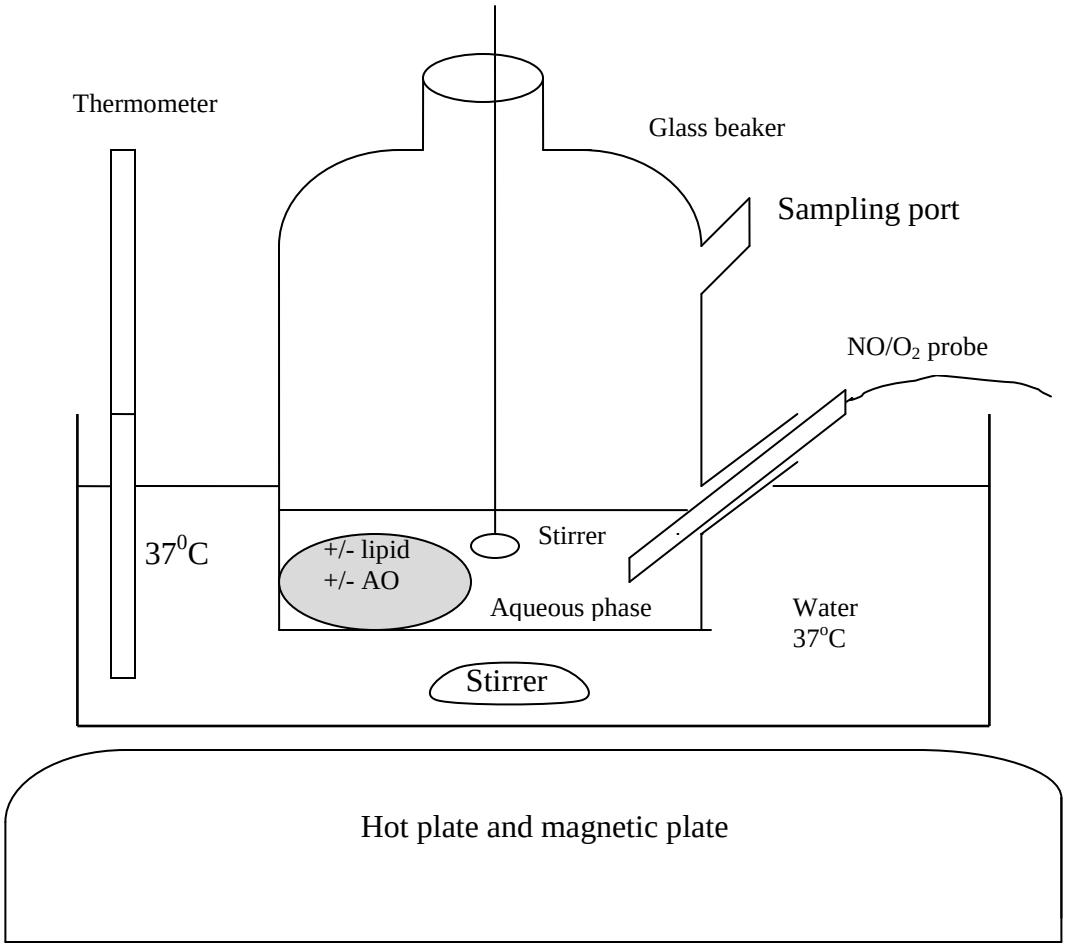


Figure 4.1: This schematic diagram represents the two-phase bench top model used to represent the gastroesophageal junction with lipid antioxidant. The aqueous phase consisted of 50 mL HCl (pH 1.5), NaSCN (1mM) and EDTA (1mM) +/- ascorbic acid (either 25µM or 1mM). The addition of nitrite (either 25µM or 100µM) initiated the experiments. The addition of the lipid tributyrin to an aqueous phase created a two-phase model. The lipid had varying concentrations of lipid anti-oxidant present. AO = antioxidant.

4.3 Results

4.3.1 Two-phase studies with presence of lipid antioxidant in lipid and ascorbic acid (1mM) in aqueous phase

4.3.1.1 A-tocopherol Present in the Lipid

The presence of α-tocopherol in the lipid component of the experimental design inhibited nitrosation at concentrations of 10 μM and greater, when nitrite (100 μM) was added to an acidic aqueous phase (figure 4.2). This was the same for all three nitrosamines measured. Addition of only 1 μM of α-tocopherol had no statistically significant effect on the concentration of any of the aqueous *N*-nitrosocompounds ($p>0.05$) with a mean NMOR concentration of 4.64 (±0.76) μM, mean NPIP concentration of 6.32 (±0.53) μM and mean NDMA concentration of 2.04 (±0.48) μM. When 10 μM of α-tocopherol was added to tributyrin, and the experiments run over 15 minutes, 1.11 (±0.24) μM NMOR, 1.28 (±0.22) μM NPIP and 0.93 (±0.13) μM NDMA were measured in the aqueous phase. This reduction in mean *N*-nitrosocompounds concentration is statistically significant ($p<0.00002$). A concentration of α-tocopherol of 100 μM and above resulted in only 0.01 (±0.01) μM of the respective *N*-nitrosamines being produced. NO and dissolved O_2 concentrations followed a pattern consistent with that found when there was no antioxidant in the lipid. When the lipid triacetin was used in place of tributyrin similar results were found (data not shown).

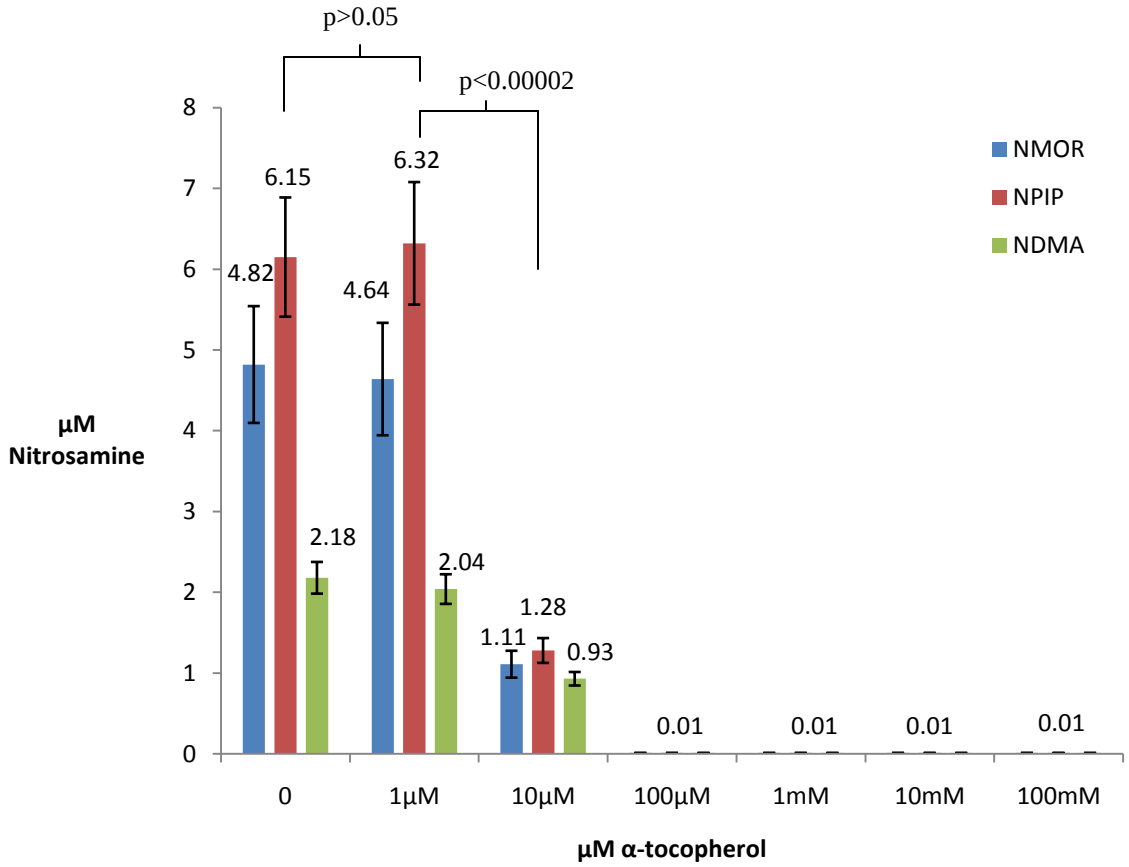


Figure 4.2: Mean aqueous *N*-nitrosamine concentrations for three different secondary amines are shown. The blue columns represent mean NMOR concentration, the red columns mean NPIP concentration and the green column NDMA concentration. In these experiments 100µM nitrite was added to an acidic aqueous phase containing 1mM ascorbic acid. The ratio of lipid to aqueous phase was 1:10. The concentration of α-tocopherol present in the lipid phase is shown on the x axis. Experiments were performed over 15 minutes. Mean values of the nitrosamine concentration are labeled. Error bars represent 1 standard deviation. (n=18)

4.3.1.2 B-carotene present in the lipid

B-carotene concentrations of 1 mM and greater were required to have a significant inhibitory effect on nitrosation. In the aqueous phase, 15 minutes after the addition of nitrite (100 μM) to HCl (pH 1.5) in the presence of thiocyanate (1mM) and ascorbic acid (1mM) and with 1mM β-carotene present in the lipid tributyrin 1.17 (±0.24) μM NMOR, 1.34 (±0.28) μM NPIP and 0.74 (±0.12) μM NDMA were measured. This compared to 4.36 (±0.23) μM NMOR, 5.85 (±0.52) μM NPIP and 2.24 (±0.41) μM NDMA when 100μM beta carotene was present in the tributyrin (figure 4.3). While these concentrations show a slight reduction from results when there is no antioxidant present, they are not statistically significant ($p>0.05$). NO and dissolved O_2 concentrations during the 15 minute experiment were similar to those obtained when no antioxidant was present in the lipid. When the lipid triacetin was used in place of tributyrin for the lipid phase, similar results were found (data not shown).

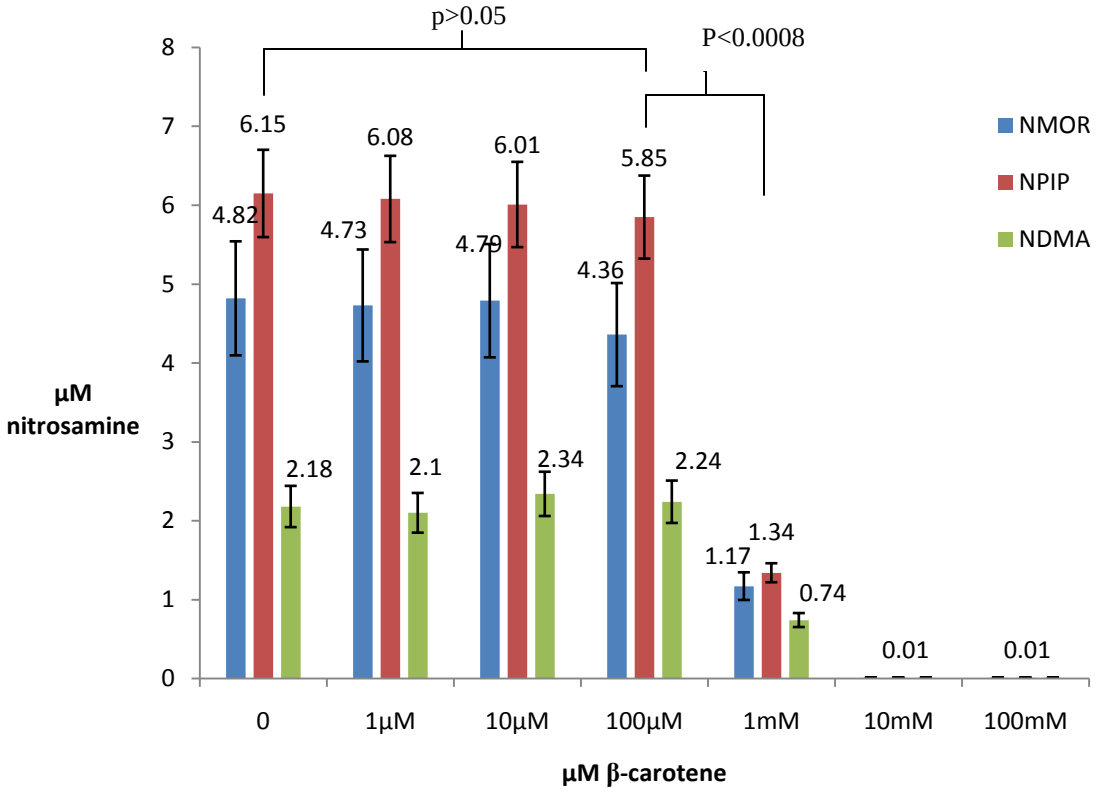


Figure 4.3: Mean aqueous *N*-nitrosamines concentration for three different secondary amines are shown. The blue columns represent mean NMOR concentration, the red columns mean NPIP concentration and the green column NDMA concentration. In these experiments 100 µM nitrite was added to an acidic aqueous phase containing 1mM ascorbic acid. The ratio of lipid to aqueous phase was 1:10. The concentration of β-carotene present in the lipid phase is shown on the x axis. Experiments were performed over 15 minutes. Mean values of the nitrosamine concentration are labeled. Error bars represent 1 standard deviation. (n=18)

4.3.1.3 BHT present in the lipid

Of the three antioxidants used in our studies, Butylated Hydroxytoluene (BHT) had the least inhibitory effect on nitrosation. Concentrations of 10mM and greater BHT in tributyrin were required to inhibit nitrosation after the addition of 100μM nitrite to HCl (pH 1.5) in the presence of thiocyanate and ascorbic acid in the aqueous phase. With a BHT concentration of 10 mM, 0.13 (±0.05) μM NMOR, 0.13 (±0.02) μM NPIP and 0.15 (±0.02) μM NDMA were measured in the aqueous phase (figure 4.4). When only 1 mM BHT was present within the lipid 3.27 (±0.54) μM NMOR, 5.26 (±0.22) μM NPIP and 1.49 (±0.26) μM NDMA were measured. The NO and dissolved O_2 concentrations measured in the aqueous phase were similar to those in the experiments where no antioxidant was present in the lipid phase. Results for the lipid phase with triacetin in place of tributyrin were similar to those seen with tributyrin.

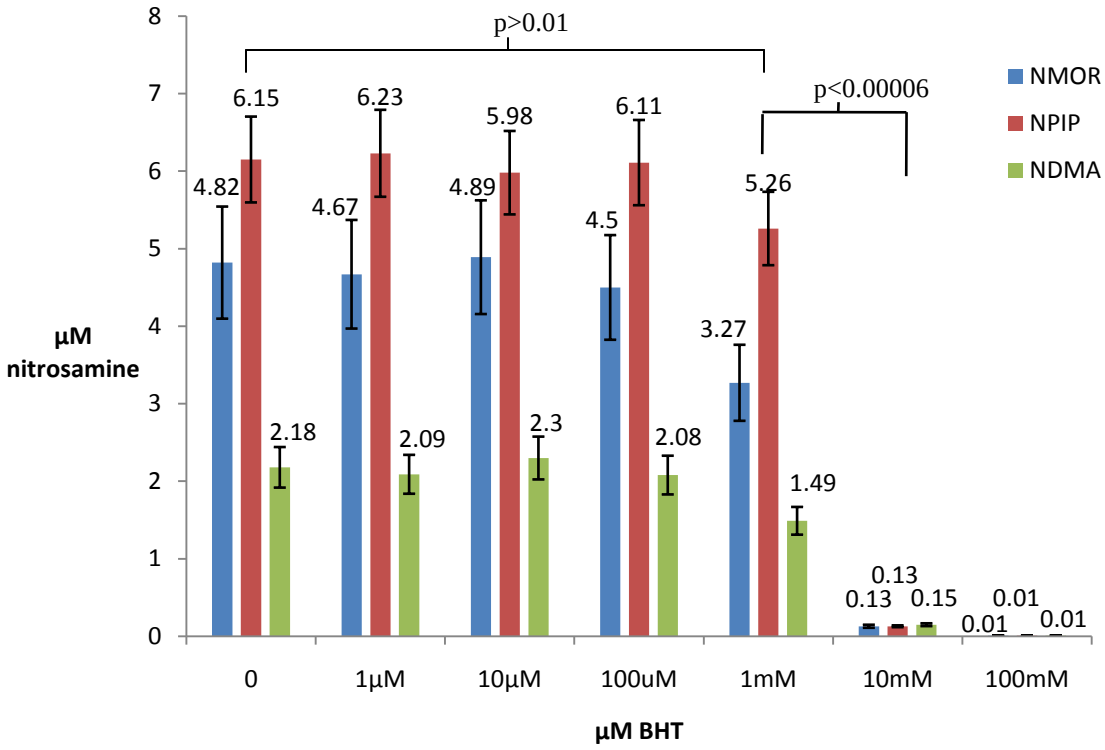


Figure 4.4: Mean aqueous *N*-nitrosamine concentrations for three different secondary amines are shown. The blue columns represent mean NMOR concentration, the red columns mean NPIP concentration and the green column NDMA concentration. In these experiments 100µM nitrite was added to an acidic aqueous phase containing 1mM ascorbic acid. The ratio of lipid to aqueous phase was 1:10. The concentration of BHT present in the lipid phase is shown on the x axis. Experiments were performed over 15 minutes. Mean values of the nitrosamine concentration are labeled. Error bars represent 1 standard deviation. n=18

4.3.2 Two-phase study with aqueous phase containing nitrite (25μM) and ascorbic acid (25μM) with a range of α-tocopherol concentrations in the lipid phase

In this experiment, there is not an excess of ascorbic acid, but rather, the ascorbic acid should all be consumed in the reaction with nitrite to generate NO. There was a mean NO concentration produced of 21 μM, maximal at 30 seconds. There was a loss of O_2 after 30 seconds, but this was measurable in the aqueous phase again just after 3 minutes (Figure 4.5). This therefore suggests there should be less nitrosative stress. Despite this, it was found in these experiments that some of the inhibition of nitrosation seen with the smaller concentrations of α-tocopherol was lost. Compared to the model where there was an excess of ascorbic acid, where a concentration of only 10 μM α-tocopherol was enough to prevent nitrosation, a concentration of 10 mM is required to achieve near complete inhibition of nitrosative stress in these experiments. In the absence of any lipid antioxidant the mean NMOR concentration produced was 1.53 (±0.26) μM. There was a statistically significant degree of inhibition with α-tocopherol concentrations of 10 μM and above but even at 1mM concentrations the mean NMOR concentration was 0.47 (±0.05) μM showing there is still nitrosation occurring (figure 4.6).

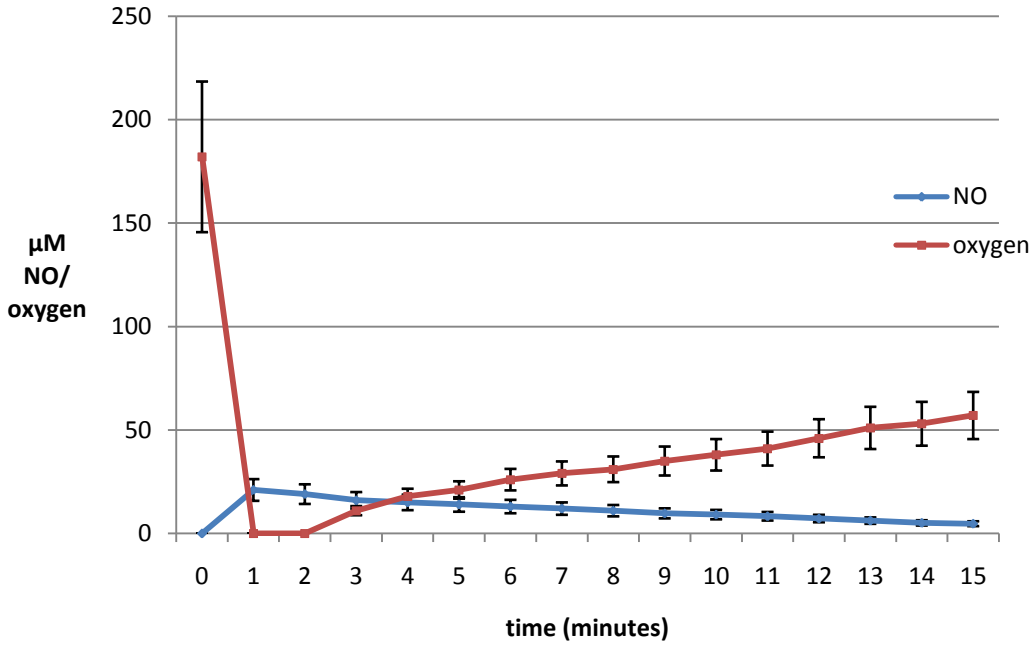


Figure 4.5: Mean aqueous NO and O_2 concentration (µM) over time after the addition of nitrite (25 µM) to an acidic aqueous phase in the presence of ascorbic acid (25µM). Error bars represent 1 standard deviation. (n=18)

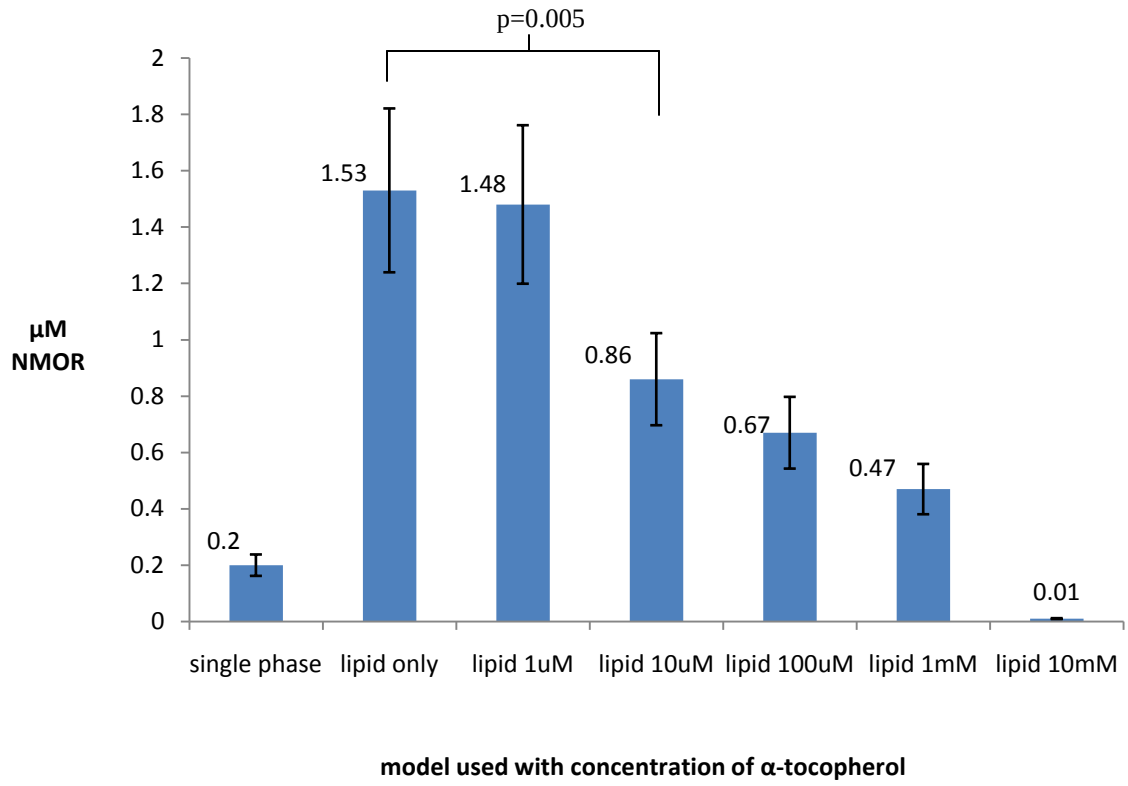


Figure 4.6: Aqueous NMOR concentrations produced over a 15 minute period in a single and two-phase system with ascorbic acid present. In a two-phase system there is either no alpha tocopherol or a range concentrations from 1 µM to 10 mM. Only 25 µM ascorbic was present in the aqueous phase and 25 µM nitrite was added. Data labels represent mean values. Error bars represent 1 standard deviation. (n=18)

4.3.3 Two-phase studies with nitrite (25 μM) but no ascorbic acid in the aqueous phase and either no, or one of a range of concentrations of α-tocopherol

In a single phase system there is generation of NMOR as expected due to acid-catalysed nitrosation with a mean NMOR concentration of 1.73 (±0.38) μM. The concentration of NMOR is not significantly lower when there is lipid without any antioxidant. However there is a trend towards there being slightly less NMOR, with a mean NMOR concentration in the aqueous phase of 1.58 (±0.20) μM. Concentrations of α-tocopherol in the lipid phase of 1 and 10 μM do not significantly change the concentration of NMOR produced ($p>0.05$). Higher concentrations of 100 μM, 1 mM and 10 mM α-tocopherol do result in significantly less NMOR concentrations of 1.08 (±0.13) μM, 0.84 (±0.05) μM and 0.74 (±0.12) μM respectively ($p=0.0007$) (figure 4.7). However these concentrations are significantly greater than those found when there was ascorbic acid present, whether in excess or only at a concentration of 25 μM ($p<0.000003$).

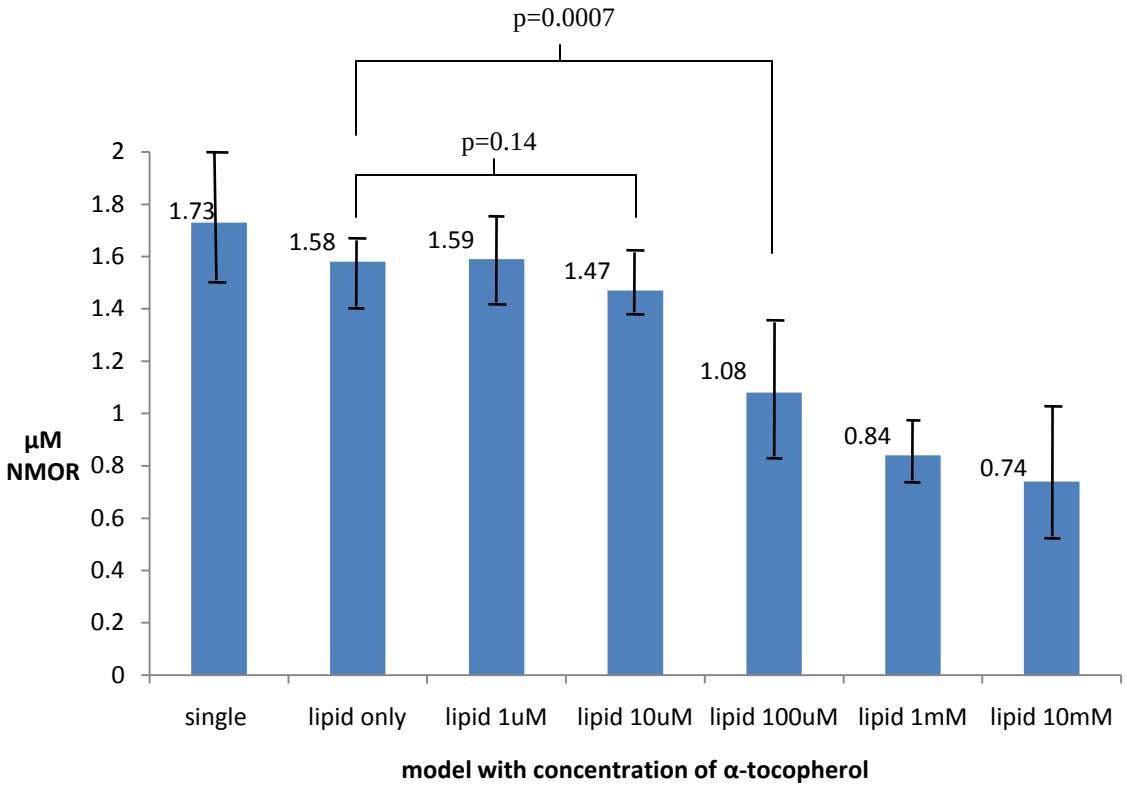


Figure 4.7: Aqueous NMOR generation in a single and two-phase system with single dose of 25µM nitrite but no ascorbic acid present. In the two-phase system there was increasing concentrations of a-tocopherol from 0µM to 10mM. Data labels show mean values of µM NMOR. Error bars represent 1 standard deviation. n=12

4.3.4 Two-phase studies (increased time interval) with α-tocopherol present and ascorbic acid (1mM) and nitrite (100μM) present in the aqueous phase

Increasing the time interval from 15 to 60 minutes increased the NMOR concentration produced, for all concentrations of α-tocopherol examined. There was a three fold increase in NMOR concentrations with increased time from 15 to 60 minutes, for α-tocopherol concentrations of 100 μM and 1 mM ($p=0.0006$). Where 10 μM α-tocopherol was present as lipid antioxidant there was a greater increase in NMOR produced, such that at 60 minutes there was a concentration of 8.46 (±0.90) μM (figure 4.8). This is 20 times the concentration of NMOR observed with 100 μM and 1 mM α-tocopherol at the same time interval. This concentration of 8.46 μM is significantly nearer the concentration found when there is no lipid antioxidant present (NMOR concentration of 10.14 μM).

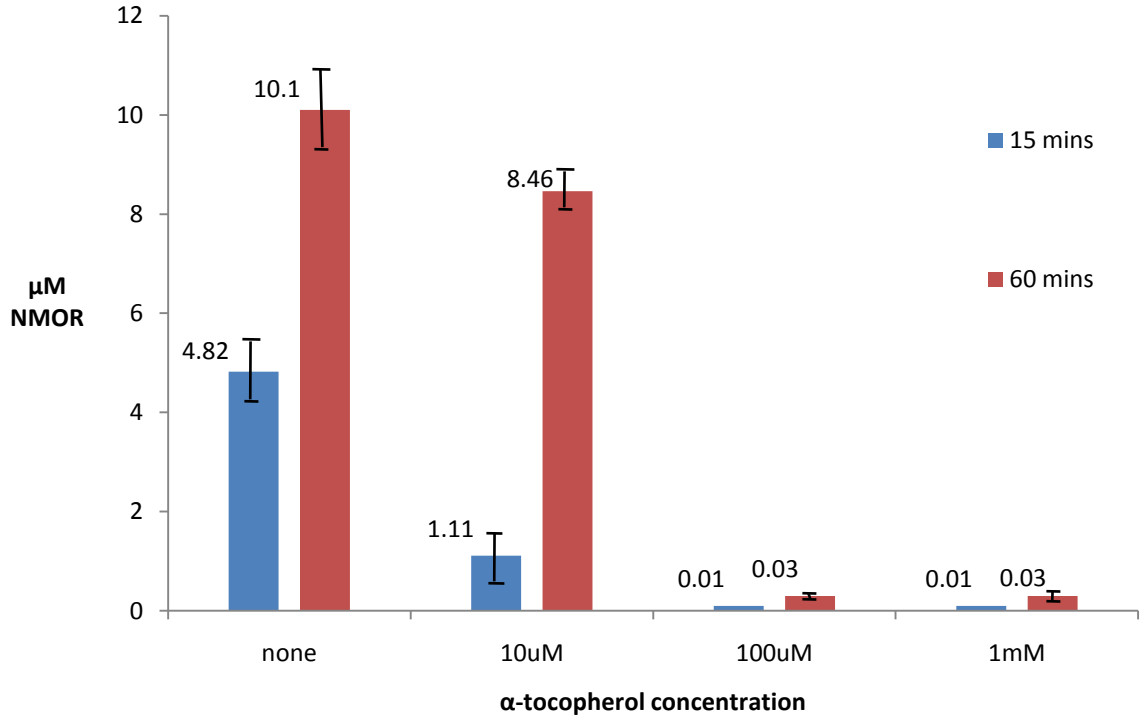


Figure 4.8: Aqueous NMOR concentrations in a two-phase model with nitrite (100 µM) added to an aqueous phase containing ascorbic acid (1mM). In the lipid phase system there was a range of concentrations of α-tocopherol (0µM to 10mM). The concentration of α-tocopherol used is shown on the x axis labels. Data is shown for both 15 minute experiments (blue column) and 60 minute experiments (red column). Data labels show mean values of µM NMOR. Error bars represent 1 standard deviation. n=12

4.4 Discussion

These results indicate that both lipid and aqueous antioxidants are required to prevent nitrosation in a model simulating the gastroesophageal junction. We have previously demonstrated an inhibitory effect of ascorbic acid in preventing acid nitrosation in a single aqueous phase system. This inhibitory effect is lost when lipid is added as a second phase into this model. However, the combination of ascorbic acid with any of the lipid antioxidants is effective in having an inhibitory effect on nitrosative stress, if sufficient concentrations are present. The concentrations required to achieve this inhibition can be very low (10μM), as can be seen for α-tocopherol. The co-operative and synergistic effect of lipid and aqueous antioxidants has been extensively researched over the past five decades (171,263-266).

The aqueous antioxidant ascorbic acid competes for acidified nitrite (240, 267,268). The reaction product from this is NO. NO is a small, gaseous, lipophilic molecule. It diffuses from the aqueous phase into the lipid phase as it is 9 times more soluble in lipid (251). In the lipid, NO meets O_2. NO reacts readily with O_2 to form the nitrosating species N_2O_3 (250). The rate of this reaction is related to the square of the NO concentration multiplied by the O_2 concentration. The reaction is therefore favoured by high NO concentrations, such as those generated when swallowed nitrite meets acidic gastric juice. The reaction of NO with O_2 is some 300 times faster in lipid than in an aqueous phase (251). The resulting nitrosating species N_2O_3 is then able to nitrosate the secondary amine to form the corresponding

N-nitrosamine (98). Previous work by Mergens et al had demonstrated that α-tocopherol may be able to prevent *N*-nitrosamine formation (269). The models used in these experiments were both an emulsified aqueous solution of α-tocopherol *in vitro* and the addition of α-tocopherol to shredded tobacco to prevent NDMA formation in tobacco smoke. Mergens et al have also shown that α-tocopherol may be able to prevent formation of nitrosamines in an *in vitro* model of the gastrointestinal system, although this was in the context of nitrosative stress from luminal acid-catalysed nitrosation in the absence of ascorbic acid (260). It is thought that, although NO does not react directly with α-tocopherol, it can react through oxidation products such as N_2O_3 (158).

The experiments performed in our model of the gastroesophageal junction confirm that α-tocopherol, as well as other lipid antioxidants, are able to prevent nitrosative stress in the presence of ascorbic acid. We found a concentration of 10 μM of α-tocopherol, 1 mM β-carotene and 10 mM BHT was sufficient to prevent nitrosamine formation in the presence of excess ascorbic acid. These findings are in keeping with the relative and absolute effectiveness of various lipid chain breaking antioxidants (270). Howard et al found that α-tocopherol was the most powerful lipid antioxidant > other vitamin E compounds > β-carotene > BHT based on rate kinetics when assessing styrene autoxidation. The antioxidant activities of these compounds are the same as their biological activities (256). Stereoelectronic factors relating to the position of the methoxy group on the aromatic ring, and also the

stabilising effect of the ethereal O_2 on the phenoxy radical, can explain the superior reactivity of α-tocopherol (145,148).

In the studies with ascorbic acid present and α-tocopherol added to the lipid compartment at concentrations of 10 μM and greater, the nitrosative stress is inhibited. With concentrations of 10 μM α-tocopherol nitrosamines are still formed (1.1 μM NMOR, 1.1 μM NPIP and 0.9 μM NDMA) but to a lesser extent than in studies in which no lipid antioxidant is added. This would suggest that the α-tocopherol is being overwhelmed to some extent. An α-tocopherol concentration of 10 μM is still, however, a surprisingly low concentration to be able to inhibit the nitrosative stress from 100 μM nitrite in this model. The stoichiometry of chain breaking lipid antioxidants is complex. In a classical inhibitory scheme two peroxyl radicals are consumed for every antioxidant molecule oxidised (148). However, radical intermediates may drain antioxidants from the system. It is still, therefore, surprising that α-tocopherol of such a low concentration (10μM) can be effective. This may possibly be explained by the recycling, or 'sparing', of α-tocopherol by ascorbic acid. Over the past 60 years there has been ongoing debate as to whether recycling between ascorbic acid and α-tocopherol exists. Tappel et al first postulated the concept that ascorbic acid is able to replenish the reducing capacity of α-tocopherol (165). *In vitro* evidence to support the concept of recycling in both homogenous solutions and liposomal membrane systems is extensive (171,173,263,264,265,271). This does not, however, seem to be borne out when some *in vivo* studies are undertaken (174) while others have postulated an *in vivo*

process of recycling does exist (175). Our own unpublished *in vitro* studies presented in Chapter 5 of this thesis, using the same model as in these current experiments, suggest that there may be recycling, as ascorbic acid is consumed to a greater extent when α-tocopherol is present.

As the incubation time was increased from 15 to 60 minutes there was a similar effect on *N*-nitrosamine generation in the presence α-tocopherol as there was in the presence of a two-phase model in the absence of a lipid antioxidant. There was a 2-3 fold increase in mean *N*-nitrosamine concentrations. The exception to this was at concentrations of only 10 μM α-tocopherol. In 15 minute experiments this concentration had only inhibited *N*-nitrosation to a limited degree. When the time interval was increased to 60 minutes there was eight times more *N*-nitrosamine generated. The final NMOR concentration, at 60 minutes, was only slightly less than the concentration of NMOR generated when there was no lipid antioxidant. This suggests that the α-tocopherol was overwhelmed in this scenario. It may be that the ascorbic acid was not sufficient to replenish the α-tocopherol, although it is unlikely that the ascorbic acid in the system was totally depleted as ascorbic acid was present in excess. Unfortunately ascorbic acid was not measured in the aqueous phase during these experiments, although in retrospect this would have been useful.

When the concentration of the nitrite entering this system was lowered to 25 μM in the absence of ascorbic acid, the mean NMOR concentration measured in the aqueous phase in the absence of α-tocopherol was 1.58 μM. This was only slightly

less than the mean concentration in a single phase system with no lipid where 1.73 μM NMOR was generated. These mean concentrations are less than half the concentrations seen when 100 μM nitrite was added to the system. This acid-catalysed nitrosation appears to be inhibited at lower concentrations of α-tocopherol when 25 μM nitrite was added as compared to 100 μM nitrite being added. Concentrations of 100 μM α-tocopherol were effective in inhibiting nitrosation when 25 μM nitrite was added compared to 10 mM being required when 100 μM nitrite was added. This shows that the generation of nitrosating compounds is less and suggests that smaller concentrations of α-tocopherol were able to compete for the nitrosating species.

In the experiments discussed thus far we have observed that either a very high concentration of α-tocopherol in the absence of ascorbic acid, or a very low concentration of α-tocopherol in the presence of excess ascorbic acid, results in inhibition of nitrosation. While in the immediate post prandial state there may be excess production of ascorbic acid relative to nitrite at the gastroesophageal junction and gastric cardia (221), this may not always be the case. There may be more of a balance between nitrite and ascorbic acid concentrations. In studies using 25μM ascorbic acid and 25μM nitrite the α-tocopherol concentration required to inhibit nitrosation was considerably greater than when there was an excess of ascorbic acid. A proposed mechanism for this is that all the ascorbic acid is oxidised to dehydroascorbic acid in the initial reaction with nitrite. When ascorbic acid is present to excess it will replenish the reducing capacity of α-tocopherol, but when all

the ascorbic acid is oxidised to dehydroascorbic acid in the initial reaction with nitrite this will not occur.

In our studies β-carotene and BHT require higher concentrations than α-tocopherol to have an inhibitory effect on nitrosamine production. B-carotene is present in many tissues throughout the human body and has an important antioxidant role to play. BHT is a synthetic antioxidant that is present in food preservatives. There is some *in vitro* and *in vivo* evidence that β-carotene together with both vitamins C and E provides synergistic protection against oxidation by peroxynitrite (266). The mechanism proposed for this is similar to the replenishment of tocopherol by ascorbic acid, in that there is an electron transfer reaction in which β-carotene radical is repaired by vitamin C. BHT has not been shown to have a capacity to be replenished by aqueous antioxidants. However, it may work in a co-operative manner with other antioxidants. Our studies, thus far, have not investigated for synergism between β-carotene and ascorbic acid, or between lipid antioxidants, although this may be worthwhile in the future.

It is interesting to note that concentrations of vitamin E in the human stomach are highest in the more proximal regions, in subjects who are *Helicobacter pylori* negative. In the presence of *Helicobacter pylori* there is a loss of proximal vitamin E, while more distal stomach concentrations are maintained. This may represent mobilization of antioxidant defenses to the sites of maximal stress (272).

The model in which we have assessed the effectiveness of antioxidants in preventing nitrosative stress is one designed to represent the gastroesophageal junction. Gastroesophageal junction and gastric cardia cancers have been increasing over the past 20 years in the Western World. The mutagen behind this rise is as yet unknown, but the carcinogenic potential from nitrite is currently being explored. It has been shown that the potential for generation of harmful *N*-nitrosocompounds exists within the area of the upper gastrointestinal tract where swallowed nitrite meets acidic gastric juice. Antioxidants, such as those studied in this model, may have a role in preventing the formation of harmful *N*-nitrosocompounds within the upper gastrointestinal tract.

Chapter 5 Interaction between ascorbic acid and α-tocopherol

5.1 Introduction and Aims

The synergistic activity between vitamins C and E was first suggested in 1941, when it was found that although ascorbic acid alone was a very poor antioxidant for lard and oil, it enhanced the antioxidant activity of tocopherols (164). It was not until 1968 that Tappel suggested that vitamin C might reduce the tocopheroxyl radical formed from tocopherol during the scavenging of free radicals *in vivo*, thus permitting a single molecule of vitamin E to scavenge many radicals (165). There has been some controversy in this field regarding the synergy between the two vitamins.

In experiments performed in Chapter 4 of this thesis, using a two-phase model, it was noticed that the concentration of α-tocopherol required to prevent nitrosation was much smaller than expected, with 10 μM of α-tocopherol inhibiting nitrosation when 100 μM of nitrite was added to a two-phase model generating NO. This could be explained by ascorbic acid reducing the tocopheroxyl radical produced in inhibiting nitrosation. This would, therefore, allow one molecule of α-tocopherol to reduce many nitrosating species. It was also found that, in two-phase experiments where we used only the same concentration of ascorbic acid as of nitrite, the concentration of α-tocopherol that had been effective in inhibiting nitrosation in experiments with an excess of ascorbic acid was now much less effective. This

suggests, that in experiments where there was an excess of ascorbic acid, ascorbic acid was replenishing the reducing capacity of α-tocopherol. In experiments where the ascorbic acid concentration was reduced, ascorbic acid became more deplete in the initial reaction with nitrite and subsequently was not so readily available to replenish the α-tocopherol.

The aim of the experiments in this chapter was to determine if there was any interaction between vitamin C and E in experimental models representing the chemistry occurring at the gastroesophageal junction.

5.2 Experimental Design

5.2.1 Single and two-phase models of the GO junction

Bench top models were used as in experiments in Chapters 3 and 4 (figures 3.1 and 4.1). These models were adjusted to provide different chemical environments.

5.2.1.1 With reduced ascorbic acid concentration

In these experiments there was not an excess of ascorbic acid using just the same concentration of ascorbic acid as that of sodium nitrite which was used to initiate the experiment. This should mean that there is no excess of ascorbic acid present to react with other compounds such as α-tocopherol. Therefore, 100 µM nitrite was added to the acidic environment containing 100 µM ascorbic acid. These experiments were performed over 30 minutes.

5.2.1.2 Several pulsed boluses of nitrite

Sodium nitrite at various concentrations (1 µM, 2.5 µM and 10 µM) was added to the aqueous phase at times 0, 7.5, 15 and 22.5 minutes. The concentration of ascorbic acid in the aqueous phase of these experiments was 100 µM. The purpose of adding several boluses of nitrite to the experimental model was to simulate the *in*

vivo situation. It has previously been shown that after a nitrate-rich meal there are boluses of salivary nitrite reaching the acidic gastric juice (213).

5.2.2 Three-phase model of the GO junction

Our experiments thus far in this thesis have only looked at the aqueous layer as a provider of ascorbic acid leading to generation of NO in an acidic environment and. It is consumption of ascorbic acid from this phase that has previously been observed. A three-phase model was constructed as a better way of representing the *in vivo* environment. This three-phase model consisted of two aqueous layers and a lipid layer (figure 5.1). The lipid layer provided complete separation of the aqueous layers but allowed the passage of NO and O_2. This allowed analysis of ascorbic acid away from the initial site of its antioxidant reactions in the luminal side of the model. It allowed for examination of ascorbic acid consumption in the epithelial compartment to see if it is consumed when there is the potential for nitrosative stress in this compartment.

A modified cylindrical Delrin model was constructed. Delrin is an engineering plastic also known as polyoxymethylene. The model was made to a total volume of 6700 µl. The model was constructed in two halves to allow the creation of all replaceable three layers. The two halves could be tightened together without any leak of aqueous contents. The lower half had a port through which sodium nitrite

could be added to initiate the generation of NO in this lower layer. This lower port was sealable with a plastic cap.

The upper layer had a total volume of 4000 µl. It consisted only of 150 µM ascorbic acid in PBS to a pH 7.4. It was from this layer that samples were taken to measure ascorbic acid and total vitamin C concentrations. WPI NO and O_2 probes, as described in Chapter 2.1.1 and 2.1.2, were placed at the bottom of this layer, just above the middle layer, to continuously monitor NO and O_2 concentrations during the experiment. The middle layer was made up of a composite membrane used in the design of NO probes (Donation from World Precision Instruments, Sarasota, Florida)), with 800 µL of the liquid lipid triacetin spread evenly over the surface of the membrane. The composite membrane was known to allow free passage of NO and O_2 through the membrane. The lipid used was triacetin as its density at 37^{o}C is such that it forms a layer. In some of the experiments α-tocopherol was added to the lipid. The concentrations of α-tocopherol varied from 10 µM. The lower layer was an aqueous layer designed to resemble the chemistry at the gastroesophageal junction. In this layer HCl provided an acidic environment with a pH of 1.5. NaSCN (1 mM) and EDTA (1 mM) were added, together with ascorbic acid (1 mM). The ascorbic acid was added in a concentration that was in excess of the concentration of sodium nitrite (100 µM) used to initiate the experiment. This layer was used to generate NO. The total volume of the lower layer was 1900 µl.

In terms of relating this *in vitro* model to conditions in the human upper gastrointestinal tract, the upper layer represents the intracellular compartment, while the middle layer represents the lipid membrane. The lower layer represents the lumen of the upper gastrointestinal tract

Experiments were performed at 37^{o}C for 30 minutes. The temperature was kept constant by performing the experiments on a hot plate. Samples (100µL) were taken at time 0, 1, 2, 5, 10, 16, 23 and 30 minutes from the upper layer of the model for ascorbic acid and total vitamin C. The usual methods for processing ascorbic acid samples had to be adapted. This occurred because the initial procedure involved diluting the samples twenty fold and it was noticed that the samples were being diluted beyond the point where the ascorbic acid was detectable. Therefore, 80 µL of upper layer aqueous sample was taken from the top layer of the model. This was equally divided and the 40 µL samples added to 120 µL of either MS or MS:DTT. This gave a final dilution of 1 in 4. In all experiments there was a control sample of MS and MS:DTT which was analysed to make sure the sample was the source of ASC. The model had an open top through the upper layer allowing oxygen to enter. Samples were then analysed as described in Chapter 2.1.8.

To ensure that the model was suitable for purpose, NO diffusion across the lipid layer was monitored in the upper phase. In all experiments NO was detected to be entering into the upper aqueous phase after diffusion through the middle lipid layer. There was a standard pattern of diffusion, with a gradual increase in the NO

concentration recordings from time 2 minutes after the nitrite was added to the lower phase. The maximum mean NO concentration achieved was 20 μM after 30 minutes (figure 5.2).

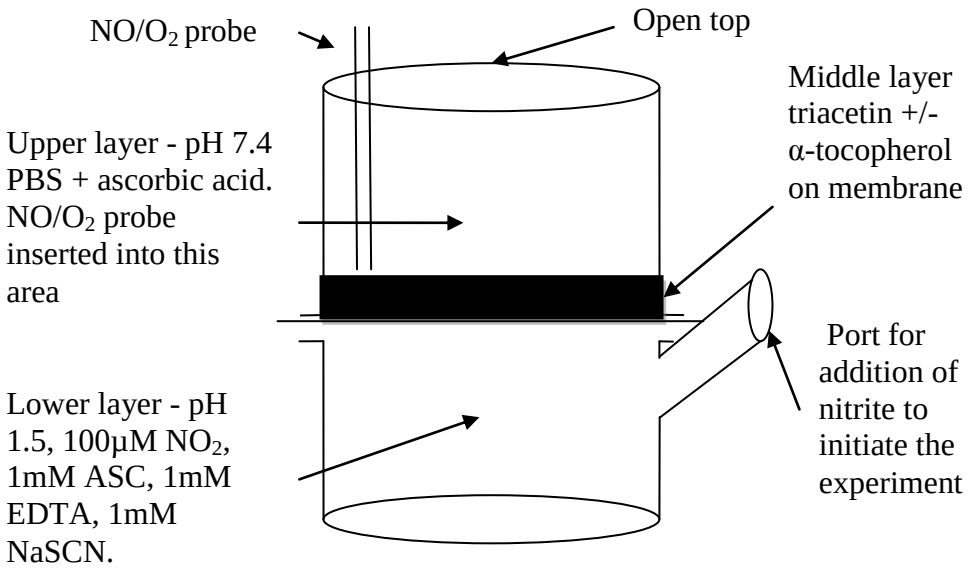


Figure 5.1: Representation of the Delrin model and constituents used to form a three-phase model. The lower layer was representative of acidic gastric juice and experiments were initiated by adding 100 µM nitrite. The middle layer was composed of the lipid triacetin sitting on a composite hydrophobic membrane. The upper layer consisted of 150 µM ascorbic acid in a neutral environment.

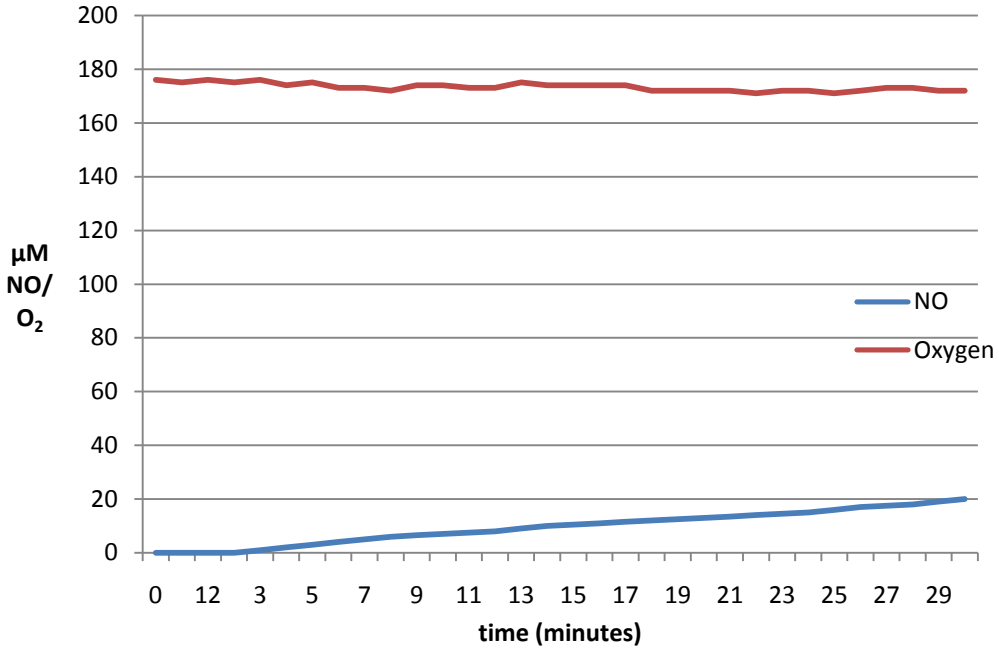


Figure 5.2: NO and O_2 concentrations in the upper phase of a three-phase model. The concentrations of NO and O_2 were measured just above the middle layer in a three-phase model, over a 30 minute period, representing the chemistry at the gastroesophageal junction after the addition of 100 µM sodium nitrite to an acidic lower layer containing 1 mM ascorbic acid. Data labels show the mean NO concentration at a given time. n=18

5.3 Results

5.3.1 Two-phase model with α-tocopherol present in the presence of excess ascorbic acid (1mM) relative to nitrite (100μM) with increased time

In 15 minute two-phase experiments in the presence of ascorbic acid it has been seen that concentrations of 10 μM α-tocopherol and greater were enough to almost fully inhibit nitrosation, as only 0.01 (±0.01) μM NMOR was produced for concentrations of 100 μM, 1 mM, 10 mM, while a mean concentration of 1.11 (±0.24) μM NMOR was produced when 10 μM α-tocopherol was present. When the time period of the experiment was increased to 60 minutes the NMOR concentration generated was significantly increased in all experiments using different concentrations of α-tocopherol. However, the biggest increase occurred with 10 μM α-tocopherol, as eight times NMOR was produced at 60 minutes ($p=0.00004$), compared to three times for greater concentrations of α-tocopherol ($p>0.0002$).

5.3.2 Two-phase model with α-tocopherol and reduced ascorbic acid (100μM) relative to nitrite (100μM)

The NMOR concentration produced in these experiments was higher for concentrations of 10 μM α-tocopherol and greater than those NMOR concentrations seen using 100 μM nitrite with an excess of ascorbic acid. In a system containing no α-tocopherol there was a mean NMOR concentration of 2.45 (±0.6) μM. A concentration of 10 μM α-tocopherol partially inhibited NMOR generation such that the mean NMOR concentration at 15 minutes was 1.23 (±0.16) μM ($p=0.0001$). Even at the higher concentration of 1 mM α-tocopherol the mean NMOR concentration was 0.38 (±0.09) μM at 15 minutes ($p=0.00006$) (figure 5.3). These results show a similar pattern to those seen in the experiments in Chapter 4.3.2, where smaller doses of 25μM nitrite and ascorbic acid were used in the experimental design.

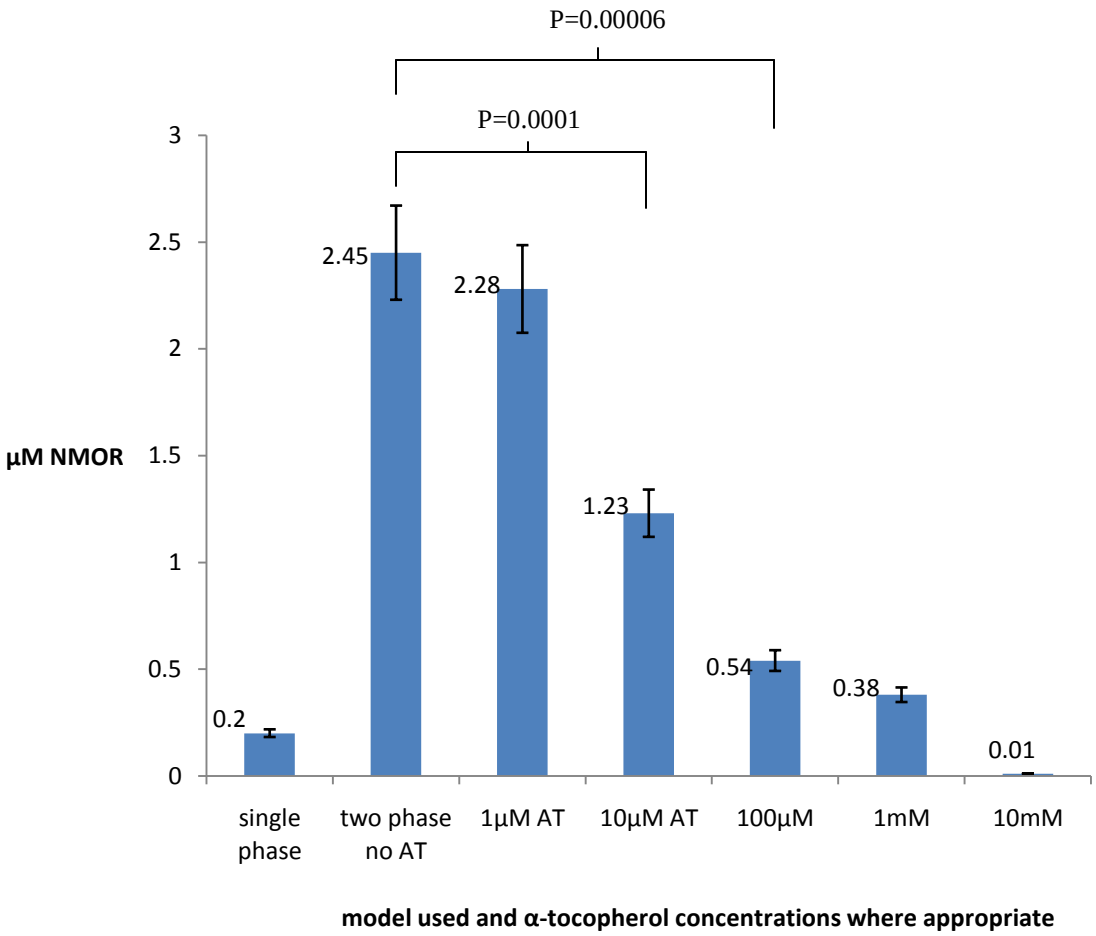


Figure 5.3: Mean aqueous phase NMOR concentrations (µM) at 15 minutes from a two-phase model where experiments were initiated by the addition of nitrite (100µM) to an acidic aqueous phase containing only 100µM ascorbic acid. In this model the lipid phase contains concentrations of α-tocopherol (AT) ranging from 0 to 10 mM. Data labels show the mean value for each experimental condition. Error bars represent 1 standard deviation. n=12

5.3.3 Two-phase model with α-tocopherol with reduced ascorbic acid (100μM) relative to nitrite (100μM) with increased time interval

NMOR concentrations changed dramatically with increased time. After 15 minutes, at concentrations of 10 μM and above, α-tocopherol had managed to provide a significant degree of inhibition of nitrosation, although the degree of inhibition was not as great as when there was an excess of ascorbic acid. However, after 60 minutes the inhibition was almost completely lost where concentrations of 10 μM and 100 μM α-tocopherol were used with mean NMOR concentrations of 9.55 (±0.71) μM and 9.28 (±1.17) μM respectively. These concentrations of NMOR were not significantly different from the mean NMOR concentration in a two-phase model in the absence of lipid antioxidant which was 10.1 (±1.1) μM.

5.3.4 Ascorbic acid consumption during 30 minute experiments in a single and two-phase model in the presence and absence of α-tocopherol when ascorbic acid (1mM) is present in excess relative to the nitrite concentration (100μM)

In single phase experiments the mean percentage of ascorbic acid present at 30 minutes compared to the starting ascorbic acid was 37.2 (±4.1) %. The biggest drop in ascorbic acid concentration occurs within the first few minutes after the addition of nitrite. Similar mean percentages of ascorbic acid remaining at 30 minutes were found in a two-phase model using tributyrin, and also in a two-phase model where the lipid was made into several globules. The mean percentages of ascorbic acid remaining at 30 minutes were 33.6 (±2.1) % for lipid in single globule and 36.2 (±3.6) % for lipid in the form of several globules. There is no statistical significance between any of the percentage ascorbic acid remaining at 30 minutes for these experiments ($p=0.068$).

In a 2 phase model where the lipid antioxidant α-tocopherol was present, at a concentration of 10 mM (a concentration shown to inhibit nitrosation), the ascorbic acid present at 30 minutes was only 14.6 (±5.1) % that of the concentration. This is a statistically significant reduction in the available ascorbic acid ($p=0.00007$). There was no statistically significant difference when the lipid was present in globules with α-tocopherol present ($p=0.29$). Similar results were found for α-tocopherol concentrations of 100μM and 1mM (figure 5.4).

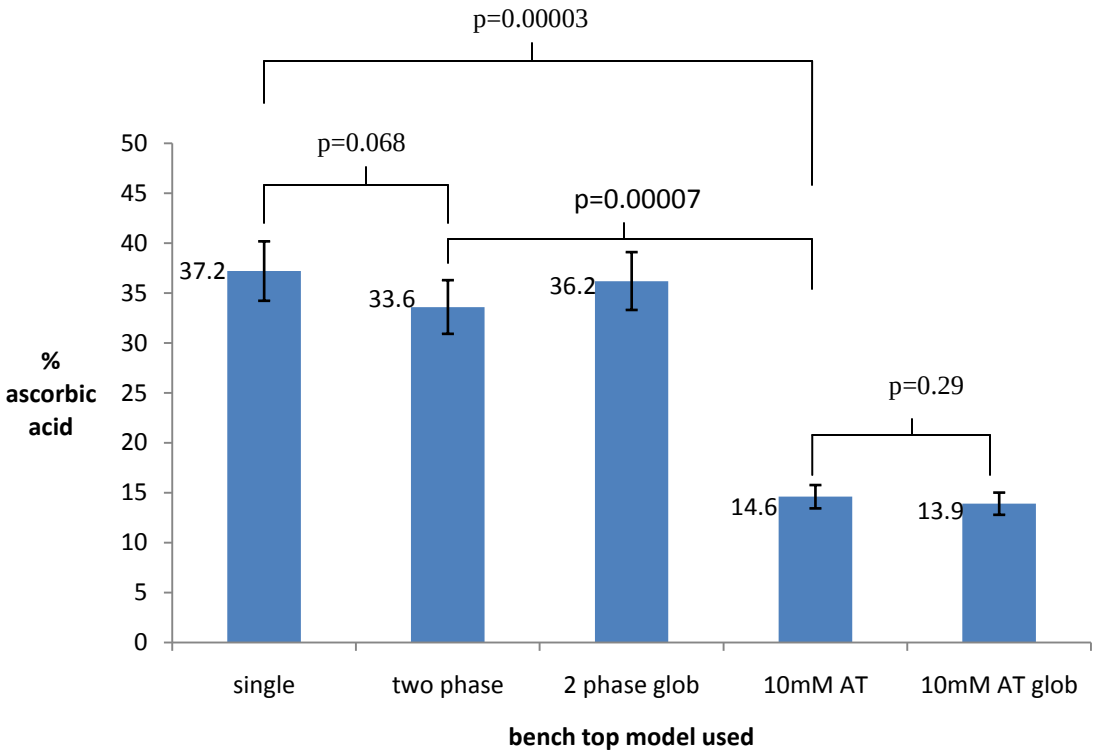


Figure 5.4: The mean percentage ascorbic acid remaining at 30 minutes in different experimental models is shown. In all experiments there was an initial excess of ascorbic acid to nitrite in the aqueous phase (1mM ascorbic acid to 100µM nitrite). In 2 phase experiments the lipid had either no α-tocopherol present or 10mM α-tocopherol (AT) present. The lipid was either present as a single phase or as multiple globules (glob). Data labels show mean percentages for each experimental condition. Error bars represent 1 standard deviation. n=18

5.3.5 Ascorbic acid consumption during 30 minute experiments using pulsed nitrite in a single phase two-phase model with and without α-tocopherol

There was rapid oxidation of ascorbic acid using several boluses of nitrite as compared to a single bolus. When 10μM nitrite was added at time 0, 7.5, 15 and 22.5 minutes, there was no detectable ascorbic acid at 30 minutes. On changing the dose to 1μM nitrite there was little difference in the ascorbic acid concentrations at the beginning and end of the experiments. A midrange nitrite concentration of 2.5μM, pulsed into the two-phase model, at the same time intervals as before, was used in the absence of lipid antioxidant. At the end of the 30 minute experiment the mean ascorbic acid percentage remaining was 61.6 (±17.2) %. There was a considerable reduction in the concentration of ascorbic acid remaining when concentrations of 100 μM α-tocopherol and higher were present in the lipid phase (figure 5.5). The mean percentage ascorbic acid remaining when 100 μM α-tocopherol was present was 8 (±4) %. With concentrations greater than 1 mM there was no ascorbic acid present at the end of the experiment and only a mean ascorbic acid percentage of 5 (±2) % at 15 minutes.

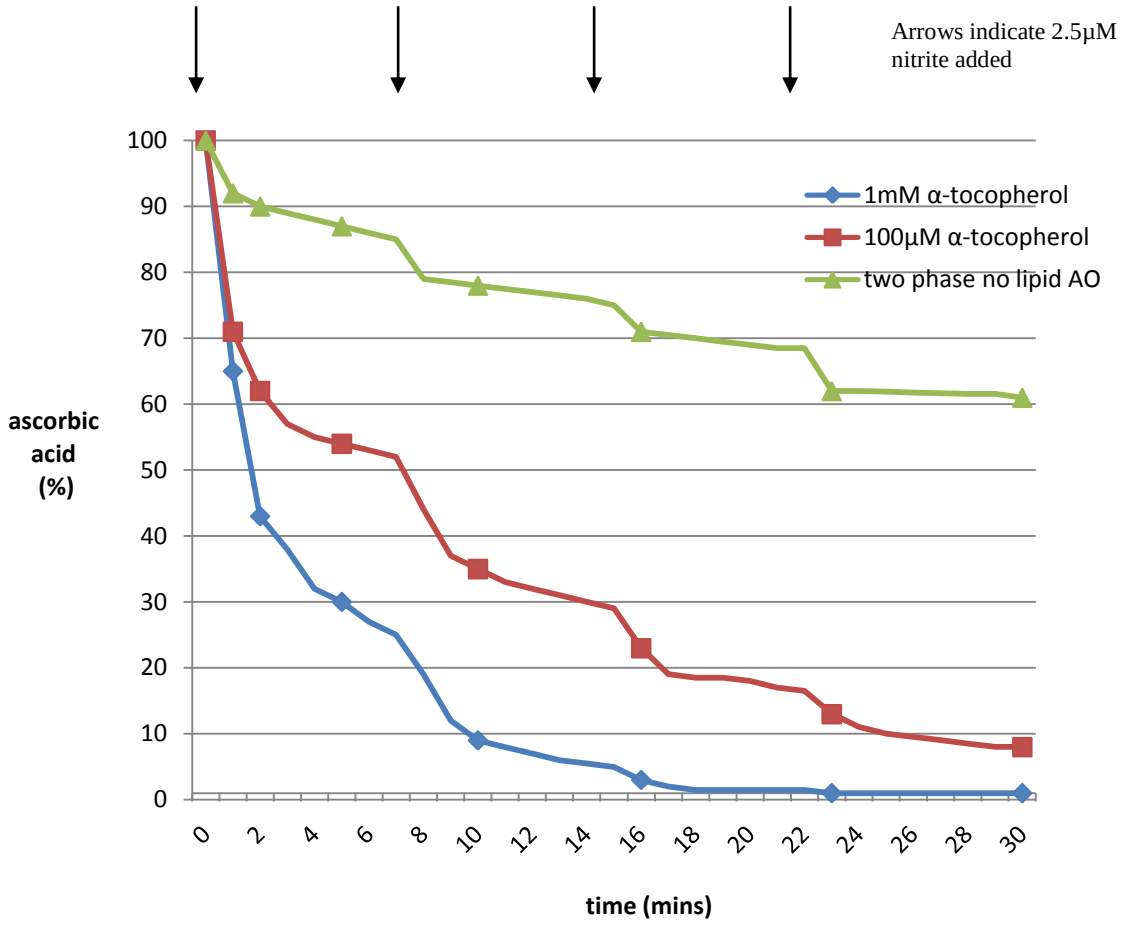


Figure 5.5: The mean % ascorbic acid remaining at given time intervals is shown for three different lipid designs in a two-phase model. Into an acidic aqueous phase containing 100 µM ascorbic acid was added a 2.5 µM bolus of nitrite at times 0, 7.5, 15 and 22.5 minutes (this is represented by the arrows at the top of the diagram). The lipid phase had one of: no antioxidant (AO) (green line), 100 µM α-tocopherol (red line) or 1mM present (blue line). n=12

5.3.6 A-tocopherol concentration from 2 phase model experiments in the presence of excess ascorbic acid (1mM) and in conditions where ascorbic acid (100 µM) will be consumed

In 30 minute two-phase experiments with 100 µM α-tocopherol present in the lipid phase in the presence of excess ascorbic acid (1 mM) relative to nitrite (100 µM), the mean α-tocopherol present at the end of the 30 minute experiment was 34 (±7) µM. In experiments where 100 µM ascorbic acid and 100 µM nitrite were added to otherwise the same experimental design, there was statistically significant reduction ($p<0.01$) in the mean α-tocopherol present, with a concentration of 8 (±6) µM. When smaller concentrations of α-tocopherol were assessed, the results were more dramatic: In experiments where 10 µM α-tocopherol was used in the lipid, with an excess of ascorbic acid (1mM), and 100 µM nitrite added, the mean α-tocopherol concentration at 30 minutes was 7.2 (±0.4) µM. However, when 100 µM nitrite was added to aqueous solution containing 100 µM ascorbic acid, there was no α-tocopherol present at 30 minutes in all twelve replicate experiments.

5.3.7 Ascorbic acid consumption in a three-phase model

In the upper layer of a three-phase system, where the lipid phase has no antioxidant present, there was a mean % ascorbic acid, remaining at 30 minutes, of 77.3 (±4.6) %. The available ascorbic acid at 30 minutes when there was 10mM α-tocopherol present was 71.9 (±3.1) % of the starting concentration. The difference in available in ascorbic acid at 30 minutes in experiments with α-tocopherol is statistically significant ($p=0.004$) (figure 5.6).

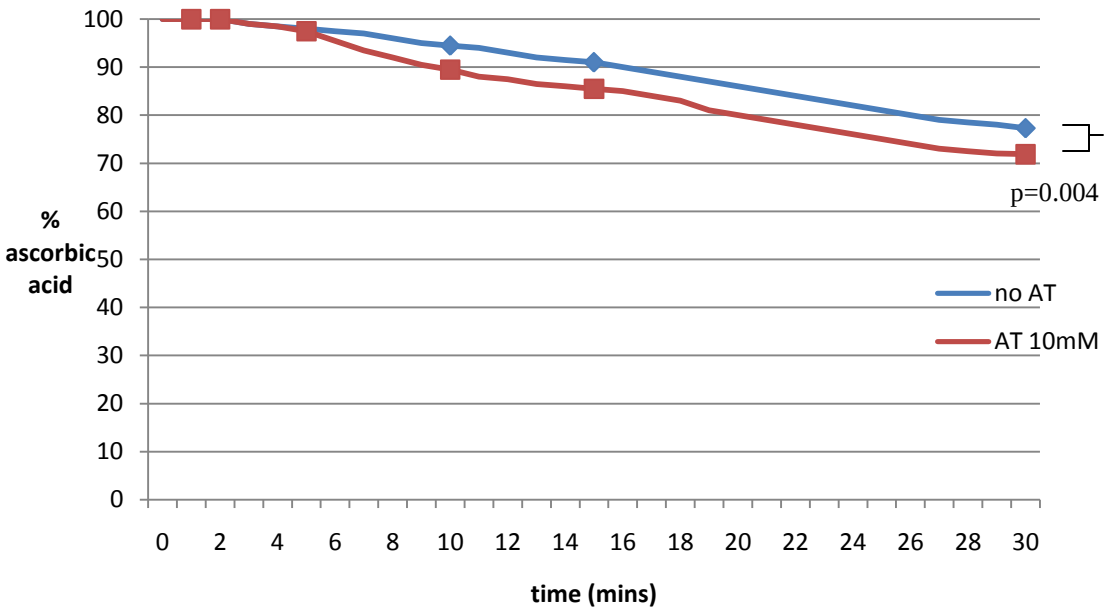


Figure 5.6: Mean percentage ascorbic acid in the upper layer of a three-phase model compared to the original concentration is shown against the 30 minutes of the experiment. The upper layer contained 150 µM ascorbic acid at pH 7.4 at the start of the experiment. Experiments were initiated by adding 100 µM nitrite into a lower phase containing 1 mM ascorbic acid in an acidic aqueous phase. NO generated in this lower aqueous phase diffuses through a middle lipid layer into the upper layer. The middle layer was composed of triacetin supported on a hydrophobic membrane, with 10 mM α-tocopherol (AT) either present (red line) or absent (blue line). n=12

5.4 Discussion

The possible replenishment of the reducing capacity of α-tocopherol by ascorbic acid has been an area of interest for five or six decades. Previous studies have provided contradictory evidence for this recycling process (174,175,263,264,265). The experiments performed within this chapter would suggest that this process does occur, at least in an *in vitro* setting representative of the chemistry occurring at the gastroesophageal junction.

Initially, our interest in this area of antioxidant recycling was stimulated by finding that the concentration of α-tocopherol required to inhibit nitrosation was only 10μM. This is a surprisingly low concentration of antioxidant to inhibit the NO concentrations of over 80μM which were generated within this system. This suggests that one molecule of α-tocopherol is able to scavenge many radicals. Normally one molecule of α-tocopherol would reduce one molecule of NO by a one or two electron transfer reaction (150). Therefore 10μM of α-tocopherol would only be expected to reduce 10μM of NO. We also found that when the time course of the experiment was increased from 15 to 60 minutes, the inhibitory effect of α-tocopherol was largely overcome, as 9.55 (±0.71) μM NMOR was produced at 60 minutes when 10μM α-tocopherol was present in the lipid phase. This loss of inhibition of nitrosative stress was also observed when a smaller concentration of aqueous ascorbic acid relative to initiating nitrite concentration was used in a two-phase model. It is known that one molecule of ascorbic acid will convert two molecules of NO (137). Therefore, in the model used, the ascorbic acid would be

expected to be consumed and this was confirmed in our experiments. In fact, ascorbic acid was consumed within 5 minutes of the addition of nitrite. A proposed explanation for the results seen in our model using low concentrations of ascorbic acid is that a small concentration of ascorbic acid was quickly consumed in inhibiting nitrosative stress within the lipid phase, so that there was little regeneration of oxidised α-tocopherol by ascorbic acid. Therefore, nitrosation of the morpholine was able to take place uninhibited after the small concentration of α-tocopherol was consumed. Consequently, this means that NMOR produced in a two-phase model with 10μM α-tocopherol, and a reduced concentration of ascorbic acid, is much greater than the NMOR concentration where ascorbic acid concentrations are in excess.

In the models used in these experiments we were able to control the aqueous and lipid phases, and also able to sample relevant concentrations of the chemical reagents from them. This allowed us to measure directly the ascorbic acid and α-tocopherol concentrations. This required minor revisions to our method of ascorbic acid measurement, as initially some of the samples were too dilute. Nevertheless, once this problem was overcome, accurate measurements of ascorbic acid concentration were able to take place.

In a single phase model, where ascorbic acid was in the aqueous phase to excess compared to the nitrite concentration added, the ascorbic acid concentrations fell quickly in the initial few minutes, and then there was only a gradual loss of

ascorbic acid and increase in dehydroascorbic acid. This reflects the fact that the ascorbic acid is initially reacting with nitrous acid to generate NO. This reaction occurs rapidly in the first few minutes and thereafter there is a recycling of this process, as NO reacts with O_2 to form RNOS such as N_2O_3, and ascorbic acid reacts with this to form further NO (248). This process gradually reduces the available ascorbic acid. In our single phase model there was initially total consumption of the O_2, but after 10 minutes there was once again a measurable O_2 concentration within the system. Despite O_2 being present there was not complete consumption of all ascorbic acid within this model. There was a mean concentration of just over a third of the starting ascorbic acid available at 60 minutes. In two-phase models, for both single globule and multiple globules of tributyrin, the ascorbic acid concentration profile with time, showed no significant difference to that found in the single phase system. Although the aqueous chemical reactants were not changed by adding the lipid phase, given that NO is very lipophilic (251) it would be expected to diffuse into the lipid. Despite this, the measurable NO and O_2 were not significantly different in the single and two-phase models. Therefore, it is not surprising that the ascorbic acid concentrations were similar in these models. However, in a two-phase model where α-tocopherol was present there was a significant reduction in ascorbic acid concentration as NMOR production is inhibited. A proposed model for the chemical reactions occurring to account for these findings is shown in figure 5.7.

At the gastroesophageal junction in humans it has been suggested that pulses, rather than a one off bolus of NO, are generated after foodstuff has been swallowed

(221). The pulsing of NO leads to pulses of nitrosative stress and this appears to be more effective than a single bolus in overwhelming the antioxidant effect of ascorbic acid and α-tocopherol. In our experiments using several boluses of nitrite there was a considerable increase in the oxidation of ascorbic acid to dehydroascorbic acid, and even at low concentrations of initiating nitrite, ascorbic acid was very quickly consumed.

The three-phase model allowed us to look at an experimental model where the ascorbic acid was present in an environment with a constant O_2 supply. We were also able to control the delivery of NO into the acid rich environment with ascorbic acid present as the NO diffused through a lipid layer into the aqueous environment from where the ascorbic acid was measured. The phase from which the ascorbic acid was being measured was representative of the epithelial compartment. This means that the ascorbic acid in this compartment was in a different aqueous phase from the ascorbic acid that was involved in the generation of NO from nitrite. This may partly explain why the consumption of ascorbic acid in this phase was not as great as had been seen in two-phase experiments with the same concentrations of α-tocopherol present. It is likely that most of this difference was due to the concentration of NO that was present in the upper layer. Nonetheless the difference in ascorbic acid oxidation between experiments where higher concentrations of α-tocopherol were present and absent was statistically significant. There was not a significant difference in ascorbic acid oxidation when the concentration of α-tocopherol was less than 1mM. This may be due to effective recycling of the α-

tocopherol by ascorbic acid from the lower compartment. It is likely that most of the nitrosative stress was occurring at the interface between the lower and middle layers.

The *in vitro* models do not fully represent the *in vivo* setting, in that there was neither the full array of chemical reactants in the epithelial compartment to generate all the possible nitrosating compounds nor the physical conditions such as blood flow. However, the experiments discussed in this chapter add to the body of evidence to support a process of recycling between ascorbic acid and α-tocopherol.

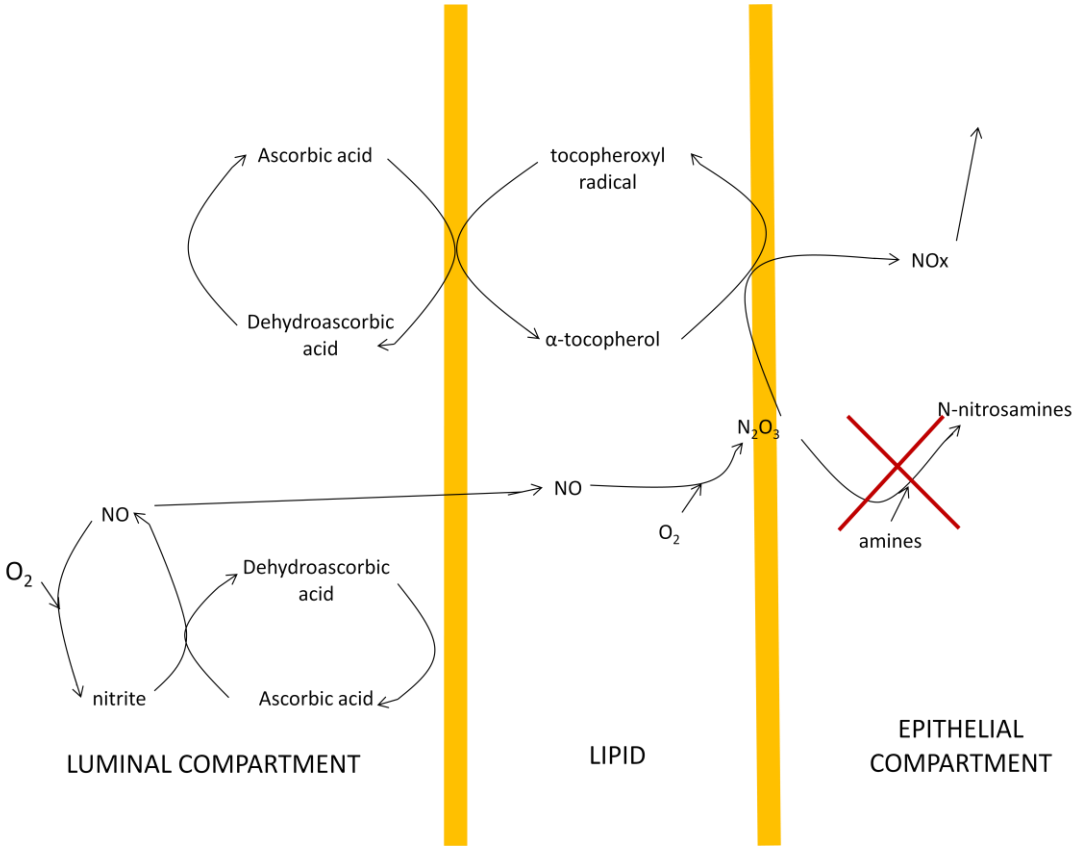


Figure 5.7: Proposed method of recycling of α-tocopherol by ascorbic acid. In the reaction of NO with O_2 in the luminal compartment nitrite is formed. Nitrite is reduced to NO by ascorbic acid, which is oxidized to dehydroascorbic acid. In the lipid NO and O_2 react to form nitrite and nitrosating species such as N_2O_3. In the presence of α-tocopherol N_2O_3 forms NOx (including NO) while α-tocopherol is oxidized to tocopheroxyl radical. The ascorbic acid is able to replenish α-tocopherol through its conversion from the tocopheroxyl radical. In this process the nitrosation of amines is inhibited.

Chapter 6 Development of silastic tubing model suitable for measuring *in vivo* nitrosative stress

6.1 Introduction and Aims

N-nitrosation from dietary nitrate may be, in part, responsible for the increasing incidence in adenocarcinoma at the gastroesophageal junction. Work presented in this thesis adds to the growing body of evidence, (44,144,221,222) from *in vitro* work, suggesting that there is the chemical potential for nitrosation at and around the gastroesophageal junction in the healthy acid-secreting stomach. However, there has not been any previous *in vivo* work that effectively assesses *N*-nitrosation, in the presence of ascorbic acid, in this region. The purpose of the following study was to develop an *in vitro* model that would measure nitrosation and could subsequently be used *in vivo*. The tubing had therefore to be suitable not only to allow nitrosation within the lumen, but also be comfortable for a human subject to swallow. The tubing would thereafter sit within the oesophagus and stomach, straddling the gastroesophageal junction. It was considered important to know what was happening chemically along the length of the tube at various sites within the upper gastrointestinal tract, therefore the tubing had to be compartmentalised into chemically distinct and intact segments of a given length. There have been previous studies examining nitrosative stress in bench top models, using silastic tubing to form an epithelial compartment (44). These studies showed that in conditions simulating the healthy proximal stomach and gastroesophageal junction, there was

accumulation of nitrite and *N*-nitrosamine within an amine rich neutral environment, contained in a silastic tube lumen. The silastic tubing used in these experiments was very thin, with an internal diameter of 500µm and wall thickness of 250µm. Such tubing would therefore not be suitable for easy passage into the upper gastrointestinal tract, and the volumes contained within a 1cm section of this tubing would be very small, resulting in too small a volume for chemical analysis. The aim of this chapter was to develop the silastic tubing model further, such that it could be used in human subjects.

6.2 Experimental design

6.2.1 Silastic Tubing

It has been shown *in vitro* that a hydrophobic silastic membrane has similar properties to an epithelial membrane, in terms of NO and O_2 diffusion (273-276). Previous work has shown nitrosative stress within the lumen of the tubing made from such material, when NO has been present outside and a nitrosatable compound inside (44). Silastic tubing from Altec was used (Altesil High Strength Silicone Tubing, Altec, UK). This is a blended silicone elastomer tubing which is non toxic and translucent, also known as polydimethylsiloxane. It withstands a maximum temperature of 220^{o}C and a minimum temperature of -55^{o}C with a density of 1.13g/cm^{3}. The core diameter had to allow a volume adequate for subsequent analysis of a contained liquid, while the thickness of the silastic wall had to be suitable for diffusion of O_2 and NO. Silastic tubing of core diameter 2.5 mm and wall diameter 0.5 mm was chosen, as this allowed internal content of 80-100 µL per 1.5 cm length of the tube, while allowing diffusion of NO and O_2 through the wall.

6.2.2 Compartmentalisation of silastic tubing

In order to determine nitrosation occurring at different sites along the upper gastrointestinal tract, the silastic tube had to be compartmentalised into chemically distinct sections. Compartments were made at approximately 1.5 cm intervals along

the tubing after filling the lumen with a solution of pH 7.4, with a range of concentrations of morpholine present. To ensure that the tubing was adequately compartmentalised and that each individual compartment would not receive contamination from neighbouring compartments, the tubing was twisted at the appropriate length (1.5 cm). Over the top of this twist a double knot was applied using vicryl suture ties (Ethicon, Gibco, Paisley, UK). The double knot was placed at both ends of the twist.

Several studies were performed to ensure that the compartmentalisation was successful, both in terms of direct fluid contamination between segments, and also as regards gaseous contamination along the length of the tubing. In order to be of any value the silastic tubing had to be compartmentalised into sections that could later provide detail on nitrosative stress related to the anatomy of the upper gastrointestinal tract. The integrity of the sections, therefore, had to be maintained both in the liquid and gaseous phases. This is particularly important as it is known that NO diffuses through lipids rapidly (251,273). Such diffusion could alter where nitrosative stress would appear to be occurring in relation to sections of the tubing, thus misrepresenting the true site. Fluid volume from within the lumen was removed using a glass syringe and this sample was subjected to analysis for nitrite and NMOR concentrations.

6.2.3 Experimental models used

Four different models were used in the following experiments. All of the models are similar but there are significant differences that allow different aspects of the chemistry, and of the silastic tubing, to be assessed.

Model 1 - This was the basic model used within this chapter. It can be used to generate NO in aqueous phase with silastic tubing present. It was set up as follows:

A 50 mL glass scintillation vial was filled with: Hydrochloric acid pH 1.5, ascorbic acid (1 mM), EDTA (1 mM), NaSCN (1 mM), and varying concentrations of $NaNO_2$ (10-100µM).

Silastic tubing, filled with varying concentrations of the secondary amine morpholine (5 mM, 10 mM, 25 mM and 100 mM) buffered to pH 7.4 using 100 mM PBS, was placed into the scintillation vial and completely surrounded by the aqueous solution within the glass vial (figure 6.1).

The model was placed in a water bath at 37^{o}C for 15 minutes. From previous studies we know that in an acidic environment in the presence of a reducing agent such as ascorbic acid, the nitrous acid formed in an acidic environment from nitrite, is converted to NO. It can therefore be expected that NO will be formed in the aqueous environment in the scintillation vial. This will diffuse through the silastic tubing wall and, through the formation of nitrosating species (principally N_2O_3), will

nitrosate the morpholine to form NMOR. A large accumulation of nitrite would also be expected intraluminally in the silastic tubing.

At the end of the 15 minute incubation, the silastic tubing was taken out of the scintillation vial and laid lengthwise along a bench. The fluid from each individual compartment was then removed, using a 100 µL steel Thomson syringe. Of this volume, 10 µL was taken for nitrite analysis and 40 µL was subjected to an extraction process for NMOR. These experiments were repeated twelve times.

Model 2 – This was a modification of the basic model to allow for assessment of diffusion of NO along the length of the tubing causing contamination of neighbouring compartments which had not been directly exposed to NO. In order to do this we modified the *in vitro* experiment to only submerge half of the tube rather than the entire length. Therefore only four out of the eight were being exposed directly to NO. Morpholine (25 mM) was present in the tube to allow assessment of nitrosation. If nitrite and NMOR were detected in the unexposed compartments it would suggest that there had been diffusion through either the wall of the tubing, or through the knots which are meant to be compartmentalising the tube. In all experiments involving model 2, the added nitrite concentration was 100 µM (Figure 6.2).

Model 3 – In this model there was a coating of α-tocopherol on either the inside or outside of the silastic tubing. To coat the outside of the tubing

concentrated ≥96% a-tocopherol was smeared along a length of it. To coat the inside of the tubing concentrated ≥96% a-tocopherol was injected into the lumen and then squeezed through the inside of the tube to form a thin film along its length. The tubing was then placed such that it passed through two beakers. In Beaker 1 it was exposed to an aqueous environment in the presence of nitrite, and in Beaker 2 the aqueous environment had ascorbic acid in addition to the nitrite. This allowed us to determine the effect of HNO_2 versus NO. The nitrite concentration used was 1mM $NaNO_2$, to allow for more in the way of α-tocopherol induced nitrosative stress, in the absence of ascorbic acid, to occur. The concentration of ascorbic acid present in Beaker 2 was increased to 5mM to allow for excess ascorbic acid over nitrite in these experiments. The silastic tubing was compartmentalised such that in each beaker there was one compartment with α-tocopherol either inside or outside the tubing. In all experiments, the incubation lasted 15 minutes before removing the silastic tubing fluid for nitrite analysis (Figure 6.3).

Model 4 - In this model experiments were performed with α-tocopherol smeared on the outside of the tubing, while varying the pH of the tubing contents through 0.6, 1.5, 2.5, to 4.5. Thiocyanate was also either present or absent within the tubing. The surrounding conditions in the aqueous phase were HCl (pH 1.5), EDTA (1 mM), $NaNO_2$ (1 mM) and NaSCN (1 mM). There was no ascorbic acid in the aqueous phase (Figure 6.4). All experiments were replicated twelve times.

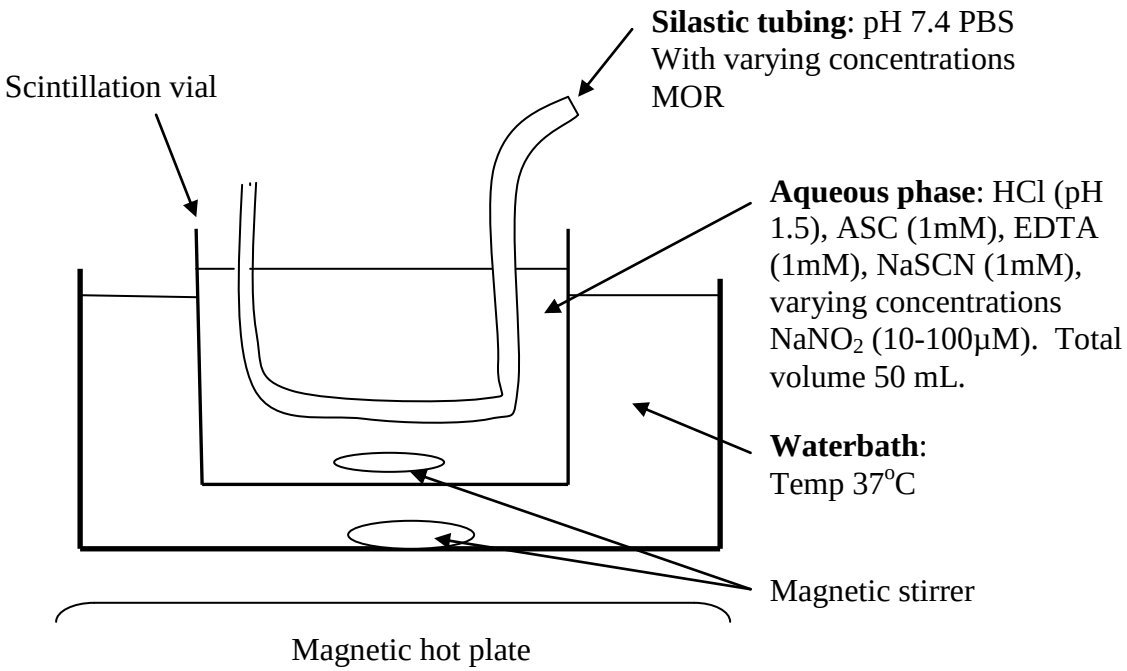


Figure 6.1: Model 1 was composed of two parts. A silastic tubing filled with a range of concentrations of the secondary amine morpholine buffered to pH 7.4 by PBS. The silastic tubing was surrounded by an aqueous phase within a glass scintillation vial. The aqueous phase consisted of HCl (pH 1.5), ascorbic acid (1 mM), EDTA (1 mM), NaSCN (1 mM), and a reaction forming NO was initiated by the addition of varying concentrations of $NaNO_2$ (10 µM, 25 µM, 50 µM, 100 µM). The model was placed on a magnetic hot plate to keep the aqueous phase at 37°C, under constant stirring.

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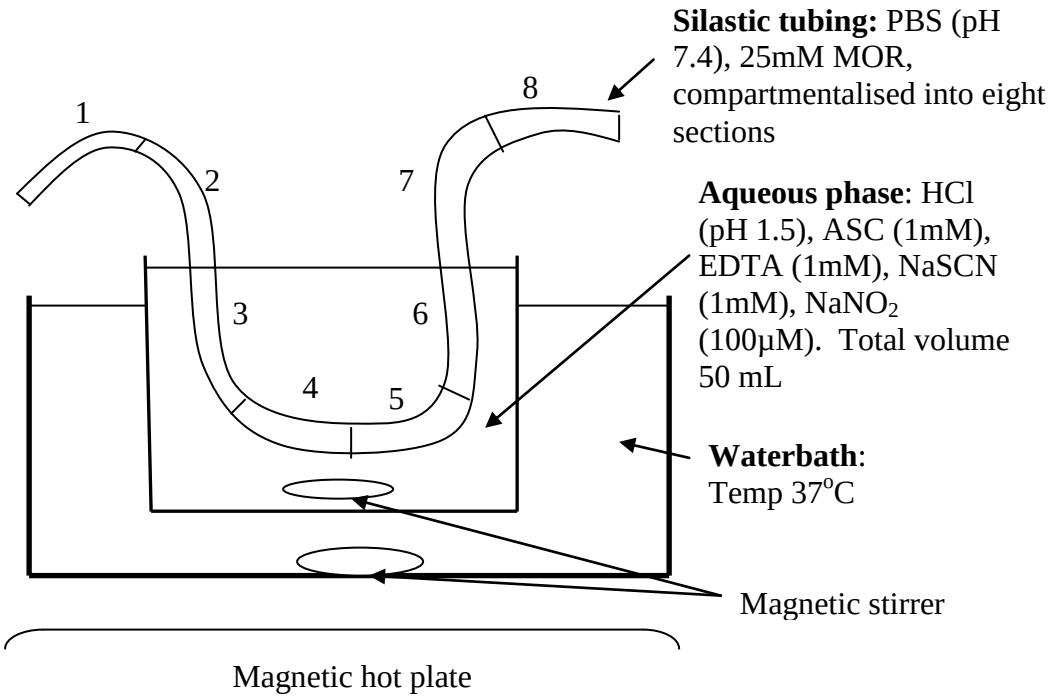


Figure 6.2: In model two the silastic tube contained 25 mM morpholine buffered to pH 7.4 by 100 mM PBS. The tube was compartmentalized into 8 sections. Only sections 3-6 of the tubing were exposed to the aqueous phase contained within a glass scintillation vial. Sections 1, 2, 7, 8 lay exposed to air only. Section numbers are shown in the above diagram. The aqueous phase had a total volume of 50 mL, and consisted of HCl (pH 1.5), EDTA (1 mM), NaSCN (1 mM) and ascorbic acid (1 mM). 100 µM $NaNO_2$ was added to the aqueous phase to initiate the generation of NO. The model was placed on a magnetic hot plate to keep the aqueous phase at 37°C, under constant stirring.

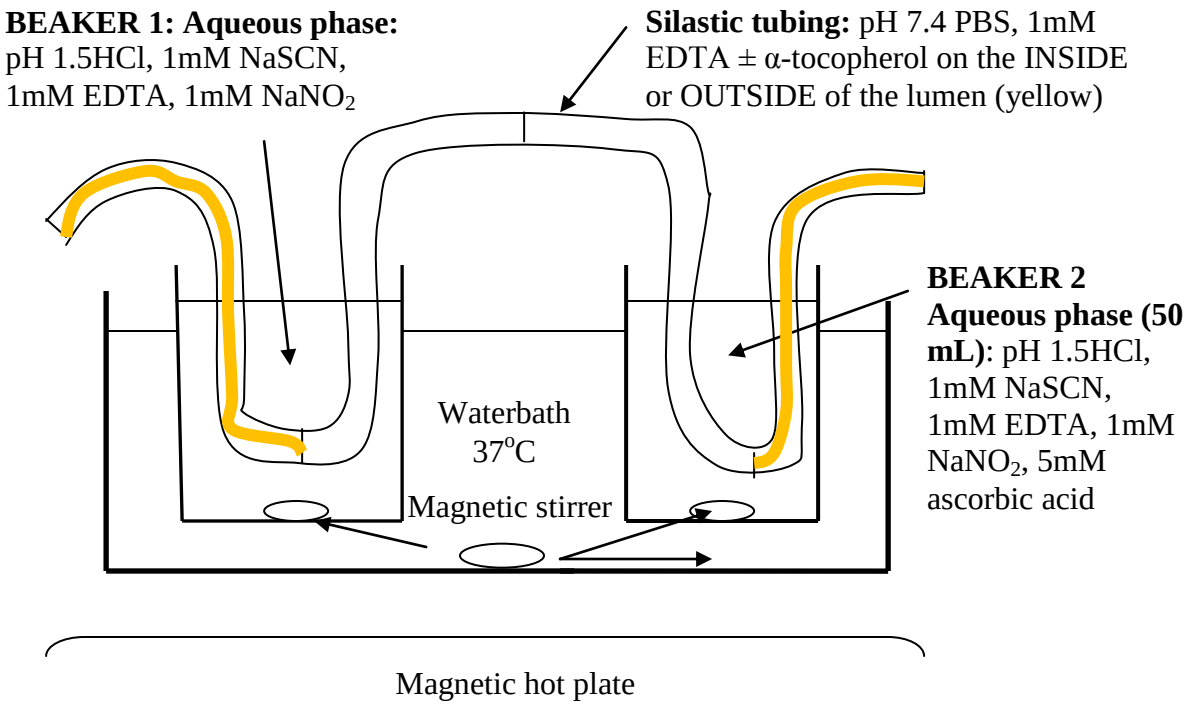


Figure 6.3: Model 3 consisted of two glass beakers of 50 ml total volume. In beaker 1 there was HCl (pH 1.5) with NaSCN (1 mM), EDTA (1 mM) and $NaNO_2$ (1 mM). Beaker 2 had the same contents as beaker 1 but with ascorbic acid. Two sections of silastic tubing were then placed into each of the beakers, while four others lay out with. The lumen of the tubing contained PBS (pH 7.4), EDTA (1 mM) and in one section of tubing per beaker a thin lining of concentrated ≥96% α-tocopherol (α-TP). In each beaker, exposed to the aqueous phase, a control length of tubing had no α-tocopherol present. The model was placed on a magnetic hot plate to keep the aqueous phase at 37°C and to allow stirring of the beaker contents through a magnetic stirrer.

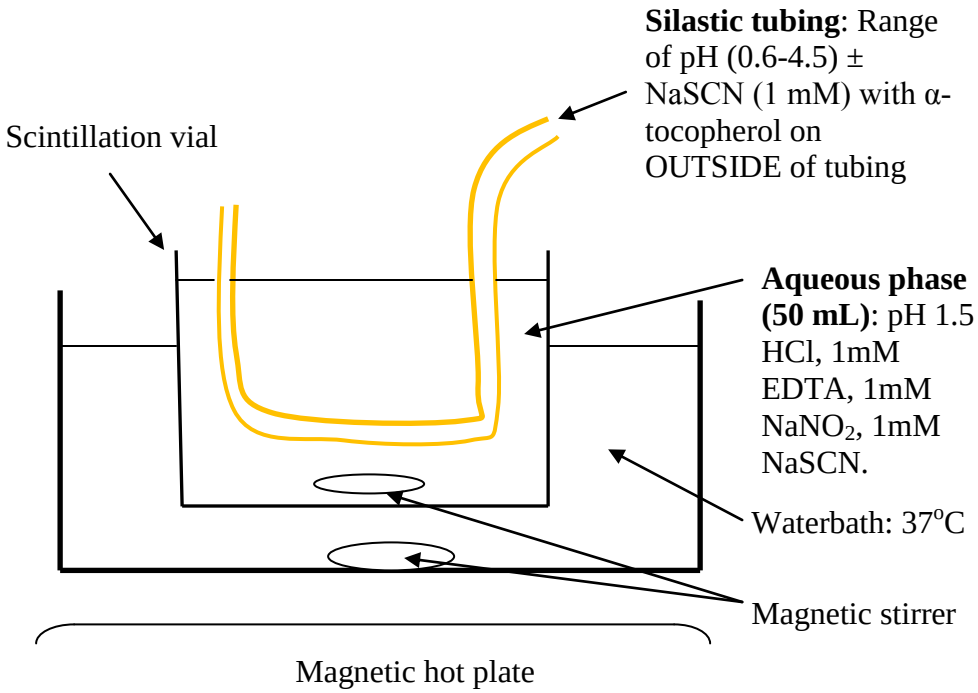


Figure 6.4: In model 4 silastic tubing was coated with concentrated α-tocopherol on the outside while in others the silastic tubing was unaltered. Silastic tube luminal pH was one of 0.6, 1.5, 2.5 or 4.5 adjusted by using HCl and PBS. NaSCN (1 mM) was either absent or present in the silastic tubing. The tube was placed into a glass beaker containing an acidic aqueous phase. The aqueous phase was made up of HCl (pH 1.5), EDTA (1mM), NaSCN (1 mM) and $NaNO_2$ (1mM) was added to initiate the reaction. In all experiments using this model there was no ascorbic acid present.

6.3 Results

6.3.1 Experiments using silastic tubing in model 1

6.3.1.1 NO concentrations within the aqueous phase

NO was generated in the aqueous phase of model 1, at a peak concentration of 89 (± 4) µM at time 30 seconds, and continued to be produced in high concentrations throughout the time course of the experiments, when the 100 µM nitrite was the concentration added to the experimentantal model (figure 6.5). The relationship of NO produced was linear to the concentration of nitrite added (r^2=0.96) (figure 6.5).

6.3.1.2 Silastic Tubing Nitrite Concentration

The mean silastic tubing nitrite concentration at 15 minutes when 100µM nitrite was added into the aqueous phase of the bench top model was 705 (±25) µM. When other concentrations of nitrite were added to the aqueous environment the silastic tubing mean nitrite concentration showed a linear relationship (r^2=0.92) to the concentration of nitrite added as shown in figure 6.6.

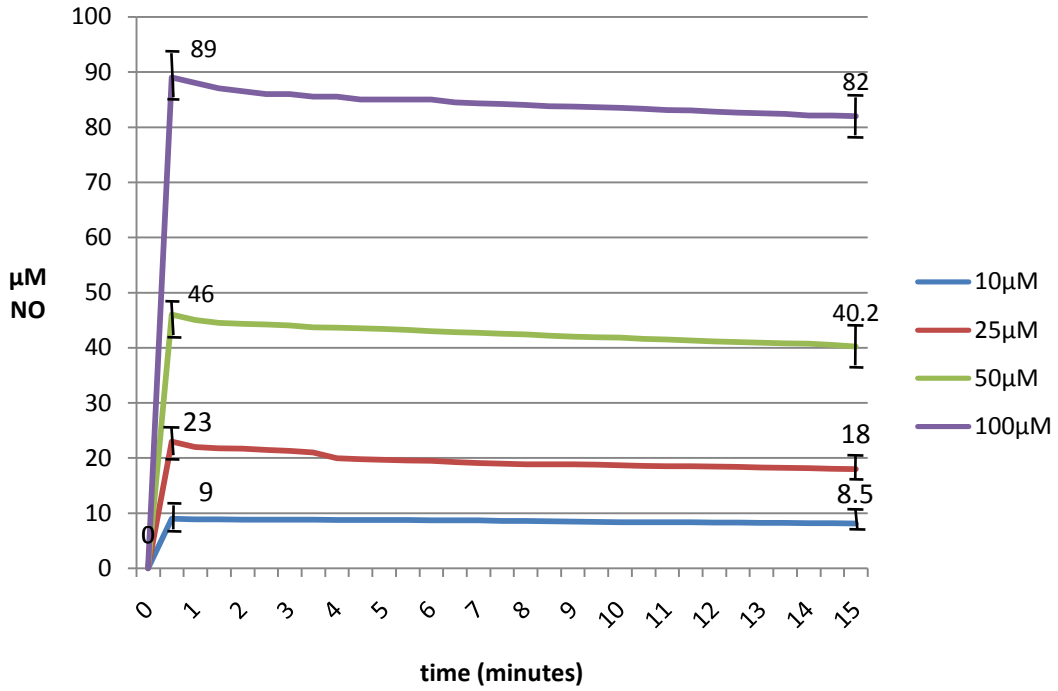


Figure 6.5: Mean NO concentrations within the aqueous phase of the model 1 are shown in relation to time (minutes). Concentrations of NO are shown for four different concentrations of nitrite (10, 25, 50 and 100µM) which were added to the acidic aqueous phase containing 1mM ascorbic acid. Data labels show the mean concentration at 1 minute (maximal concentration) and 15 minutes. Error bars represent 1 standard deviation. n=12

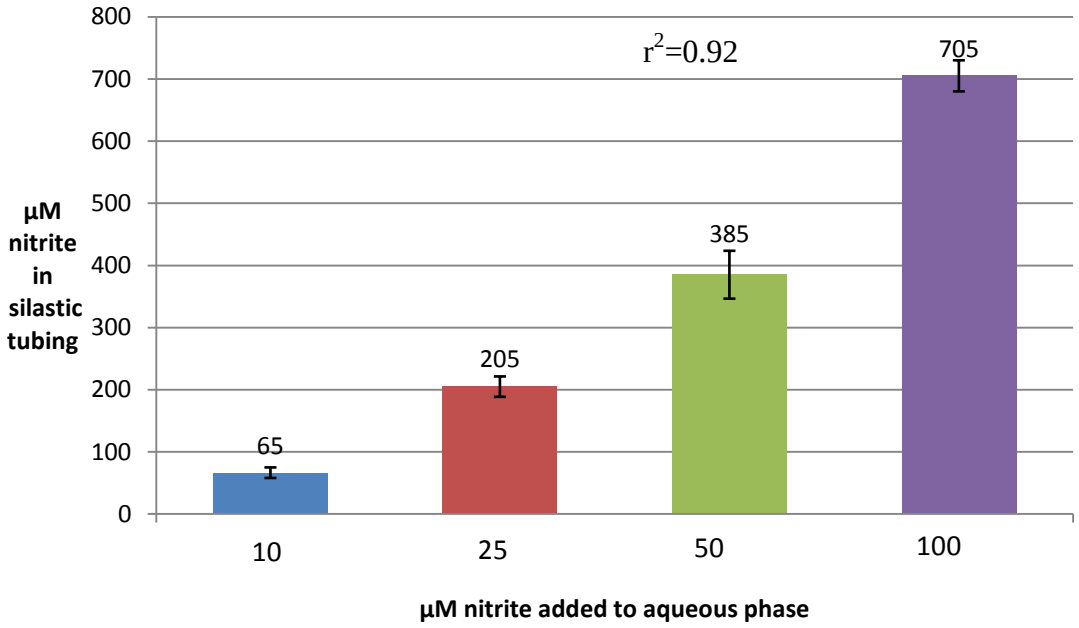


Figure 6.6: Mean silastic tubing concentrations are shown at the end of a 15 minute experiment, where silastic tubing with a neutral luminal compartment was exposed to an acidic phase containing ascorbic acid, to which was added one of four nitrite concentrations (10, 25, 50 and 100µM) at time 0 minutes. Data labels show the mean nitrite concentration within the lumen. Error bars represent 1 standard deviation from the mean value. n=12

6.3.1.3 Silastic tubing NMOR concentrations with different silastic tubing morpholine concentrations

Model 1 was used but on this occasion varying concentrations of morpholine were placed within the silastic tubing (5 mM, 10 mM, 25 mM, 33 mM, 50 mM and 100 mM). Keeping all other experimental conditions the same, there was an increase in the mean NMOR concentration formed as the morpholine concentration was increased (figure 6.7). However, this relationship was not linear (r^2=0.69). When 100 µM nitrite was added to the aqueous phase surrounding the silastic tubing, the mean concentration of NMOR at 15 minutes, when 5 mM morpholine was present within the tubing, was 8.24 (± 3.11) µM. The mean silastic tubing NMOR concentrations at 15 minutes was 49.0 (±13.9) µM, when 25 mM morpholine was present within the tubing at the start of the experiment. This represents a nitrite to NMOR ratio of 1:14.

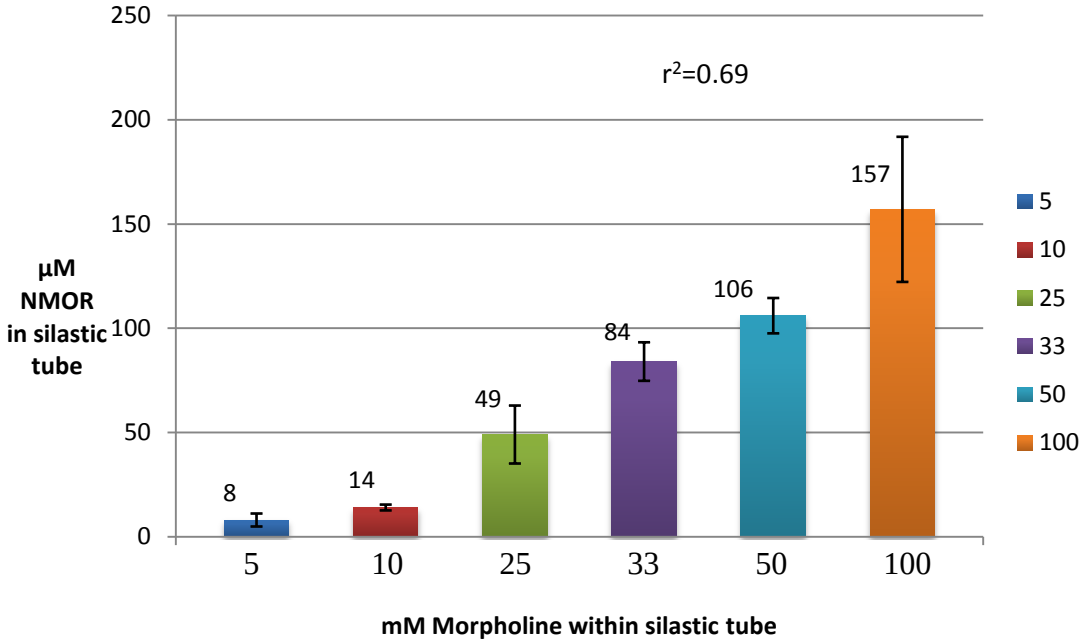


Figure 6.7: NMOR concentrations (µM) in the silastic tubing at the end of a 15 minute incubation, according to the concentration of secondary amine present (5, 10, 25, 33, 50, 100 mM). The tube was exposed to an acidic aqueous phase in the presence of ascorbic acid, with 100 µM sodium nitrite added to initiate the experiment. Data labels show the mean NMOR concentration within the tube. Error bars represent 1 standard deviation from the mean. n=12

6.3.2 Compartmentalisation of silastic tubing using Model 2

Two separate experiments were performed to assess for compartmentalisation. The first of these experiments looks at physical compartmentalisation in the aqueous phase, while the second experiment determines the effectiveness of compartmentalisation as regards the diffusion of gaseous NO.

6.3.2.1 pH Studies to determine successful compartmentalisation of silastic tubing in Model 2

This experiment was performed to assess for liquid compartmentalisation of the silastic tubing into sections. Into one open end of the silastic tube was introduced a solution of HCl (pH 1.5) using a Thomson injector needle, while into the other end was added PBS (pH 7.4). The pH was recorded at the start of the experiment, and also at the end of twelve hours to allow for any diffusion. This experiment was repeated six times. There was no recordable-statistical difference between the starting pH and the final pH in any of the experiments, indicating that there was no liquid diffusion contamination (table 6.1).

Table 6.1: Starting and finishing pH in 12 hour aqueous diffusion experiment to assess compartmentalisation of silastic tubing

	Starting pH (average of 6 experiments)	Finishing pH (average of 6 experiments)
Acid Compartment	1.5 ± 0	1.51 ± 0.01
Neutral Compartment	7.4 ± 0	7.38 ± 0.03

6.3.2.2 Studies to Assess Gaseous Contamination of Neighbouring Sections of Tubing

Given that NO can diffuse through the membrane, these studies were undertaken to determine whether NO could diffuse through the length of the wall of the tubing and contaminate neighbouring sections. Model 2 allows us to assess for nitrosation, by measuring nitrite and NMOR from sections deliberately exposed to NO, as opposed to neighbouring sections deliberately not exposed to NO. In this model 100 μM nitrite was added to the aqueous phase containing ascorbic acid. Results from this model show that only those compartments exposed to NO had significant concentrations of nitrite and NMOR (table 6.2). The concentration of

nitrite in those compartments not exposed to NO was 5 (±2) µM, while the average concentration in the compartments exposed to NO was 689 (±36) µM. The mean NMOR in the compartments exposed to NO was 50.13 (±11.5) µM while no detectable NMOR was found in any of the compartments not subjected to NO.

Table 6.2: Luminal NMOR (µM) and NO_2 (mM) ± SD according to the section of silastic tube analysed at 15 minutes

Compartment	Submerged in NO containing aqueous phase	Mean nitrite µM (±SD)	Mean NMOR µM (±SD)
1	No	3 (±2.5)	N.D.
2	No	8 (±3.0)	N.D.
3	Yes	705 (±35)	53.8 (±9.5)
4	Yes	657 (±28)	48.1(±10.6)
5	Yes	721 (±42)	46.3 (±11.0)
6	Yes	676 (±39)	52.3 (±14.7)
7	No	7 (±1.5)	N.D.
8	No	5 (±0.5)	N.D.

This series of results demonstrates appropriate compartmentalisation such that there is no aqueous diffusion through the twists and no diffusion of NO along the length of the silastic tubing.

6.3.3 Studies using model 3 with α-tocopherol on the outside and inside of the tube with luminal pH 1.5

Comparison between tubing smeared with α-tocopherol on the inside and the tubing not so treated showed that, in the presence of ascorbic acid, when the tube was exposed to 1 mM acidified nitrite, there was a significant reduction in the nitrite concentration where α-tocopherol was present (p=0.0000002) (figure 6.8). The mean nitrite concentration in the tubing without α-tocopherol was 1831 (± 60.5) μM whereas the mean nitrite concentration was 1172 (± 65.6) μM when α-tocopherol (1mM) was present. In the absence of ascorbic acid there was a doubling in the mean nitrite concentration when α-tocopherol was present inside the tubing (p=0.000006). In experiments where there was no ascorbic acid there was a mean nitrite concentration of 229 (± 32.7) μM in tubing without α-tocopherol compared to 478 (± 20.1) μM when α-tocopherol was present inside the tubing. For experiments with α-tocopherol smeared on the outside of the tube, in the absence of ascorbic acid, the mean nitrite concentration from the inside of the tubing was 838 (± 45) μM. This reflects a four fold increase in nitrite concentration as compared to studies where there was no ascorbic acid and no α-tocopherol (p=0.0000004), and is double the concentration compared to when α-tocopherol is on the inside of the tubing.

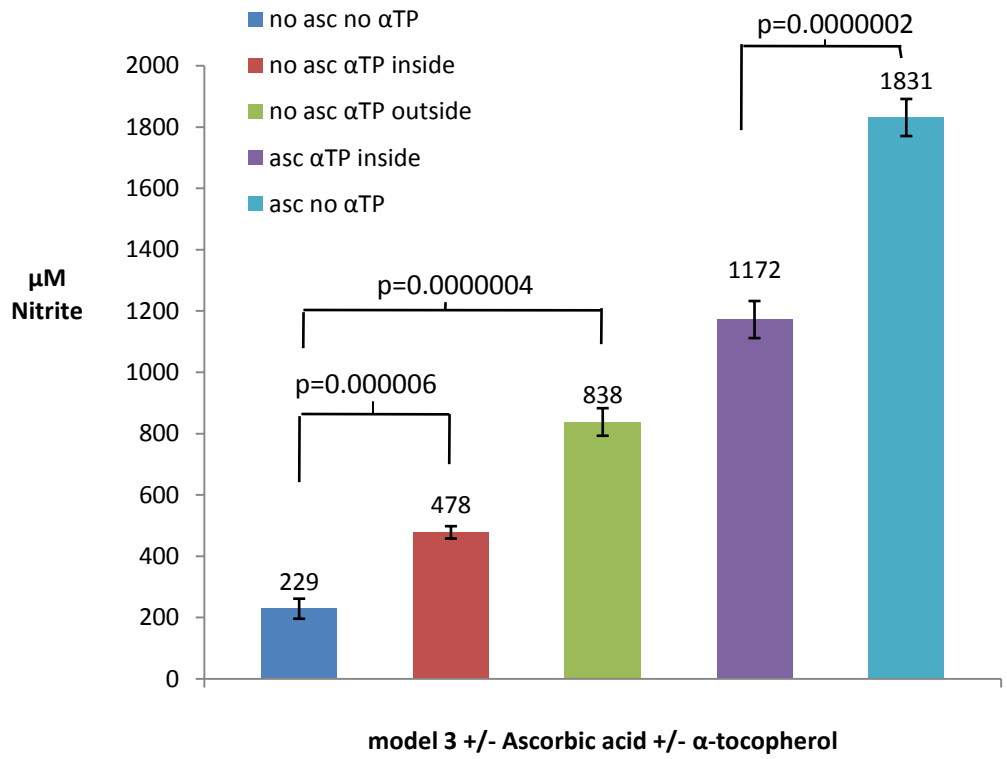


Figure 6.8: The mean silastic tubing nitrite under different experimental conditions after the addition of 1mM sodium nitrite into an acidic aqueous phase surrounding silastic tubing. The different experimental conditions are in the absence of ascorbic acid (asc) and α-tocopherol (α-TP) (dark blue column), absence of ascorbic acid with concentrated α-tocopherol smeared on the inside of the tubing (red column), in the absence of ascorbic acid with concentrated α-tocopherol smeared on the outside of the tubing (green column), 5mM ascorbic acid present in the aqueous phase with concentrated α-tocopherol smeared on the inside of the tubing (purple column), 5mM ascorbic acid present within the aqueous phase with no α-tocopherol present (light blue column). Data labels show mean values. Error bars represent 1 standard deviation.

6.3.4 Model 4 with A-tocopherol on the outside of silastic tubing with varying pH of the luminal silastic tube compartment and no ascorbic acid in the aqueous phase

At pH 4.5 no nitrite was found within the tubing whether thiocyanate was present or not. In experiments with reduced pH, there was increased nitrite formation within the tubing in the presence of α-tocopherol, with six times the nitrite concentration present at pH 0.6 compared to pH 2.5 ($p<0.001$) (figure 6.9). Thiocyanate made the greatest percentage increase in silastic tube nitrite, in the presence of α-tocopherol when the pH was 1.5, as it led to over a doubling of nitrite concentration compared to when α-tocopherol was present alone (326 μM to 823 μM). At a pH of less than 4.5 the acidity of the solution made no difference to the effect of thiocyanate on silastic tube mean nitrite concentrations, in the absence of α-tocopherol ($p>0.2$). There was also no difference in silastic tube mean nitrite across the range of pH's less than 4.5 in the absence of both thiocyanate and α-tocopherol ($p>0.05$).

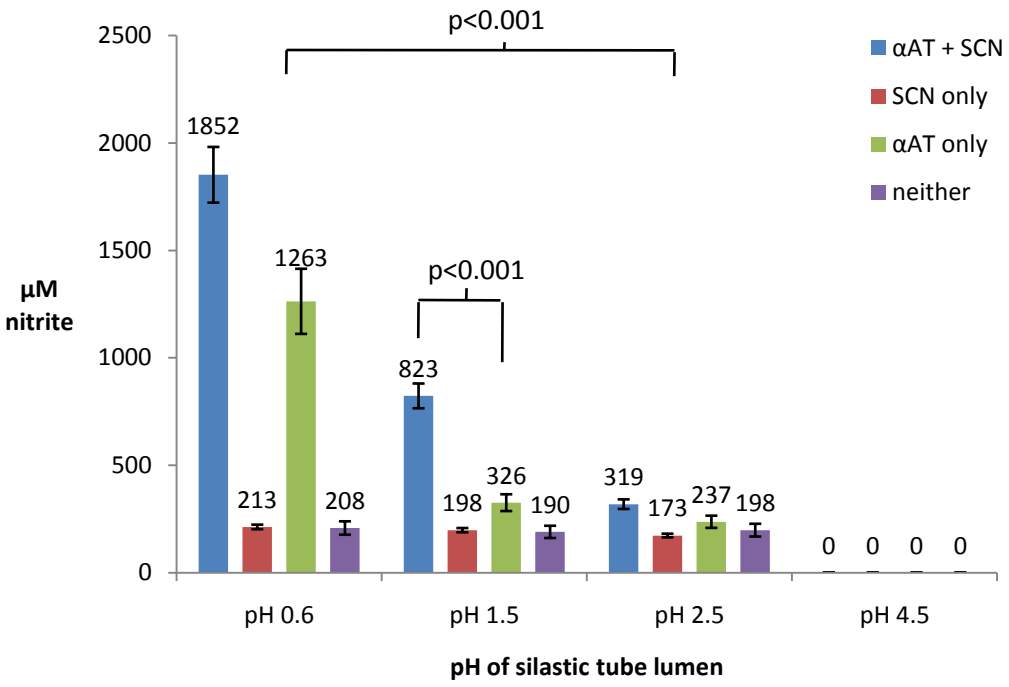


Figure 6.9: The mean nitrite concentration within silastic tubing with different pH of the silastic tubing compartment. Within the silastic tubing there was one of four experimental conditions. There was a combination of the presence or absence of α-tocopherol (αTP) and thiocyanate (SCN). The tubing was bathed in an acidic aqueous phase which always contained EDTA (1 mM), NaSCN (1 mM) and ascorbic acid (5 mM), and to which was added $NaNO_2$ (1 mM) to initiate the generation of NO. Data labels show the mean nitrite concentration. Error bars represent 1 standard deviation. n=12

6.4 Discussion

This chapter describes the design of a useful model for nitrosative stress *in vitro* which can be transferred to the *in vivo* setting. Silastic membranes have been known to behave in a similar way to epithelial lipid membranes, in terms of NO and O_2 diffusion, for some time, and have been successfully used in several different experimental models (44,273-277). In previous studies, silastic tubing had been used to show nitrosative stress in bench top models of the chemistry occurring in the upper gastrointestinal tract, where salivary nitrite meets acidic gastric juice (44). We have taken this model and made adjustments to provide a potentially useful tool for *in vivo* studies. This model has had to be developed in stages to show robustness, allowing its use *in vivo,* while still demonstrating nitrosative stress. The model has been adapted further still, to create a more physiologically representative situation where lipid antioxidants are also present. The use of lipid antioxidants in this setting has provided information relating to the chemistry of nitrosative stress, secondary to the generation of NO from nitrite.

In model 1, after the addition of nitrite to the acidic aqueous phase in the presence of ascorbic acid, NO is generated. NO will rapidly diffuse into and through the silastic membrane (120,251). As NO passes through the silastic tubing, it reacts with O_2 to form N_2O_3, (273). N_2O_3 can then nitrosate secondary amines such as morpholine (120,278). N_2O_3 also reacts with water in the neutral pH of the lumen of the tubing, to form nitrite (279). The nitrite formed in this way is trapped within the

lumen and is indicative of the flux of NO into the tubing. The exact NO concentrations generated within the lumen of the human upper gastrointestinal tract, at any one time, depend mainly on the delivery of nitrite from saliva. There is evidence that after a nitrate rich meal there, high concentrations of NO in excess of 100μM are produced (43,45,280). This study, showed that across a range of NO concentrations from 10-100μM detectable levels of nitrite are generated within the neutral lumen of silastic tubing. This is particularly useful information, as the thickness of the wall of tubing used in these experiments was considerably greater than those used in a previous model developed by Iijima et al (44). It may have been that with the increased thickness, there was not sufficient passage of NO to generate nitrosative stress, at the concentrations of NO we expect to find *in vivo*. The thickness of the tubing was increased to facilitate passing it nasogastrically, without coiling in the oropharynx. This also allowed us to use a tube with a greater internal capacity, and thus provide us with more volume for subsequent nitrite and NMOR analysis. The silastic tubing nitrite concentrations increased linearly with the increase in aqueous phase NO concentrations used in these experiments making the tubing a useful tool for measuring the amount of nitrosative stress produced over the range of NO concentrations we would expect to encounter at and around the gastroesophageal junction.

There was an increase in the NMOR concentration generated as the concentration of morpholine was increased within the silastic tubing, although this relationship was not linear. At the concentrations of NO we may expect to find *in*

vivo after a nitrate meal, the most suitable morpholine concentration was 25mM as this provided a NMOR concentration which was within the range of concentrations which were easily measurable, using the same nitrosamine analysis and calculations as were already in place for previous experiments. The simultaneous increase in NMOR and nitrite suggests this is largely mediated by NO produced by the reaction of nitrite with ascorbic acid, in the acidic aqueous phase of model 1. Previous work has shown that in the absence of ascorbic acid in the aqueous phase, there was accumulation of nitrite within the luminal compartment of silastic tubing, although the concentration found was not as high as when ascorbic acid was present. However, only very low concentrations of silastic tubing *N*-nitrosamine were formed when there was no ascorbic acid present. The accumulation of some nitrite can be partly explained by nitrous acid passing through the membrane, and also by chemical species such as N_2O_3 passing through the membrane in small concentrations into the lumen of the tubing, where they can then be oxidised to nitrite (44).

In order to be useful for defining anatomical areas of increased nitrosative stress there had to be compartmentalisation of the silastic tubing into small sections. To provide enough volume from the lumen, the tubing was divided into individual sections every 1.5 cm. This section length was also the smallest, technically feasible, with the diameter of tubing and the tying required. Several different methods of forming suitable compartments were attempted before the method described above was proven to be successful. The compartmentalisation had to be

intact, so that there was no aqueous diffusion between neighbouring sections. pH studies confirmed that there was little if any diffusion of aqueous contents of the tubing between sections. As NO is very lipophilic (251), we also had to ensure that there was no diffusion of NO along the length of the tubing, into neighbouring sections. The results presented confirm that there is minimal, if any, NO diffusion along the length of the tubing. Therefore, if a section of the tube is situated within a certain anatomical region, then the nitrite and NMOR concentrations from that section represent the nitrosative stress arising at that anatomical location.

The last part of this chapter looks at the presence of α-tocopherol inside and outside the silastic tubing. The intention in using α-tocopherol, had been to build up a physiologically relevant model, which was more representative of the conditions *in vivo*. There were limitations using models 3 and 4. The most important is that controlling the exact concentration of α-tocopherol smeared along the tubing was not possible. It may also be that where α-tocopherol was smeared on the outside of the tubing there was some of the lipid antioxidant distributed into the aqueous phase as small particles. There were, however, some interesting results from these studies. When ascorbic acid was present in an aqueous phase, α-tocopherol within the tubing resulted in a reduction in silastic tube nitrite of approximately 35% compared to when there was no α-tocopherol. However, in the absence of ascorbic acid in the aqueous phase α-tocopherol inside the tubing led to more than a doubling of the silastic tube nitrite concentration compared to when there was neither ascorbic acid

or α-tocopherol. The silastic tube nitrite concentration was higher still when α-tocopherol was smeared on the outside of the tubing, in the absence of ascorbic acid.

The reaction of α-tocopherol with NO was an area of much controversy in the mid 1990s. Reports of a direct oxidant effect of NO with α-tocopherol generating α-tocopheroxyl radical were suggested by several researchers (153-155). Other groups found that α-tocopherol was stable in the presence of NO (157,281). It appears that NO does not directly react with α-tocopherol, but rather α-tocopherol oxidation is due to oxidised metabolites of NO such as N_2O_3. The oxidised products of α-tocopherol include α-tocopheroxyl radical and α-tocopheryl quinine (158). It has been suggested that, in this way, α-tocopherol acts as an antioxidant to an even greater extent than aqueous antioxidants. Other studies have gone on to suggest a complex and as yet undefined oxidation process between α-tocopherol and NO, resulting in compounds such as the nitrite ester of α-tocopherol (282). It is known that when nitrite reacts with tocopherol NO is generated (283,284). This chemistry has relevance to the findings in our studies. In the presence of ascorbic acid in the aqueous phase, NO is generated, and diffuses across the silastic membrane, where it reacts with O_2 to form N_2O_3. In the absence of α-tocopherol, nitrite will accumulate from the reaction of this N_2O_3 with water. When α-tocopherol is present inside the tubing it will compete for the reaction with N_2O_3, resulting in less nitrite accumulating within the tubing. In an acidic aqueous environment, but in the absence of ascorbic acid, and with no α-tocopherol associated with the silastic tubing, there is still a small accumulation of nitrite within the tubing. This can be

explained by the fact that the pK_a of nitrous acid is 3.23 and that in acidic environments nitrous acid is produced. This nitrous acid will diffuse across the silastic membrane, although the rate of diffusion of this molecule will be considerably slower than the highly lipophilic NO molecule.

When α-tocopherol was smeared on the outside of the silastic tubing nitrite, and nitrous acid, will react with α-tocopherol in the aqueous phase of the model, producing NO which then diffuses across the silastic membrane into the aerobic neutral environment, producing nitrite within the lumen of the tubing. This can explain the high nitrite concentrations in silastic tubing when α-tocopherol was smeared on the outside of the tubing in the absence of aqueous ascorbic acid. That proposed mechanism of the production of α-tocopherol derived NO facilitates its diffusion from aqueous phase to silastic tubing is supported by results showing an altered intraluminal silastic tubing pH and the presence or absence of thiocyanate. A silastic tubing pH of 4.5 is above the pK_a of nitrite (pK_a 3.23). This explains why there was no accumulation of nitrite in any of the experimental conditions where α-tocopherol was smeared on the outside of the tubing. As the pH within silastic tubing became more acidic there was a proportional rise in silastic tubing nitrite concentrations when α-tocopherol was present on the outside of the tubing. There was no change in the silastic tubing nitrite concentrations with thiocyanate in the absence of α-tocopherol as the pH was more acidic. The presence of thiocyanate does however lead to higher nitrite concentrations in the presence of α-tocopherol. These findings suggest that the increase in epithelial nitrite, when α-tocopherol was

smeared on the outside of the tubing in the absence of aqueous ascorbic acid, was influenced by the presence of nitrosating species and therefore largely NO mediated.

It was also found that, even when α-tocopherol was smeared on the inside of the silastic tubing, in the absence of aqueous ascorbic acid, there was an increase in nitrite accumulation. In these experiments however, the concentration of nitrite within the tubing was only half that seen when α-tocopherol was smeared on the outside of the tubing. This is a more difficult observation to explain but may be due to α-tocopherol incorporating its structure into the silastic membrane and thus presenting its polar antioxidant head into the aqueous nitrite rich environment where NO can be formed and subsequently diffuse over the membrane.

The studies with α-tocopherol show the versatility of the silastic tubing model and how it could be manipulated to answer questions *in vivo*. However, given that the concentrations of α-tocopherol cannot be accurately determined, and that some of the mechanisms behind the NO α-tocopherol interaction are yet to be defined, it would be premature to use this model *in vivo* at present.

There is good evidence from the studies presented within this Chapter that the adapted silastic tubing with compartmentalisation into 1.5 cm segments, will potentially be able to provide accurate information on nitrosative stress at different sites in the upper gastrointestinal tract.

Chapter 7 *In vivo* studies to determine sites of nitrosative stress at and around the gastroesophageal junction in subjects with a healthy acid secreting stomach.

The work presented within this Chapter was carried out in collaboration with Dr J Winter.

7.1 Introduction

This thesis has, so far, concentrated on bench top models to evaluate the chemistry occurring at and around the gastroesophageal junction, in relation to dietary nitrate. It is however recognised that these models do not necessarily fully represent *in vivo* conditions. Thus far, it has not been possible to determine the dynamic effects of changing the luminal environment from normal to being rich in nitrite on human tissue. It has been established that increased dietary nitrate leads to increased NO levels at the gastroesophageal junction (45). It is proposed that the high concentrations of NO generated, at and around the gastroesophageal junction, will lead to nitrosative stress in those individuals with a healthy acid secreting stomach. The compartmentalised silastic tubing, designed in Chapter 6, provides a tool for measuring nitrosative stress along the length of the oesophagus and stomach, and in particular around the gastroesophageal junction in human subjects. The following studies were therefore performed to assess any nitrosative stress in the upper gastrointestinal tract *in vivo* in healthy volunteers.

7.2 Materials and Methods

7.2.1 Silastic tubing

Compartmentalised silastic tubing similar to Model 2 described in Chapter 6 was created. This tubing was filled with 25mM morpholine, which was buffered at intracellular pH 7.4 using 2mM phosphate buffer solution. Compartmentalisation of the tube into 21 compartments was performed as outlined previously (Chapter 6.2.2). In order to simultaneously monitor luminal pH, a customised four channel pH catheter was attached to the silastic tubing by tying with Vicryl sutures and securing with Sleek waterproof tape. To assist passage of the tubing, a 5 cm length of 10F gastric tubing containing a radio-opaque marker was secured to the distal end of the pH catheter. The radio-opaque marker was also used to locate the end of the tubing and ensure the tubing was sitting in the appropriate place in the stomach by radiography (Figure 7.1).

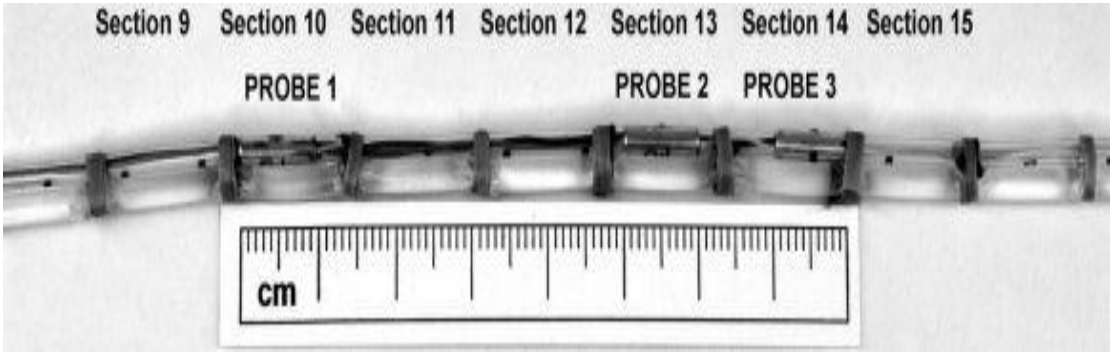


Figure 7.1: Silastic tubing used in *in vivo* studies. The sections shown represent the region that traversed the gastroesophageal junction. The pH probe 1 was in line with section 10. This was the probe that was used to determine if there was significant reflux of acidic stomach contents into the oesophagus. The pH probes 2 and 3 were tied beside sections 13 and 14. These probes were used to determine the pH step up point where the acidic stomach contents met the neutral pH environment of the oesophagus, and thus helped to define the gastroesophageal junction in these healthy volunteers. The individual sections were 15mm in length each containing approx 80-100 µL of PBS (pH 7.4) with 25 mM morpholine.

7.2.2 Healthy human subjects and protocol

Six male volunteers were recruited, with a median age of 42 years. Ethical approval for the studies was granted by the West Glasgow's University NHS Trust Ethical Approval Committee. The subjects were healthy volunteers with no history of reflux disease and were *Helicobacter pylori* negative on carbon urea breath testing. These subjects attended, fasting, on two separate days. On each day, they had the silastic tubing passed nasogastrically, such that sections 1-13 lay within the oesophagus and sections 14-21 lay within the stomach. This was achieved using the pH sensors and confirmed radiologically (figure 7.2). The tip of the tube lay 12 cm within the stomach. Thirty minutes after the insertion of the tube, the subjects were administered one of the two study drinks. On Day 1, the volunteer was given a control drink of 60 mL of water. On Day 2, the volunteer was given 60 mL of water containing potassium nitrate (2 mmole). This amount of KNO_3 equates to the total in a standard meal of fish and chips

The tube remained *in situ* for two hours after the drink had been ingested. During this time, the pH was monitored, using the four channel pH catheter, at each site every four seconds, giving a detailed assessment of the acidity around the gastroesophageal junction, and at points within the oesophagus and stomach. Immediately before the drink and at 15 minute intervals after it, a 1 mL saliva sample and a 10 mL blood sample were collected for measurement of serum nitrate and salivary nitrate concentrations.

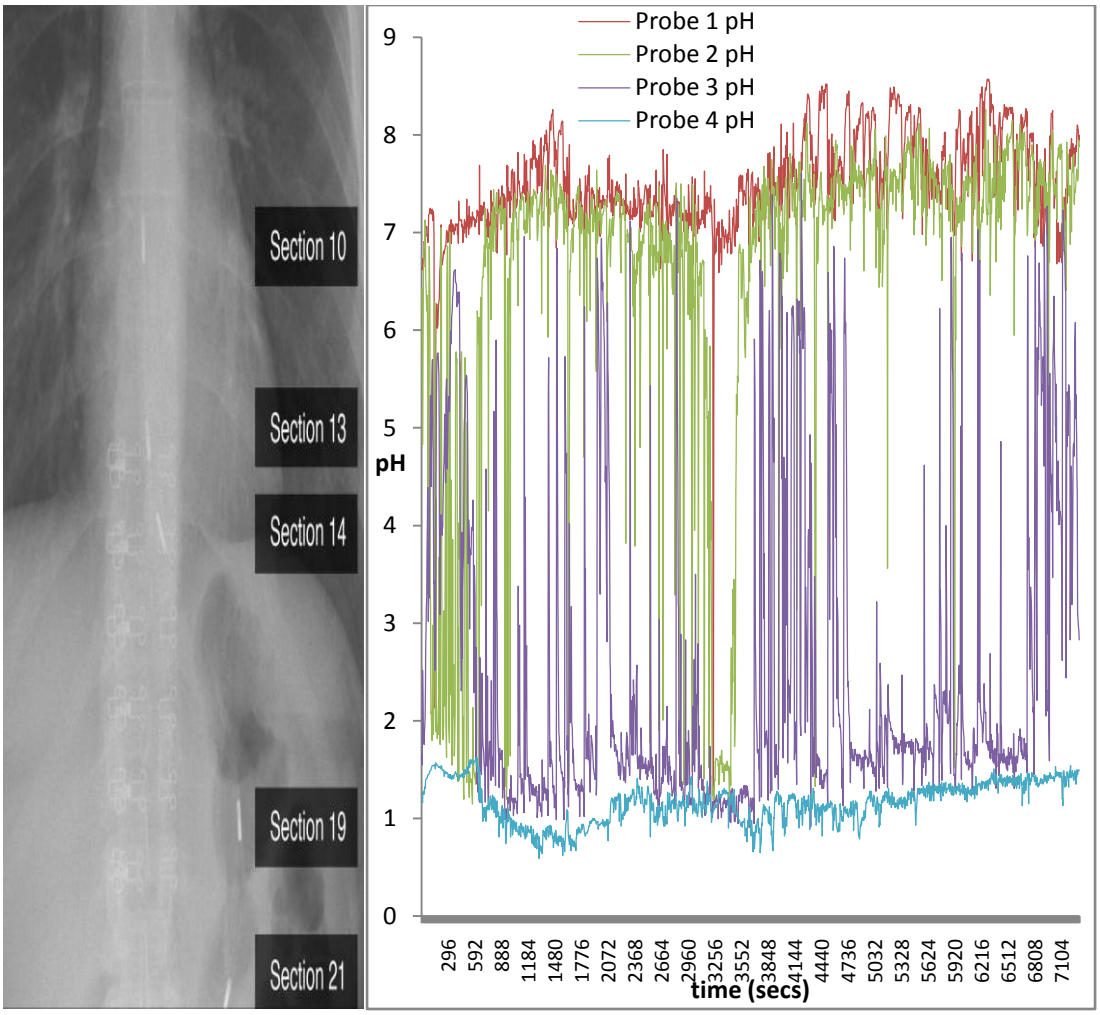


Figure 7.2: Radiograph of the lower chest and upper abdomen, and 4 channel continuous pH study of a healthy volunteer with silastic tube in situ. The pH channel probes are radio-opaque and are shown together with a radio-opaque marker secured at the most distal end of the pH catheter. Sections 13 and 14 lay either side of the gastroesophageal junction, as recognised by the pH step up point between probes 2 (green) and 3 (purple). Probe 1 (red), adjacent to section 10, lay in the neutral pH oesophagus. Probe 4 (blue), adjacent to section 19, lay within the more distal acidic stomach.

7.2.3 Serum nitrate analysis

Blood samples (10 mL) were taken from a 20G i.v cannula sited in the antecubital fossa at times 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes. The first 5 mL of this sample were discarded to allow for dead space within the cannula. The serum sample (2 mL) was stored at –20°C prior to analysis. On thawing, 400 µL of sample was centrifuged through a 10-kilodalton ultrafilter to remove high-molecular-weight proteins. Samples were then mixed with a nitrate reductase in reaction buffer and analysed as described in Chapter 2.1.5.

7.2.4 Salivary nitrite analysis

Salivary samples of 1 mL were obtained, passively without stimulation of salivation, at times 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes. Samples were mixed with 10 µL of 5.0 M NaOH. This rendered the samples alkaline and prevented loss of nitrite. Samples were stored at 4°C, then centrifuged and analyzed the same day, on 96-well microplates using the modified Griess reaction described in Chapter 2.1.4.

7.2.5 Silastic tube nitrite and NMOR concentrations

Immediately after removal of the tube after 2 hours, each section of tubing was carefully aspirated using a 100 µL glass syringe. A total of 10 µL of sample removed from each section of tubing was used for measurement of the silastic tube nitrite concentration, while 40 µL was used for analysis of NMOR. Analysis for concentrations of nitrite and NMOR were carried out as described in Chapter 2.1.10.

7.3 Results

7.3.1 pH studies

During the study, only small pH variations at the electrodes lying either side of the gastroesophageal junction were recorded. There were changes in pH at these sites mainly related to movement with respiration and on swallowing. There was excursion of the tubing at the gastroesophageal junction of 3-4 cm with normal respiration as detected by fluoroscopic screening. Actual reflux was recorded as a pH of less than 4 in the electrode placed 5 cm above the gastroesophageal junction (see figure 7.1 – probe 1 lying beside section 10 of the silastic tubing). There was only one volunteer in whom reflux was observed and this was for a very short period of time. It occurred only on the day that the control drink was given. Overall, reflux was seen for a median of only 0.1% of the total time (interquartile range 0%-1.0%). The pH of section 13 which was primarily on the oesophageal side of the gastroesophageal junction had a pH of less than 2 for only 7% of the time, while section 14, which was mainly on the gastric side of the gastroesophageal junction, had a pH of less than 2 for 63% of the time. Section 19 was always exposed to an acidic environment.

7.3.2 Serum nitrate

The median serum nitrate concentration prior to the administration of the control drink was 16.9 (interquartile range, 7.9 - 20.3) µM. 45 minutes after the administration of the control drink, the serum nitrate concentration did not significantly change ($p>0.05$) from the basal reading, with a median concentration of 14.1 (interquartile range, 7.2-17.7) µM and this continued to the case up to the end of the experiment at 120 minutes. However, after ingestion of a 2 mmole potassium nitrate drink, a significant increase ($p<0.005$) in the median serum nitrate concentration was observed, from 12.7 (interquartile range, 6.9-16.1) µM on sampling prior to administration of the drink to a peak of 47.1 (interquartile range, 23.2-57.9) µM 45 minutes after the drink was given. The serum nitrate concentration remained at this higher level concentration for the duration of the study (figure 7.3).

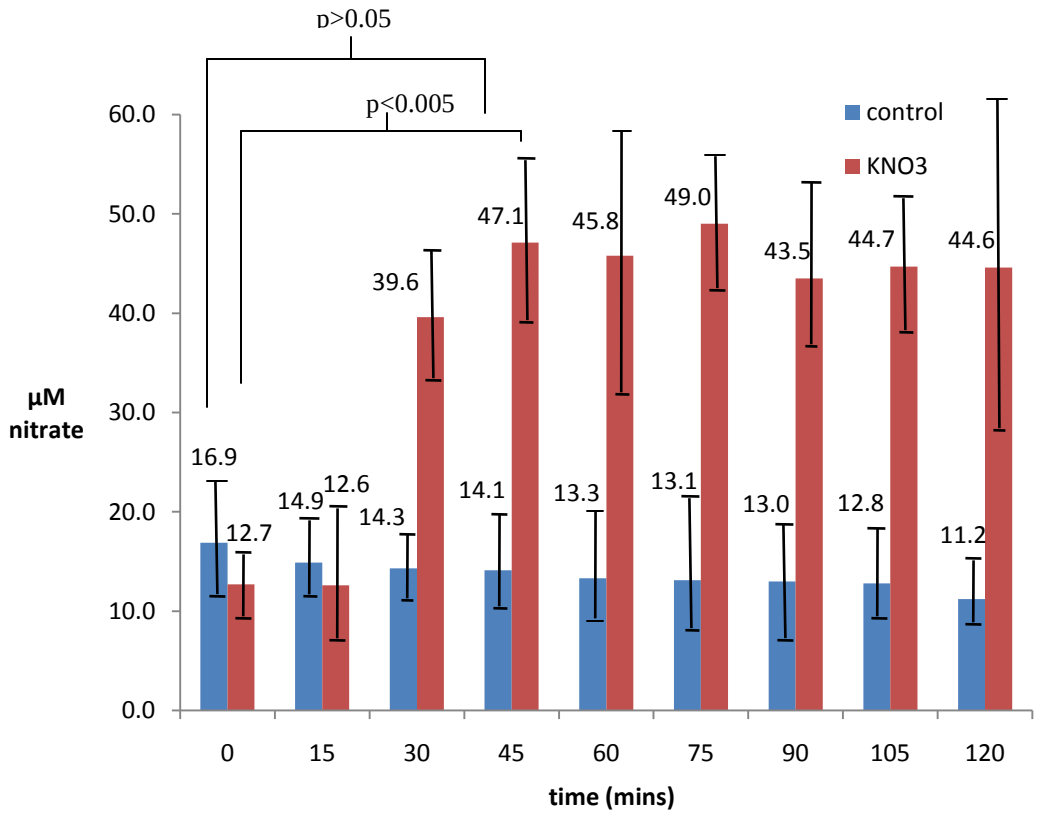


Figure 7.3: Median serum nitrate concentration (µM) in healthy volunteers at the given time intervals (minutes) during the 2 hours of the experiment after either a control drink or 2 mmole KNO_3^- drink has been ingested at time 0 minutes. Interquartile ranges are shown by the error bars.

7.3.3 Salivary nitrite

For volunteers, on the day they consumed the control drink, the median salivary nitrite concentration prior to the administration of the control was 63.7 (interquartile range, 43.5 – 89.4) μM. 75 minutes after the administration of the control drink, the salivary nitrite concentration did not significantly change ($p>0.05$) from the basal reading, with a median concentration of 57.3 (interquartile range, 38.6 – 87.5) μM. However, on the day the volunteers were given a 2 mmole potassium nitrate drink there was a significant increase ($p<0.0005$) in the median salivary nitrite concentration from 30.5 (interquartile range, 21.6 - 38.3) μM on sampling prior to administration of the drink to a peak of 330.9 (interquartile range, 250.2 – 398.5) μM 75 minutes after the drink was ingested. The salivary nitrite concentration reached peak concentrations at 45 minutes (311.1 μM), and remained significantly elevated for the remainder of the study (figure 7.4).

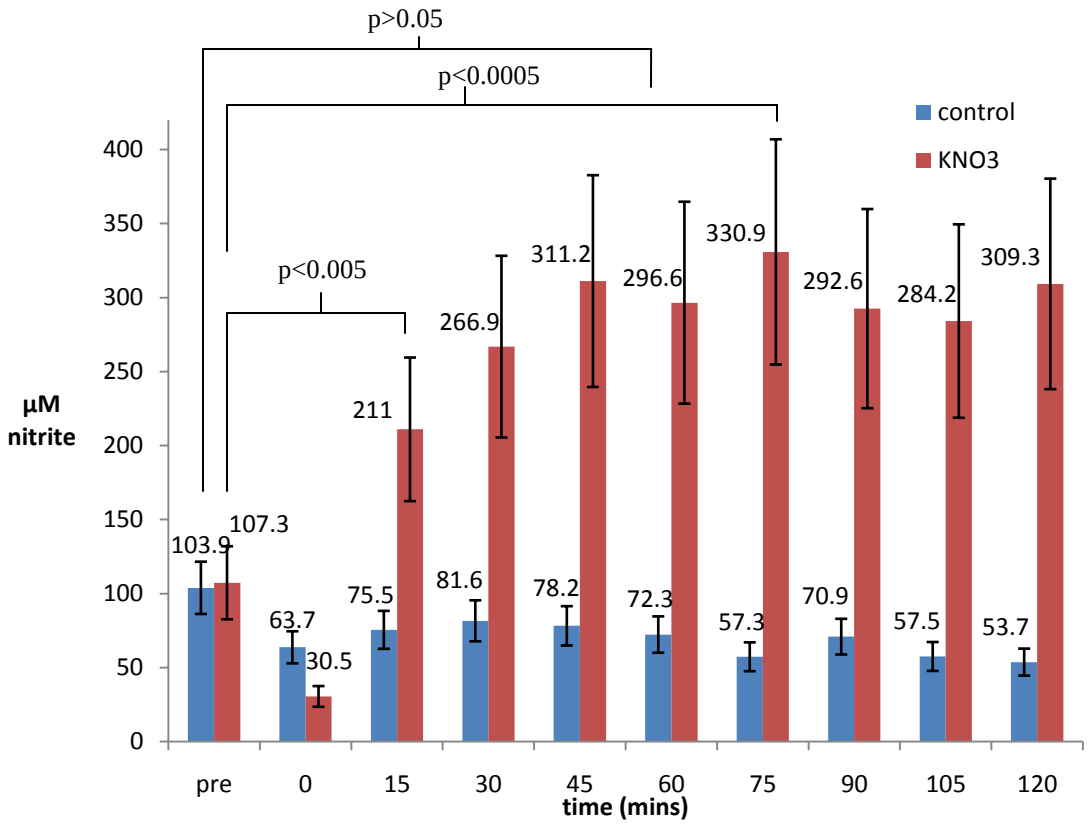
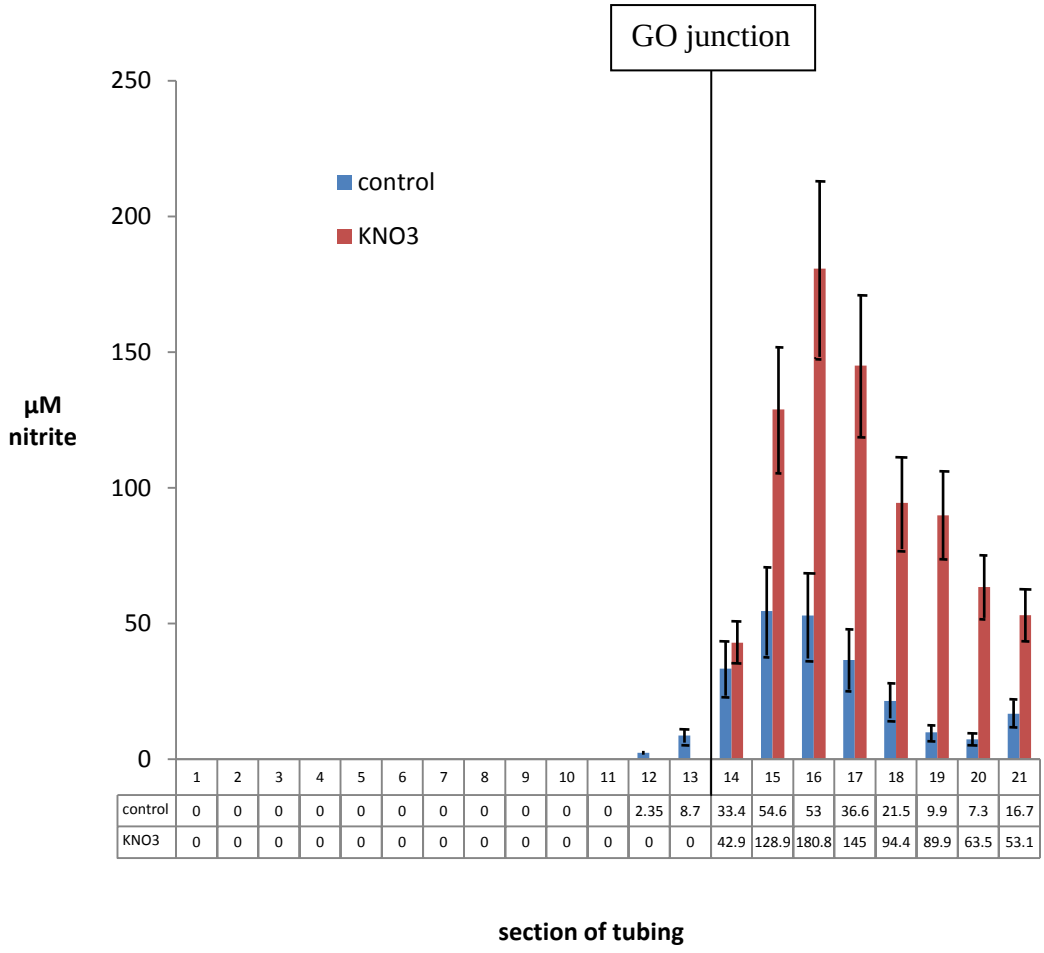


Figure 7.4: Median salivary nitrite concentrations (µM) in healthy volunteers taken at the given time intervals over the 120 minute time course of the experiment. Red columns represent the mean concentrations on the day the KNO_3 drink was ingested, while the blue columns represent the control day. The concentration on the day a control drink was given is compared to a day on which a 2mmole nitrate drink was given. Error bars represent the interquartile range. (n=6)

7.3.4 Silastic tube nitrite

Nitrite was not detected within the tube sections lying within the oesophagus, whereas, nitrite was present in tube sections exposed to gastric acid, on both control and KNO_3 administered days. In the tubing from the control day the median total nitrite formed within the tube was 13.1 (interquartile range, 6.9-22.2) nmoles whereas there was significantly more ($p=0.001$) nitrite formed within the tubing of volunteers when they were administered the KNO_3 drink with the median value of 42.1 (interquartile range, 27.8 - 60.3) nmoles. The concentration profile of the sections of tubing was similar on both study days, with the highest concentration being detected in sections 15 and 16 (figure 7.5). These sections are the first sections to be exposed constantly to intragastric acidity. Peak median concentrations of nitrite were detected in section 15, being 54.6 (interquartile range, 35.0-65.2) µM after the control drink and increasing to 128.9 (interquartile range, 46.1-148.7) µM after nitrite. The peak median concentration of tubing nitrite was found in section 16 on the day the nitrate drink was given, with a concentration of 180.8 (interquartile range, 95.0-229.7) µM. This compares to a median concentration of 53.0 (interquartile range, 35.6-67.1) µM for the same section on the day a control drink was given. This is therefore over a three fold increase in nitrite concentration between the control drink group and those administered the nitrate drink (figure 7.5).

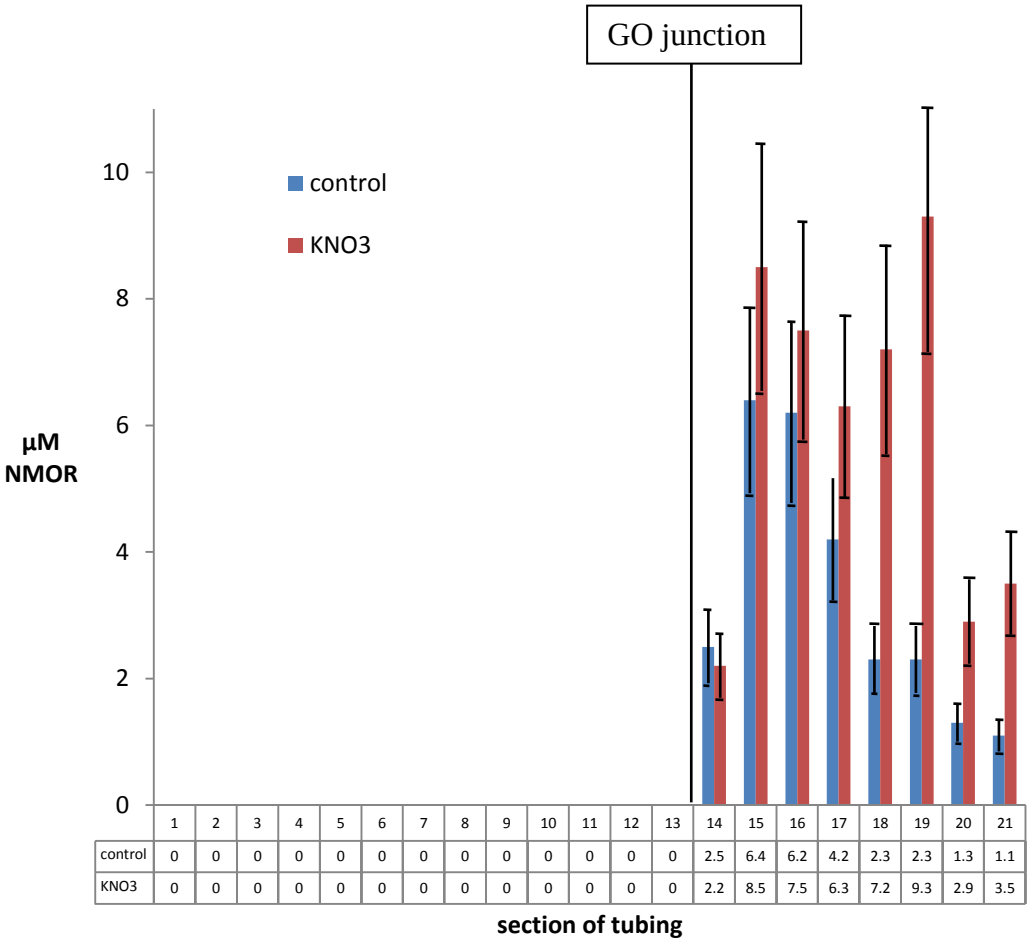


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
control	0	0	0	0	0	0	0	0	0	0	0	2.35	8.7	33.4	54.6	53	36.6	21.5	9.9	7.3	16.7
KNO3	0	0	0	0	0	0	0	0	0	0	0	0	0	42.9	128.9	180.8	145	94.4	89.9	63.5	53.1

Figure 7.5: Median silastic tubing nitrite concentrations for individual sections of tubing at the end of the 2 hour experiment. The gastroesophageal (GO) junction is represented by the vertical line between sections 13 and 14. Results are shown after administration of either the control drink or nitrate drink. The data values shown below the graph are median values for each section after both control and KNO_3. Median concentrations are shown with interquartile range indicated for each section by the error bars.

7.3.5 Silastic tube NMOR

Throughout the sections of silastic tubing lying within the oesophagus, there was no NMOR detected. However, NMOR was detected in the sections of tubing exposed to gastric acid in volunteers after administration of both control and nitrate drinks. There was a significant difference in the NMOR formed in the tubing after administration of potassium nitrate (2 mmole) compared to the control, with a near doubling in the median concentration of NMOR (p=0.008) (Figure 7.6). The median NMOR formed within the tube was 2.1 (interquartile range, 0.8-2.9) nmols on the control day and 4.3 (interquartile range, 2.3-6.6) nmols on the day the potassium nitrate drink was given. In the control experiments, the peak NMOR concentration was 8.2 (interquartile range, 1.9-9.8) μM, measured in section 15 of the tubing. However, the concentration profile was different on the day the volunteers were given the potassium nitrate drink. There was a plateau of increased concentrations in the gastric lying sections of the tubing, rather than a peak concentration. The peak NMOR concentration after nitrate drink was measured in section 19, where the concentration of 9.3 (interquartile range, 4.0-15.7) μM was almost four times the 2.3 (interquartile range, 0-4.1) μM detected in section 19 on the day of the control drink. In fact, the most striking differences between the control day and nitrate drink NMOR concentrations occurred in the most distal section of gastric tubing. Even in section 21, the most distal section of gastric tubing, there was 3.5 (interquartile range, 0 – 7.3) μM NMOR formed on the day the volunteers had a nitrate drink compared to 1.0 (interquartile range 0 -2.1) μM on the control day



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
control	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5	6.4	6.2	4.2	2.3	2.3	1.3	1.1
KNO3	0	0	0	0	0	0	0	0	0	0	0	0	0	2.2	8.5	7.5	6.3	7.2	9.3	2.9	3.5

Figure 7.6: Median silastic tube NMOR concentrations at the end of a 2 hour experiment. The gastroesophageal (GO) junction is represented by the vertical line between sections 13 and 14. Results are shown after administration of either the control drink or nitrate drink. The data values shown below the graph are median values for each section after both control and KNO_3. Median concentrations are shown with interquartile range indicated for each section by the error bars.

7.3.6 Correlation of silastic tube nitrite with NMOR

There was a near four fold increase in the peak silastic tube nitrite concentration after the administration of nitrate. However, there was only a doubling in the peak NMOR concentration. The ratio of NMOR to nitrite was therefore calculated for each gastric tube section. The median of all ratios of NMOR:nitrite concentration was 0.1258 (interquartile range, 0.0903-0.1691) μM after the control drink. There was a significantly lower ratio of 0.0580 (interquartile range, 0.0445-0.0711) μM after the nitrate drink (p=0.0047). For oesophageal tube sections there were small concentrations of nitrite found in the lower two oesophageal sections in one of the subjects. This subject was the same volunteer in whom there was a small degree of reflux recorded on the day the control drink was given. There was no recordable NMOR found within these sections and therefore no ratios have been calculated.

7.4 Discussion

A novel mechanism whereby dietary nitrate can produce nitrosative stress at and around the gastroesophageal junction has been proposed. This has been supported by the results of *in vitro* experiments using bench top models, described in this thesis, and by others working within this area of research (43,44,144,285,286). In this hypothetical model, NO is generated in extremely high concentrations where salivary nitrite, generated from dietary nitrate, meets acidic gastric juice in the presence of ascorbic acid, as has been shown to be the case in human subjects after a nitrate meal (44,280). The model suggests that NO then diffuses into the surrounding epithelial compartments or lipid where it can form nitrosating species such as dinitrogen trioxide through the reaction of NO with O_2.

This study indicates that the hypothetical model for *N*-nitrosation proposed above has relevance in an *in vivo* situation in subjects with a healthy acid secreting stomach. Overall, healthy volunteers showed an increase in nitrosative stress in the upper gastrointestinal tract after a nitrate rich drink was given. This was indicated by an increase in the total silastic tube nitrite and NMOR concentrations. The site of all of the nitrosative stress was within the stomach with the exception of one volunteer who had a short lived period of proven acidic reflux into the oesophagus.

The highest concentrations of nitrite were seen within the sections of tubing that lay in the proximal stomach. This was seen on both the control day and on the

day a nitrate rich drink was given. These findings would be in keeping with the anatomical site where high concentrations of salivary nitrite are first meeting with acidic gastric juice containing ascorbic acid (221). Nitrite can enter into the lumen of the silastic tubing by one of two routes (44). The first, and in these experiments the one likely to be more important, is the generation of NO from the reaction of nitrosating species with ascorbic acid. The NO can then diffuse through the wall of the silastic tubing and both within the wall and within the lumen of the tubing, react with O_2 to form N_2O_3. N_2O_3 can then either react with nitrosatable species such as the amine present in the lumen or it can react with water to form nitrite as shown in the reaction:

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$$

In an acidic environment nitrite will largely form nitrous acid as shown by the reaction:

$$HNO_2 \leftrightarrow H^+ + NO_2^-$$

The second mechanism through which nitrite can accumulate within the lumen of the tube depends on the pK_a of nitrous acid being 3.34. At the acidic pH found in subjects with a healthy acid secreting stomach nitrous acid is in the relatively non polar form HNO_2. This HNO_2 can then slowly diffuse through the wall of the

silastic tube. Once within the neutral environment of the tube lumen HNO_2 will quickly form nitrite.

One of the more surprising results from this study was the plateau effect of the distribution of NMOR within the sections of tubing extending into the more distal regions of the stomach. The tubing extended into the stomach some 12.5cm. It had been expected that the nitrosative stress in this region would be less than at the gastroesophageal junction and cardia region of the stomach. These results can, however, be explained by the availability of chemical reactants within the lumen of the healthy acid secreting stomach. We found that the peak nitrite concentration was in the sections that lay within the proximal stomach, while the NMOR concentrations appeared in the proximal stomach and concentrations began to plateau in the more distal sections in the stomach. The peak concentration of NMOR was seen several centimetres below the gastroesophageal junction in section 19. Therefore, there was a tendency for the ratio of nitrite to NMOR to be lower in the proximal stomach after the nitrate drink. The ratio of NMOR:nitrite within the silastic tube is an expression of NO:nitrite in the lumen of the gastrointestinal tract. NMOR only can be formed, within the epithelial compartment, by the diffusion of NO, whereas nitrite can be formed via the diffusion of either NO or nitrous acid, as discussed above. The administration of nitrate resulted in a 230% increase in peak silastic tube nitrite concentrations, but only a 45% increase in peak NMOR concentrations. Therefore, the nitrate meal actually reduced the ratio of NMOR:nitrite, although not the amount of NMOR produced. This suggests that the

entire increased amount of swallowed salivary nitrite was not being converted to NO in the proximal stomach, but rather some of it remained in the form of nitrous acid and nitrosating species in the lumen of the stomach itself. This could occur if the high proximal stomach nitrite concentrations have exhausted the ascorbic acid concentration. This would then allow nitrite to enter the tubing as nitrous acid rather than NO. There was intragastric variation in concentrations of ascorbic acid, with the concentrations being lower in the cardia region of the stomach than the more distal region (221). The reason for ascorbic acid concentrations varying within different regions of the stomach is not clear, although it is known that in the fasting state vitamin C is actively secreted into the stomach (96,134). It has previously been observed that the ratio of nitrite to ascorbic acid is highest in the most proximal stomach, with the authors proposing that this was potentially the maximal site within the stomach for the well recognised acid-catalysed nitrosation (221). The current studies would support this argument, and would suggest that there are two mechanisms for nitrosation due to salivary nitrite in the human subject. The first of these is the well established and analysed acid-catalysed nitrosation, the second being NO production in the presence of an antioxidant and a lipid phase. The factor determining which mechanism of nitrosative stress is dominant in the human subject is the availability of ascorbic acid.

Further studies using this silastic tube *in vivo* model have been performed by one of my colleagues (Winter J), with whom the initial studies were performed. Data from these studies are now published (286). A further 12 ‘healthy’ volunteers

were studied by the same methods described in this chapter. The results for silastic tubing nitrite, NMOR and the correlation between them are reassuringly similar to the results presented within this thesis. There was good correlation between the studies in terms of the sections of tubing identified with peak concentrations of nitrite and NMOR, and the median values for each section of tubing are remarkably consistent. The additional 12 patients did however provide an interesting observation that helps support the argument for acid-catalysed nitrosation and NO mediated nitrosative stress. One of the twelve volunteers was not included in the results of the studies, as they were found to be achlorhydric through recording from the pH probes in this study. The silastic tube luminal contents from this volunteer did not contain any detectable nitrite or NMOR. This is not to say that this person is at no risk of nitrosative stress. There is another well recognised mechanism for nitrosation within the human stomach and that is through denitrifying bacteria generating nitrosating species and *N*-nitrosocompounds. These bacteria can colonise an achlorhydric stomach mucosa. This mechanism is thought to be relevant to patients who develop noncardia gastric cancer, secondary to atrophic gastritis (68,287).

Further studies were also performed using the silastic tube model to examine patients who were known to have Barrett's oesophagus (286). This group of patients was chosen as they usually have severe acid reflux disease. Within this group, nitrite and NMOR were found in the oesophageal sections of the silastic tube lumen as well as the gastric sections. The extent to which the nitrite and NMOR were

detected was directly related to the degree of reflux as recorded on the pH probe lying within the oesophagus (286).

Overall the studies in this chapter indicate that, in an *in vivo* setting, there are two mechanisms through which there is the potential for nitrosative stress within the upper gastrointestinal tract from dietary nitrate. There is the well established luminal acid-catalysed *N*-nitrosation in the absence, or exhaustion, of ascorbic acid, and there is NO mediated stress in the healthy acid and ascorbic acid secreting stomach. These studies have allowed us to determine where there are the different types of stresses, as the ratio of nitrite to nitrosamine allows us to determine whether the stress is from NO, where there is both nitrite and NMOR formed, or from acid-catalysed nitrosation from nitrous acid, where there is accumulation of nitrite in the tubing but there is not the nitrosating potential of this to generate nitrosamine within the tubing. It has previously been established that NO has mutagenic potential to a cell through DNA base deamination, inactivating DNA repair enzymes, inactivating DNA repair enzymes and through the generation of *N*-nitrosocompounds such as nitrosamines. It is also known that within cells there is a range of compounds that could be nitrosated to potentially mutagenic products. These compounds that are potentially nitrosated include secondary amines, polyamines, amino acids, peptides and amides (200,288-290). With particular relevance to the upper gastrointestinal tract are conjugated bile salts glycocholic acid and taurocholic acid. These are amides that are potentially present within the refluxate of bile. It is known that bile and acid reflux are irritants to the lower oesophagus causing oesophagitis. With

oesophagitis as a background, there is then also the potential of nitrosated bile salts adding insult to the already exposed and damaged epithelium.

Chapter 8 Studies to determine intraepithelial nitrate and nitrite from human upper gastrointestinal tract tissue

8.1 Introduction

While *in vitro* studies suggest a potential mechanism for nitrosative stress from dietary nitrate, with the possible implication of this chemistry contributing to the increasing incidence in adenocarcinoma at and around the gastroesophageal junction, there had been no confirmation of this stress from human tissue studies. As has been discussed previously within this thesis, an influx of nitrite is a good marker of increased nitrosative stress. Therefore, if increased nitrite and related chemistry, such as nitrate, could be measured in the intraepithelial compartment of cells from human tissue biopsies, this would be good evidence for nitrosative stress at an *in vivo* cellular level.

The intraepithelial measurement of nitrite and nitrate from biopsies from the upper GI tract has not previously been described, therefore new techniques were attempted to obtain this information. This chapter describes the process of obtaining human biopsy specimens from specific anatomical regions of the oesophagus and stomach, cleaning of these samples, lysis of the cells within these samples and specific analysis of nitrite and nitrate from the samples. The subsequent Chapter, Chapter 9, will describe anatomical variation in nitrate and nitrite concentrations from biopsies.

Previous studies within this thesis have analysed nitrite from a simple medium in bench-top models. Human tissue is more complex and therefore analysis of two different methods of determining nitrite concentration was undertaken. These methods are the Greiss Reagent and the release of NO recorded using an NO probe, and are described in this chapter.

As well as measuring nitrite from upper gastrointestinal tract biopsies, it is also important to measure nitrate *in vivo*. This is because human tissue is more complex than previously used bench top models. It has been shown that in biological tissue the most important metabolic reaction of NO is with iron in oxyhaemoglobin to produce methaemoglobin and nitrate (291), as shown in the following reaction:

$$[HbFe(II)O_2]_2 + NO \rightarrow [HbFe(III)]_3^+ + NO_3^-$$

For example, the uptake of NO into the red blood cells with subsequent oxidation is the major metabolic pathway for endogenously formed NO (292). Also, it is known that NO reacts with superoxide in aqueous solution to form peroxynitrite. Superoxide ion has been shown to be ubiquitous in mammalian cells (293). The peroxynitrite/peroxynitrous acid system is known to be very labile, and the decomposition products from this are nitrate, nitrite and dioxygen (294), as shown by the following reactions:

$$O_2^- + NO \rightarrow OONO$$

$$4OONO \rightarrow 2NO_3^- + 2NO_2^- + O_2$$

It has also previously been shown that in the presence of peroxynitrite, nitrite is converted to nitrate. Therefore, in the following studies nitrate concentration is also measured.

Upper gastrointestinal tract biopsies require a cleaning process, as surrounding mucus and the haem components of blood would interfere with subsequent analysis of intraepithelial nitrite/nitrate. Within the lumen, oesophageal defences against acid include pre-epithelial compartments such as mucus and bicarbonate (295,296). Mucus itself is not an effective barrier to hydrogen ions (297,298) but provides mucosal protection by contributing to an area of low turbulence. Adjacent to the mucosa is the unstirred water layer (299). This layer acts as a sink for bicarbonate ions, creating an alkaline environment close to the mucosal surface (298-300). Mucus, lining the epithelial surfaces, will be the first point of contact of the luminally generated NO. Nitrite is formed from the reaction of NO with water at a more neutral pH, so therefore nitrite can be expected to be formed here when NO diffuses into the mucus. This would mean that unclean biopsies could record high nitrite concentrations from the mucus, without this being a reflection of intraepithelial nitrite content.

There are several mucolytic agents that are used in cleaning mucus from biopsy samples. Mucolytic agents that were explored as possible cleaning agents for the studies presented in the chapter were N-Acetylcysteine (NAC) (301), pepsin, and pronase E. Unfortunately, these cleaning agents are normally used in a setting where rupture or leak of the cell membranes would not be important. For the proposed measurement of intraepithelial contents, it was thought that the ideal solution would be isotonic and non toxic to the cell membrane. Pepsin works best at lower pH and from pH 6 and above is unstable. Therefore, this wasn't suitable for use in these studies where there is a neutral pH. NAC is known to be a mucolytic agent that has been previously used to clean surrounding mucus from gastrointestinal biopsy specimens. Solutions between 3 and 10% NAC have been used effectively to clean samples from the upper gastrointestinal tract (302,303). A solution of NAC is acidic and the pH of a solution of 3.1% NAC is 3. "Pronase E" is the name given to a group of proteolytic enzymes produced by Streptomyces griseus K-1. At least 10 proteases are in the mixture, five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases and one zinc carboxypeptidase. Digestion with the product has been useful when extensive or complete degradation of protein is required. It has previously been used to digest mucus from rat stomachs in experimental models (304-307). In previous studies this has always been used as part of a harvesting of cells and has not been used simply to clean around biopsy specimens. It has stable activity across a range of pH from 5.0-9.0. Cleaning methods are described in this chapter.

After cleaning of the surrounding mucus, lysis of the cell membranes within the biopsy sample had to be performed as the intracellular concentrations are the measurements that are expected to be important. A suitable method of cell lysis, to release intracellular nitrite and nitrate into surrounding PBS, had to be developed and is discussed.

8.2 Materials and Methods

8.2.1 Obtaining and storing upper gastrointestinal tract samples

Epithelial biopsies from the oesophagus and stomach were obtained from volunteers undergoing upper gastrointestinal endoscopy in Gartnavel General Hospital. Ethical approval for the studies was granted by the West Glasgow's University NHS Trust Ethical Approval Committee. All patients were confirmed *Helicobacter pylori* negative by urease CLO testing, and were not receiving antacid therapy. Biopsies were taken using standard endoscopy forceps with large capacity. The gastroesophageal junction was identified as the top of the mucosal folds and coincided with the squamo-columnar junction in these healthy subjects. Oesophageal biopsies were taken 5 cm above the gastroesophageal junction. Proximal stomach biopsies were taken immediately below the gastroesophageal junction. Distal stomach biopsies were taken from the distal body of the stomach. Immediately after removing the forceps from the scope portal, the sample was transferred from the forceps using tweezers, and placed into a 1 mL plastic Eppendorf test tube after undergoing a cleaning process. PBS (80 µL) was added to the sample, which was placed in liquid nitrogen for transfer to a -70^{o}C freezer. All samples were analysed within four weeks.

8.2.2 Determining the optimal method for cleaning biopsy samples

Cleaning Solutions assessed:

NAC - In order to prevent osmotic and acidic lysis of our cells during the cleaning stage, it was important to clean samples at a neutral pH, with a physiological osmolarity. Therefore, sodium bicarbonate (200 mM) was added to 3.1% NAC to give a final pH of 7.2 and osmolarity of 320 mM. Biopsy samples were left in this solution for 30 minutes. After 30 minutes the sample was taken out and placed on benchguard paper, and any obvious surrounding mucus removed.

Pronase E – 1% pronase was added to PBS (pH 7.4). The biopsy samples were placed in a plastic Eppendorf test tube containing 2 mL of this solution for 30 minutes. Thereafter, the samples were placed on benchguard paper and any obvious surrounding mucus was cleaned.

In experiments used to assess the efficacy of cleaning using these solutions, twelve biopsy samples were placed in an Eppendorf test tube containing 2 mL KNO_3^- (5mM) enriched with ^{15}N. These were compared to control samples exposed to the same contaminant but which had no cleaning. ^{15}N content of samples was then analysed.

8.2.3 Protein concentration as a measure for weight of biopsy samples

Upper gastrointestinal tract biopsy samples vary largely in size. Multiple biopsy samples were taken and analysed for wet and dry weight after heating in an incubator overnight. It is particularly important to do this given that oesophageal biopsies are more difficult to obtain and generally are smaller. This revealed that wet weight could vary by up to five times between the biopsy sample weights. The dry weight was approximately 10-15% of the wet weight. The protein content may be used as a standard against size for each sample. This would allow standardisation of the nitrite and nitrate content of each sample, according to concentration per unit of protein.

8.2.4 Releasing intraepithelial contents by cell membrane lysis

A comparison of four different methods of cell lysis was made by measuring total nitrite and protein concentrations from each biopsy sample. Each method was performed on each sample from each of the three areas of interest in the upper gastrointestinal tract. There were thirty-six times samples taken to obtain mean values (± 1 standard deviation).

Snap freezing: Immediately after the cleaning process the biopsy sample was placed in 80 µL PBS in a 1 mL plastic Eppendorf test tube. It was then immediately

placed into liquid nitrogen to snap freeze the sample, thus bursting the cell membranes.

Homogenisation: After snap freezing the samples were homogenised in a glass, 1 mL, hand held homogeniser for 120 seconds.

Sonication: After snap freezing, the samples were defrosted and sonicated at 6 microns amplitude for three minutes before being centrifuged for three minutes at 13000 rpm in a microcentaur. The supernatant was thereafter analysed.

Homogenisation and Sonication combined: samples were snap frozen then homogenised, sonicated and centrifuged as in the above descriptions.

8.2.5 Biopsy nitrite concentration analysis

Within the laboratory we had access to, and experience of using, several methodologies for nitrite concentration measurement. Previous assessment of the HPLC method suggested it did not provide any more accurate measurement compared to the relatively simple Greiss reagent. For the studies in this chapter the Greiss reagent was compared to measurement using a NO probe.

a. NO probe
b. One step Greiss reagent

a. NO assessment is based on using NO production in an acidic environment and measuring NO with a WPI NO probe. The method used was based on

the same principles, and used the same probe, as discussed in Chapter 2.1.1. There was however several differences from the basic methodology and these will be described here. These differences arise mainly due to the small volumes and small concentrations of nitrite expected to be found.

The NO probe was calibrated at room temperature using known standards. As the sample was diluted by surrounding PBS, the expected concentration of nitrite, and therefore NO generated was low. The standards used to calibrate the probe were therefore, 10 nM, 50 nM, 100 nM, 500 nM and 1 µM nitrite. To the standards, an acidic reaction solution (Solution A) was added to generate NO. Solution A was made up of 30mls dH_2O, 0.55 g of potassium iodide (KI) and 200 µL of concentrated H_2SO_4. This had to be stored in the dark until it is used to prevent the KI reacting with light. 80 µL of each standard was added to 80 µL of solution A to obtain a graph of standard concentrations against the pA reading of each and to provide a y value from which we were able to calculate the NO concentration produced from the samples with unknown nitrite content. As with the standards, we added 80 µL of sample to solution A to liberate the nitrite as NO and took a pA reading which was converted into a concentration of NO released. In this experiment there was gross excess of KI in acid this reflects the nitrite concentrations as all nitrite will be converted to NO.

Given that the approximate intracellular volume of the samples was only 3 µL, the total volume of acidic solution that could be used to initiate the release of NO was very small, and this led to several technical difficulties. There has to be sufficient volume to cover the membrane of the probe. Also, due to the fact that the reading was based on a current, when the NO probe was touched the baseline amplitude of electrical current changed. Although the amplitude changes due to this interference were only a matter of tens of pA they were still significant because the samples being analysed were very dilute and the readings we expected to see were very small.

b. One step Greiss Reagent – Nitrate and nitrite have previously been measured within human biological material by the simple Greiss reaction (308-310). This method of measuring is outlined in Chapter 2.1.4.

After deliberately exposing 12 biopsy samples to high concentrations of NO generated in the model shown in figure 8.1, nitrite concentrations were measured using both of the methods described above. Of the total 80 µL volume half was used for analysis by the NO probe method, and half was used for the one step Greiss reagent method.

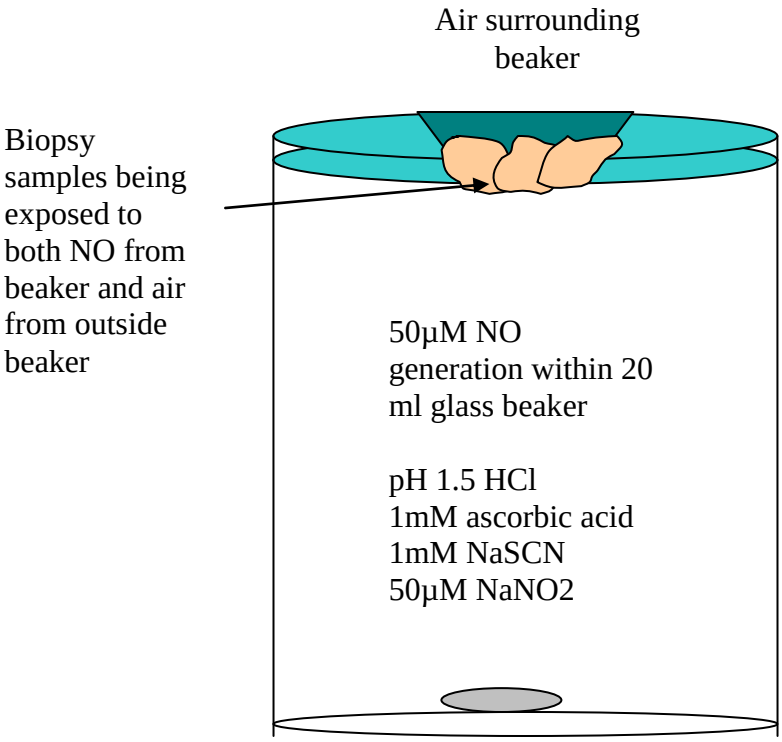


Figure 8.1: Schematic diagram of the model used to expose human upper gastrointestinal tract biopsy samples to NO. A 20 mL glass vial was filled with HCl (pH 1.5), ascorbic cid (1 mM), NaSCN (1 mM) and $NaNO_2$ (50 µM) to allow generation of approximately 50 µM of NO. The lid of this vial was cut in the middle to establish a small hole. Into this hole were placed three biopsy samples, so that the samples were both in contact with, both the lower layer to form a seal, and also the air outside the vial. Concentrations of NO were 45 µM and above in the aqueous phase of this model after 30 seconds, and were maintained at this high concentration for the 15 minutes that the biopsies remained inside.

8.3 Results

8.3.1 Cleaning with NAC and pronase E

These experiments revealed that after cleaning with 3.1% NAC only 7% ^{15}N remained while 25% ^{15}N remained when Pronase E was used as the cleaning solution. These experiments showed that while cleaning was not 100% effective with NAC, it was the best available solution and was more effective than Pronase E.

8.3.2 Protein as standardisation for the weight of a sample

There was a linear relationship between the weight of a biopsy and protein content (figure 8.2). When these results were charted there was a direct correlation between protein content and sample weight, with the slope of best fit a linear line $r^2 = 0.954$:

$y\ (\mu g) = 3.7(mg) \times X$ (where y is the protein content in mg and X is the unknown weight of a sample in mg)

This allowed use of the protein concentration as a standardising unit for nitrite and nitrate concentrations. The units for nitrate and nitrite are then given against the protein concentration of a sample (µg).

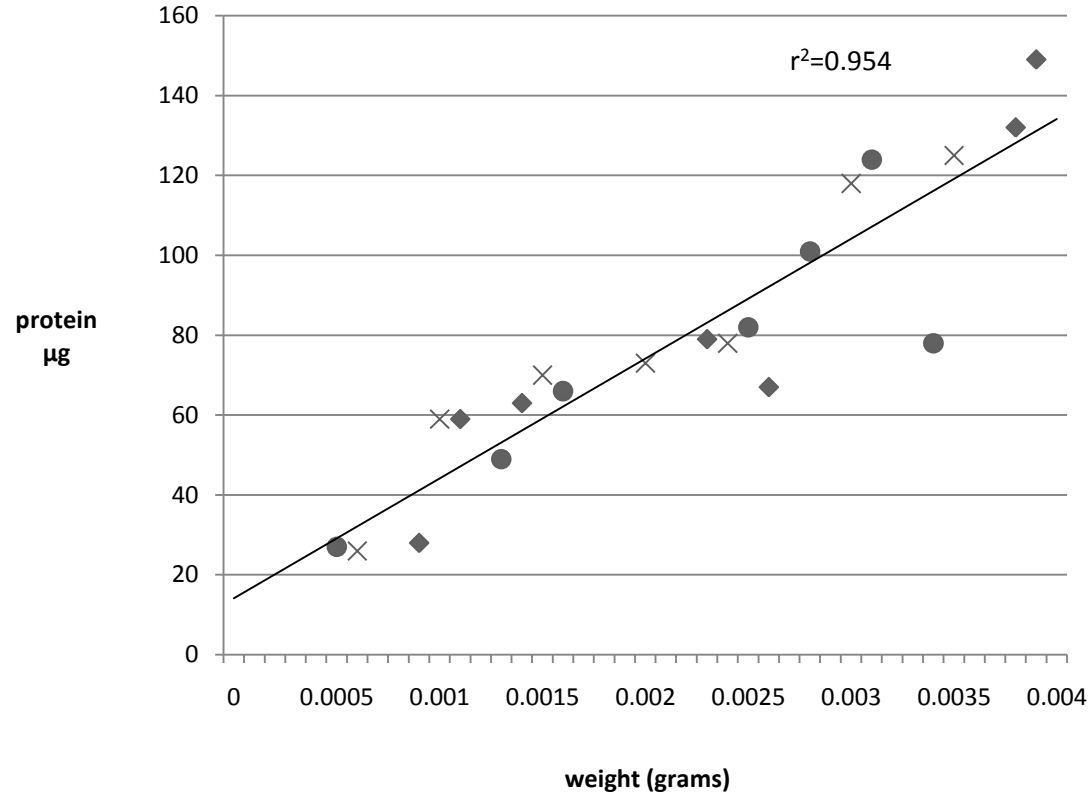


Figure 8.2: This shows the linear relationship between the weight of a biopsy sample and the protein measurement between the weight and the protein measurement of a sample from the human upper gastrointestinal tract. Samples from the oesophagus are labelled ×, samples from the proximal stomach ◆, and samples from the distal stomach ✹. The protein concentration of a biopsy sample added to an Eppendorf test tube containing 80 µL PBS was plotted against the wet weight of the same sample. The number of samples evaluated was twenty-one and these were taken in equal number from oesophagus, proximal stomach and distal stomach.

8.3.3 Releasing intraepithelial contents

Perhaps not surprisingly, the combined method involving all three approaches gave results with the highest concentrations of protein and nitrite per biopsy specimen (figure 8.3). The mean nitrite concentration of samples for each method was 12 (± 4.7) µM for snap freezing alone, 40 (± 7.3) µM for snap freezing and sonication, 46 (± 8.1) µM for snap freezing and homogenization and 66 (± 11.7) µM for a combination of all three methods. The difference between a combination approach and each individual method was statistically significant ($p = 0.0001$) The mean protein content for each method was 49 (± 10.2) µg for snap freezing alone, 87 (± 16.0) µg for snap freezing and sonication, 92 (± 16.4) µg for snap freezing and homogenization and 110 (± 20.1) µg for a combination of all three methods ($p = 0.001$). Performing all three of these actions on the biopsy samples represented the most effective method, and was therefore employed for all subsequent analysis.

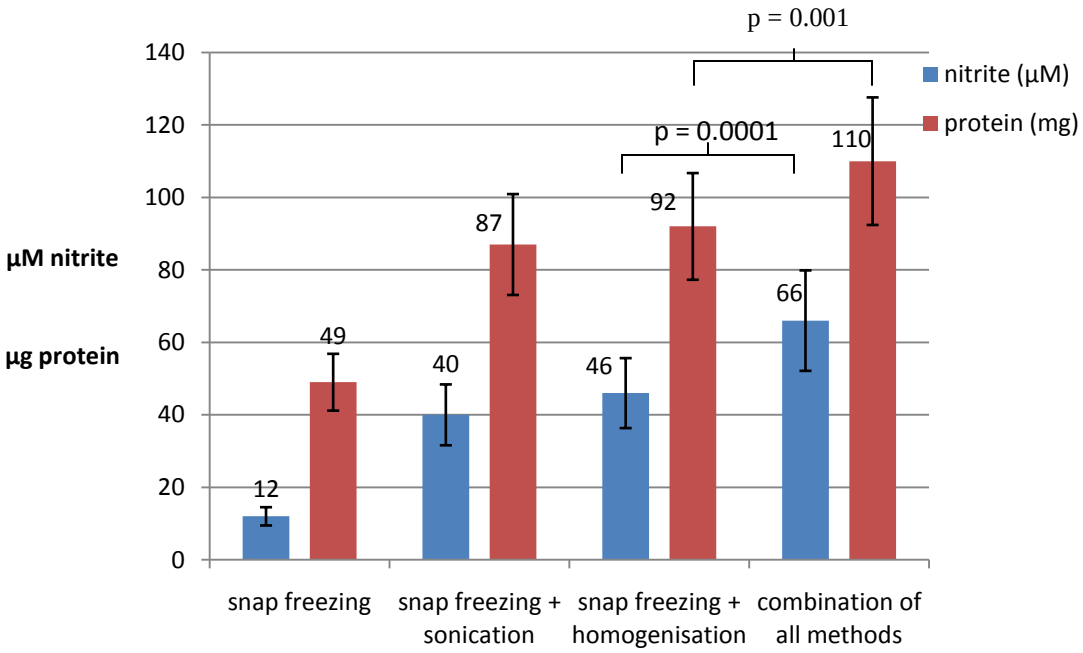


Figure 8.3: Mean nitrite (µM) (blue column) and protein (µg) (red column) concentrations in each sample when analysed by the different methods. Methods were snap freezing only, snap freezing and sonication, snap freezing and homogenization or a combination of all procedures. Data labels show mean values. Error bars represent 1 standard deviation. Statistical significance is shown.

8.3.4 Nitrite analysis

8.3.4.1 Results to determine the better method of nitrite analysis in human studies

The results for each sample when analysed by both the Greiss reagent method and NO probe method, are shown in figure 8.4. The average nitrite concentration from the NO probe method was 12.9 (± 2.1) μM, while the average from the greiss reagent was 18.0 (± 4.3) μM. There was a statistically significant difference between the two methods (p=0.00004). On all but two samples, the amount of nitrite detected was greater with the Greiss reagent.

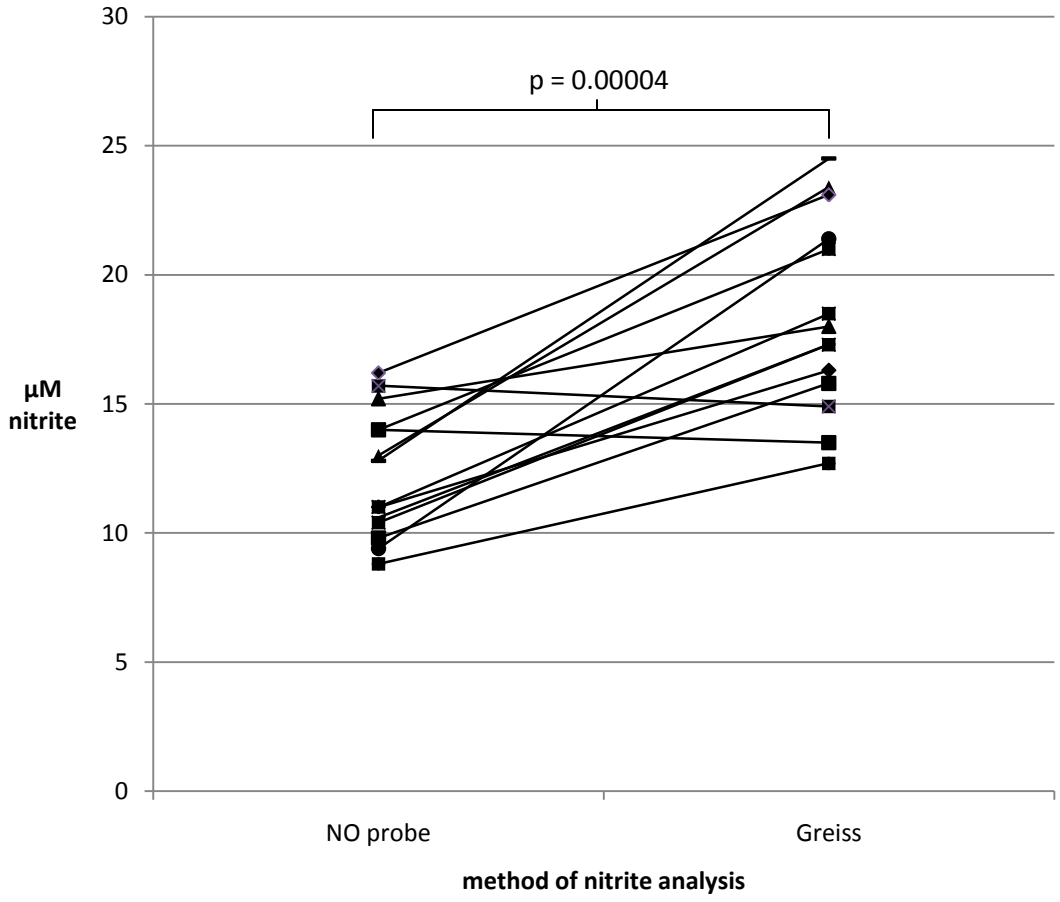


Figure 8.4: This graph compares the µM nitrite concentration measured using both NO probe recording and Greiss reaction in human biopsy samples. For each biopsy sample, both methods were performed and compared. The line between points in the NO probe column and Greiss column is to display the measurements for one sample assessed by the different methods.

8.3.4.2 Assessment of interference with the Greiss reaction

It has been established above that the one step Greiss reaction was the better of the methods for nitrite analysis from biopsy samples. However, a previously published paper by Tsikas et al (308) had suggested that, in the presence of the thiol groups on glutathione and NAC, there would be interference with the Greiss reaction, since the reaction works on the basis of acidifying the solution to allow the reaction to occur. It was suggested that this would result in nitrosothiols being formed from nitrite. In an attempt to clarify this further, a series of experiments was set up using standard concentrations of nitrite, and subjecting them to the Greiss reagent in the presence of varying concentrations of glutathione and NAC. Ascorbic acid was also assessed during these experiments, for any interference. The concentration of nitrite standards used was 25 µM, 50 µM and 100 µM. The percentage nitrite recorded after addition of ascorbic acid, glutathione and NAC was then measured by the one step Greiss reagent method.

Across a range of concentrations from 10 µM to 500 µM there was no interference from glutathione, NAC or ascorbic acid (Table 8.1). At glutathione and ascorbic acid concentrations of 1 mM and above there was interference with the Greiss reaction. Only at NAC concentrations of 5 mM and 10 mM was there a recorded reduction in nitrite concentration and the reduction was only 2-3%. Assessment for synergy between glutathione and ascorbic was also made, at the concentrations shown to significantly interfere with the reaction (10 mM). There did

not appear to be any synergy. Instead the combined effect of these two antioxidants led to an 18% reduction in nitrite concentration.

Within the samples that were being assessed the concentrations of the solutions would be considerably smaller than the concentrations required to interfere with the Greiss reaction. The studies performed by Tsikas et al suggested that smaller concentrations of these antioxidants would lead to interference (308). However, in their studies they did not use a one step Greiss reaction, but rather, they left the solution acidified for two hours prior to adding the dye component. This could have allowed time for the thiol groups to cause more interference by forming nitrosothiols from acidified nitrite.

Table 8.1: Percentage of nitrite measured using the one step Greiss reaction, after a known concentration of nitrite standard was added to one of three antioxidants, ascorbic acid, NAC and glutathione, to assess for any interference with the Greiss reaction by these antioxidants.

	% of standard nitrite concentration measured by greiss reagent		
Antioxidant concentration	Glutathione	Ascorbic acid	NAC
10 µM	100	100	100
50 µM	100	100	100
75 µM	100	100	100
100 µM	100	100	100
250 µM	100	100	100
500 µM	100	100	100
1 mM	98	99	100
5 mM	95	96	98
10 mM	88	77	97

8.3.4.3 Comparison of nitrite to nitrosothiol content of biopsy samples

It has previously been suggested that, in the acidic environment generated by the Greiss reaction, all the nitrite would react with intracellular thiol groups to form nitrosothiols (308). Nitrosothiols will also be formed from nitrosation of thiol groups *in vivo*, although the percentage of the total nitrite ending up as nitrosothiols would be expected to be small. To assess this further, we measured both nitrosothiols and nitrite from twelve biopsies obtained from an equal mix of the oesophagus, proximal stomach and distal stomach. We measured the nitrosothiols in the presence of an excess of glutathione, to both donate thiol groups, and act as a reducing agent. Therefore, when we measured nitrosothiols the total intracellular nitrite would be measured. Nitrosothiol measurement was through the NO probe method described in Chapter 8.2.5 and Chapter 2.1.7.

The results show that there is no significant difference between the nitrosothiol concentration measured and the nitrite concentration measured by the Greiss reagent ($p=0.60$) (Figure 8.4). The mean total nitrosothiol concentration was 12.1 (± 5.7) μM while the nitrite concentration was 13.8 (± 5.1) μM. Therefore, the contribution of nitrosothiols to total biopsy nitrite was not detectable by the methods adopted in this study and therefore were not measured in further studies.

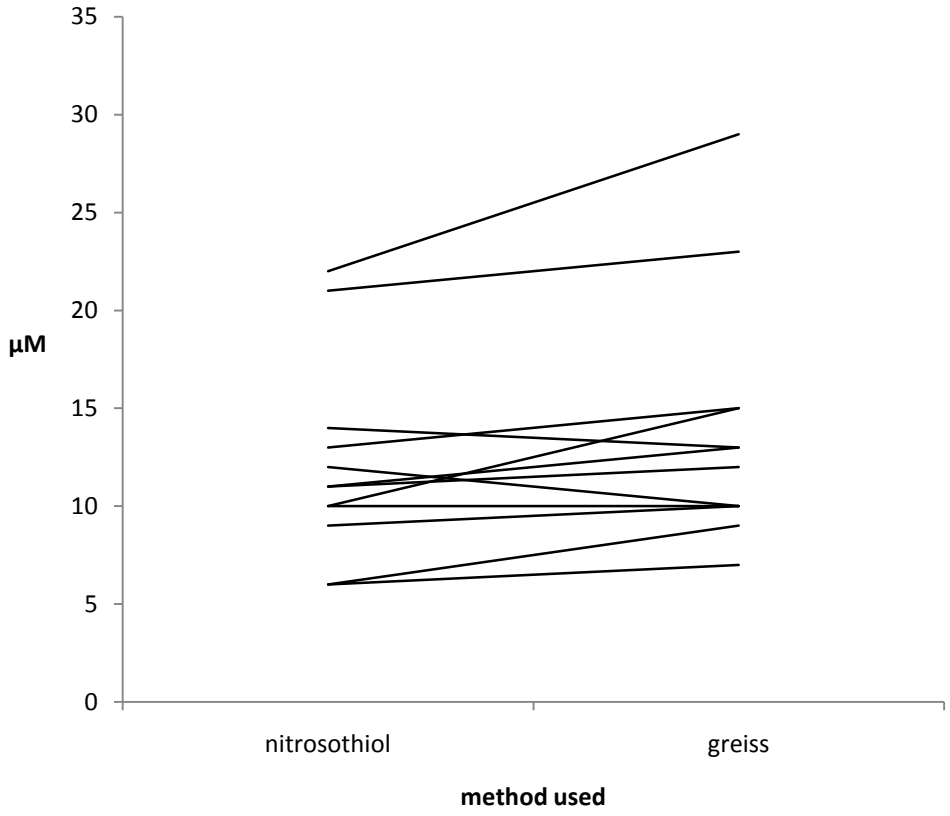


Figure 8.5: Human upper gastrointestinal tract biopsy specimens were analysed for nitrite concentration (µM), using the Greiss reagent method, and for nitrosothiol concentrations (µM), using a method whereby all nitrite would also be converted to nitrosothiol. The concentration of nitrosothiol (µM) is therefore a reflection of sample nitrite + nitrosothiol concentrations. Comparison is made between the sample concentration of nitrite, measured using the different methods, to assess any additional concentration recorded in the nitrosothiol samples which would reflect true nitrosothiol concentration.

8.4 Discussion

The intention, in performing the studies in this chapter, was to develop a method of assessing human upper gastrointestinal tract intracellular nitrate and nitrite concentrations, to enable assessment of nitrosative stress. Previous studies using silastic tubing as an epithelial compartment had shown that nitrite was a marker of nitrosative stress.

It was possible to standardise sample nitrite and nitrate concentrations relative to the protein content of each biopsy sample. This allowed for the varied sizes of biopsy that are taken from the upper gastrointestinal tract. Although the biopsy samples would have protein contained within the mucus layer the cleaning effect, of over 90%, was consistent between samples from the three anatomical regions sampled and between samples from the same anatomical region. This means that if the protein content was being overestimated it would be so constantly. Protein content, therefore, is a useful standardising measurement for nitrite and nitrate.

Processing of samples was effective in terms of releasing intracellular content into PBS by a combination of snap freezing, sonication and homogenisation. Although a combination of these techniques yielded the highest concentrations of nitrite and protein the actual degree of cell membrane destruction was not determined. This could mean that the concentrations recorded are an underestimate of the true intracellular concentrations. An independent control, such as electron

microscopy, could be employed to assess the degree of cell membrane lysis. Measurement of nitrite by the one step Greiss reaction was validated in our studies, which also show that the contribution of nitrosothiols from the samples was not relevant.

To enable accurate assessment of intracellular contents it was apparent that the mucus barrier present in the upper gastrointestinal tract would have to be removed. Unfortunately, mucus in the upper gastrointestinal tract is relatively adherent to the epithelium. This is particularly the case in the stomach. The best mucolytic cleaning solution was found to be NAC. This has been used previously to clean the upper gastrointestinal tract from mucus, but not in the context of measuring intracellular content concentrations. In our studies, the cleaning achieved was removal of over 90% of the surrounding mucus from biopsy samples.

NAC appeared to be the best cleaning solution. However, acetylcysteine is the *N*-acetyl derivative of the amino acid L-cysteine and exhibits direct and indirect antioxidant properties. Its free thiol group is capable of interacting with the electrophilic groups of ROS (310,311). This interaction with ROS leads to intermediate formation of NAC thiol, with NAC disulphide as a major end product (312). In addition, NAC can exert an indirect antioxidant effect related to its role as a GSH precursor *in vivo*. It has been shown that thiols enhance NO generation from NO releasing agents (313,314). This could, theoretically, result in the cleaning solution used in our experiments releasing NO from nitrite within the mucus layer.

NO would then be free to pass into the epithelial compartment and form nitrite and nitrosating species. However, the reaction of nitrite with NAC will take place in the neutral environment of the mucus lining of the oesophagus. The NAC produced for the studies was also made up in 200 mM sodium bicarbonate to neutralise the pH of the solution and to make it more isotonic. This means that although NO would be produced from this reaction in an acidic environment its production in a neutral environment would be minimal.

Overall, measurement of upper gastrointestinal tract biopsy nitrate and nitrite is possible using the methods described. The major problem with these methods is cleaning of adherent mucus from the biopsy sample prior to subsequent analysis for nitrite and nitrate. The mucus is likely to have a high concentration of nitrate and nitrite. The cleaning methods do appear to be able to clean over 90% of the surrounding mucus and although it may not be possible to extrapolate data relevant to intraepithelial concentrations of nitrate and nitrite, the nitrosative stress within the biopsy sample of an anatomical region can be determined.

Chapter 9: Upper gastrointestinal tract biopsy sample nitrite and nitrate concentrations

9.1 Introduction

The hypothesis, that intraepithelial nitrite and nitrate reflect where NO generation is maximal, would mean that on analysis of samples from three areas of the upper gastrointestinal tract, oesophagus, proximal stomach and distal stomach, we would expect samples from the proximal stomach of healthy subjects to have the highest concentrations of nitrite and nitrate. In previous studies it was not possible to remove all adherent mucus from biopsy samples. This means that measuring intraepithelial nitrate and nitrite is not possible with the methods described. However, studies were performed to look for biopsy sample nitrite and nitrate concentrations. While it is not possible to extrapolate from this series of experiments whether this increase is related to intracellular nitrate and nitrite, it could still provide information regarding nitrosative chemistry within the upper gastrointestinal tract. The working hypothesis was that biopsy sample nitrite and nirrate concentrations would be higher in proximal stomach biopsies, as compared to oesophageal and distal stomach biopsies, due to generation of NO from salivary nitrite meeting acidic gastric juice. To show that the higher concentrations were related to ingested nitrate, the studies were performed before and after the ingestion of a nitrate drink.

9.2 Methods

9.2.1 Healthy volunteers

A total of 17 healthy volunteers were enrolled, 9 of whom were male. The median age was 52 years. All volunteers were *Helicobacter pylori* negative, as detected by urease CLO testing, and had no history of antacid ingestion. Endoscopy with biopsy was performed on two separate days. On day one, the volunteer had 60 mL of control drink which was dH_2O. On the second day the volunteer was given a 60 mL drink containing 2 mmole KNO_3. Endoscopy with biopsy sampling was performed 2 hours after either the control or nitrate drink was given. On each day six biopsies were taken from each of, the oesophagus (5cm above the GO junction), proximal stomach (just below the gastroesophageal junction) and the distal stomach. A 1 mL salivary sample was collected at times 0 and 120 minutes for nitrite analysis. At the same time intervals as salivary samples were taken, the volunteers had blood samples taken for analysis of serum nitrate from an i.v cannula, placed in the antecubital fossa prior to the start of the experiment.

9.2.2 Processing of biopsy samples

Biopsy samples were immediately placed in a 2 mL solution of 3.1% NAC in NaOH (pH 7.4) and left for 30 minutes before being subjected to the cleaning process and cell lysis described in sections 8.2.2 and 8.2.4. Of the cleaned sample,

snap frozen, homogenised and sonicated in 80 µL PBS, 20 µL was then analysed for each of protein, nitrite and nitrate concentrations. Biopsy nitrate and nitrite concentrations were recorded as concentration per µg protein.

9.2.3 Serum nitrate analysis

Blood samples (10 mL) were taken at times 0 and 120 minutes. The first 5 mL of this sample was discarded to allow for dead space within the i.v cannula. 2 mL of serum sample was stored at –20°C until it was analysed. On thawing, 400 µL of sample was centrifuged through a 10-kilodalton ultrafilter to remove high-molecular-weight proteins. Samples were then mixed with nitrate reductase in reaction buffer and analysed as described in Chapter 2.1.5.

9.2.4 Salivary nitrite analysis

Salivary samples (1 mL) were obtained at times 0 and 120 minutes. This was mixed with 10 µL of 5.0 M NaOH. This rendered the samples alkaline and prevented loss of nitrite. Samples were stored at 4°C, then centrifuged and analyzed the same day on 96-well microplates using the modified Griess reaction as described in Chapter 2.1.4.

9.3 Results

9.3.1 Salivary nitrite

For volunteers on the day they consumed the control drink, the median salivary nitrite concentration prior to the administration of the control was 42.5 (interquartile range, 28.5 – 97.3) µM. 120 minutes after the administration of the control drink, the salivary nitrite concentration did not significantly change (p=0.09) from the basal reading, with a median concentration of 52.0 (interquartile range, 35.8 – 86.2) µM. However, on the day the volunteers were given a 2 mmole potassium nitrate drink, there was a significant increase (p=0.0000003) in the median salivary nitrite concentration from 39.7 (interquartile range, 29.6 – 65.8) µM on sampling prior to administration of the drink to a concentration 264.8 (interquartile range, 160.1 – 358.6) µM 120 minutes after the drink was given.

9.3.2 Serum nitrate

On the day the volunteers consumed the control drink, the median serum nitrate concentration prior to the administration of the control was 11.6 (interquartile range, 4.3 - 17.3) µM. At 120 minutes after the administration of the control drink, the serum nitrate concentration did not significantly change ($p=0.19$) from the basal reading, with a median concentration of 12.2 (interquartile range, 7.2-15.7) µM. However, in those who were given a 2 mmole potassium nitrate drink, there was a significant increase ($p=0.00004$) in the median serum nitrate concentration from 13.5 (interquartile range, 5.6-18.3) µM on sampling prior to administration of the drink to 54.7 (interquartile range, 31.2-69.8) µM 120 minutes after the drink was given.

9.3.3 Nitrate and nitrite concentrations at different anatomical sites

Samples from the proximal stomach had the highest concentrations of both nitrite and nitrate. The median nitrite concentration from the proximal stomach was 36.7 (interquartile range 19.5 – 48.2) µM per µg protein, which was a significantly higher concentration than the concentrations from the oesophagus (14.5 µM) (p=0.00001) and distal stomach (24.0 µM) (p=0.0003) (figure 9.1). For all three regions of the upper GI tract from which biopsies were taken, there was a significantly higher nitrate than nitrite concentration. The median nitrate concentration in the proximal stomach was 114.1 (interquartile range 64.5 - 222.1) µM per µg protein, which is significantly higher than the concentration in the oesophagus (65.2 µM) (p = 0.00004) and distal stomach (65.7 µM) (p=0.00002).

After ingestion of a 2 mmole KNO_3 drink, there was a significant increase in the median nitrite and nitrate concentrations for all anatomical sites sampled, compared to control drink ingestion samples. The greatest percentage increase in concentrations was seen in the proximal stomach samples, where there was a three fold increase in nitrite concentration with a median nitrite concentration of 124 (interquartile range 86.2 – 176.2) µM per 100 µg protein (p=0.000002), and 2.5 fold increase in nitrate concentrations with a median nitrate concentration of 285 (interquartile range 167.2 – 341.9) µM per 100 µg protein (p=0.00007) (Figure 9.2).

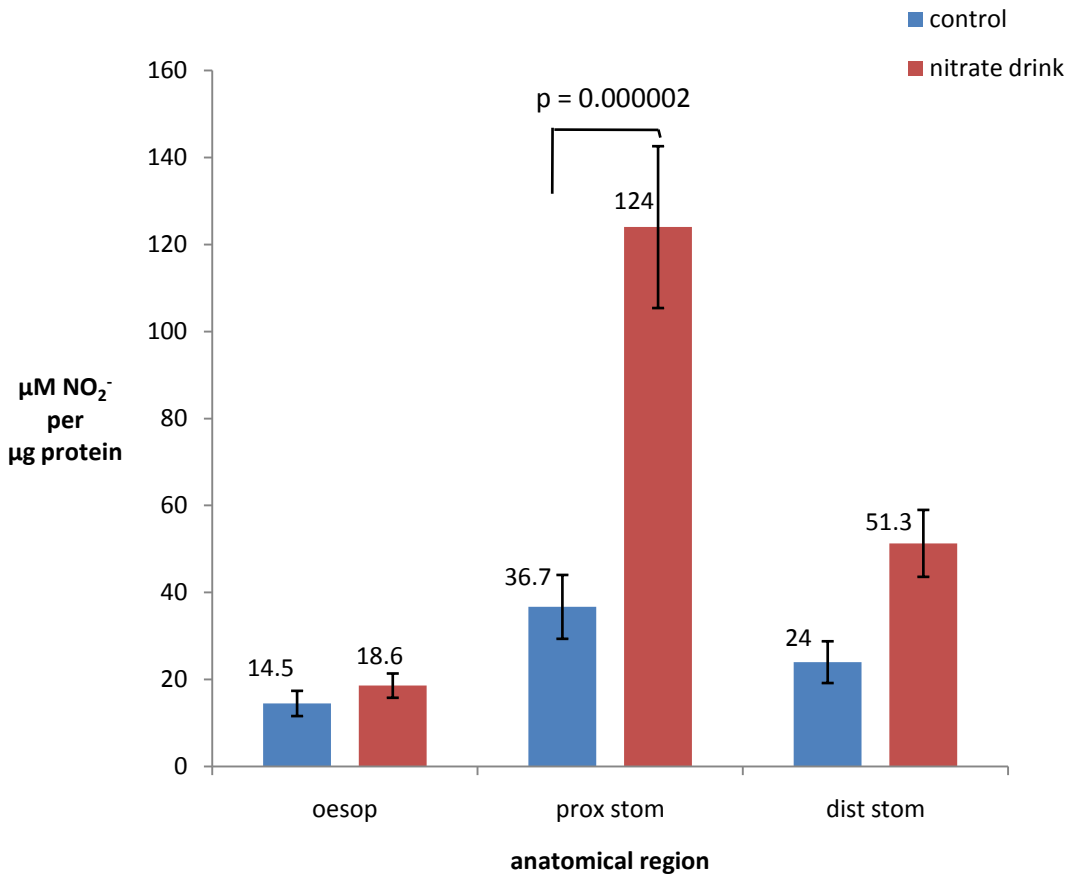


Figure 9.1: Median nitrite concentrations expressed as µM per µg protein are shown for three regions of the human upper gastrointestinal tract (oesophagus, proximal stomach and distal stomach) after a control drink (blue columns) and after a drink containing 2 mmole nitrate (red columns). Data labels show the median value. The error bars represent the 1st and 3rd interquartile range.

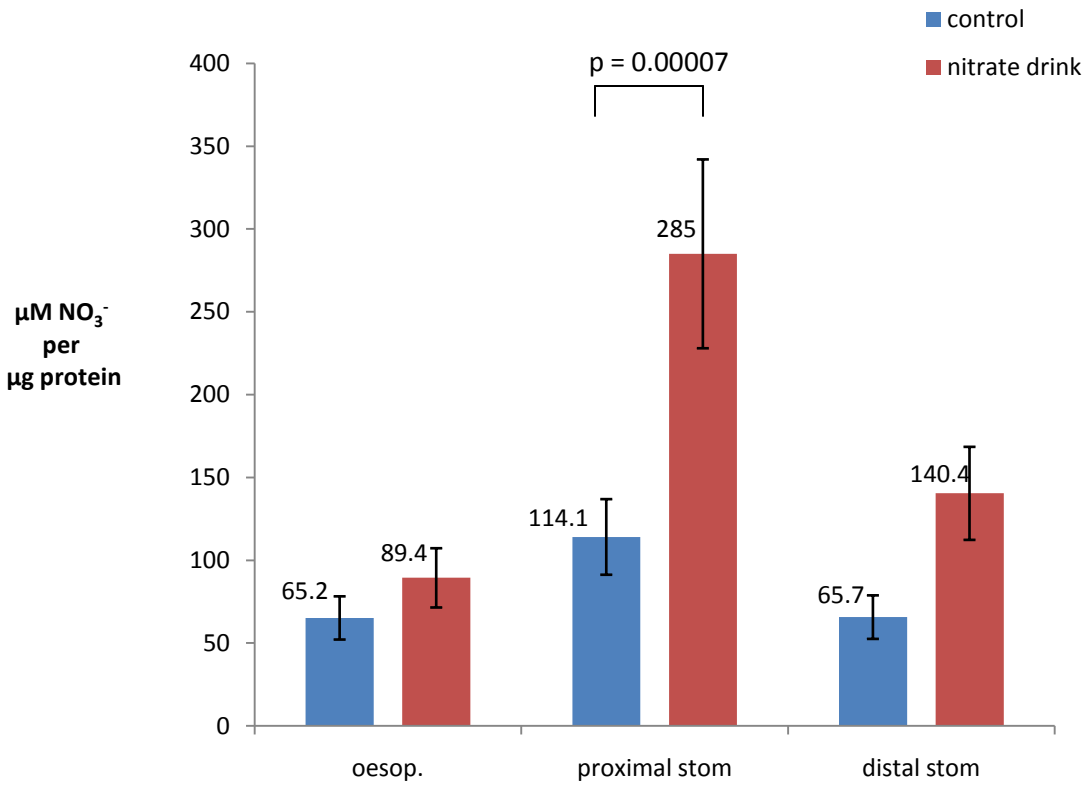


Figure 9.2: Median nitrate concentrations expressed as µM per µg protein are shown for three regions of the human upper gastrointestinal tract (oesophagus, proximal stomach and distal stomach) after a control drink (blue columns) and after a drink containing 2 mmole nitrate (red columns). Data labels show the median value. The error bars represent the 1st and 3rd interquartile range.

9.4 Discussion

This study suggests that, *in vivo*, there is an increase in nitrosative stress within the human upper gastrointestinal tract after nitrate ingestion. The mechanism proposed for the increase in this stress is through the generation of NO where salivary nitrite, produced in greater concentrations after a nitrate meal due to bacterial conversion and enterosalivary recirculation, meets acidic gastric juice containing ascorbic acid. NO can then rapidly cross the lipid cell membrane into the neutral epithelial compartment forming nitrosating species and nitrite.

After the control drink was given, the proximal stomach was the site of the highest concentrations of nitrite and nitrate. This is likely due to this region being the first point of contact of salivary nitrite with acidic gastric juice in the presence of ascorbic acid. Even in the control group there will be carriage of nitrite in saliva as shown by the concentration of salivary nitrite being 40 – 50 μM (221). It has previously been shown that the concentrations of nitrite along the length of the oesophagus are similar to the concentrations in saliva (221). NO will therefore be generated in the proximal stomach, as nitrite meets ascorbic acid in acidic gastric juice.

After ingestion of a nitrate drink the nitrite concentration of biopsy samples from all three regions of upper gastrointestinal tract was higher than concentrations found in the control samples. The greatest increase in nitrite concentrations was

observed in samples from the proximal stomach where this was three fold. There are two potential mechanisms for this. There is the generation of NO as discussed for the control group. The maximal point of NO production will be the proximal stomach, where salivary nitrite meets gastric juice containing ascorbic acid. The second mechanism is through acid-catalysed nitrosation, where the ascorbic acid within the proximal stomach is exhausted after reaction with nitrite (221). Nitrite will then form nitrous acid in the proximal stomach and this may diffuse into the neighbouring intraepithelial cellular compartments.

In our study, the biopsy concentration of nitrate was higher than nitrite in the control samples and increased after a nitrate drink. There are two possible mechanisms to explain the increase in nitrate from NO generation. The first of these is the well recognised reaction of NO with the iron contained within oxyhaemoglobin to produce methaemoglobin and nitrate (117). NO can also be expected, within a cell, to react with the superoxide to form peroxynitrite. Nitrate is a decomposition product of peroxynitrite. These chemical reactions are shown below:

$O\text{-}O^- + N{=}O \rightarrow OONO^-$	the reaction of superoxide with NO
$4OONO^- \rightarrow NO_3^- + 2NO_2^- + O_2$	the decomposition of peroxynitrite to nitrate, nitrite and oxygen

There are a few potential problems in concluding that the increases in biopsy sample nitrite and nitrate are all due to generation of NO. The main problem relates to the cleaning of the biopsy specimens. It had been the intention in this study that measurement of nitrite and nitrate concentrations would have been from the intracellular compartment. Enrichment studies using ^{15}N suggested that 93% of adherent mucus was cleaned from the biopsies using NAC. While this suggests reasonable cleaning, it is not enough to provide confidence that the increases in biopsy sample nitrite and nitrate are all due to an increase in intracellular compartment concentrations of these compounds. It is possible that the increases observed were mainly due to an increase in the adherent mucus concentrations of these compounds. The mucus overlying the epithelium of the upper gastrointestinal tract has a neutral pH and when NO meets this environment the NO can be expected to form nitrite. Whether there is enough of a mucus barrier to prevent any of the very high concentrations of NO seen to be produced at the gastroesophageal junction and proximal stomach (43,45,280) is unknown.

As well as nitrosative stress from NO, there is also the potential for acid-catalysed nitrosation to be having an effect in these studies. Previous work by Suzuki et al has shown that after a nitrate rich meal there is a change in the nitrite to ascorbic acid ratio such that acid-catalysed nitrosation is favoured (221). This could be due to exhaustion of available gastric juice ascorbic acid such that nitrous acid is formed in acid from nitrite. This can then pass into the epithelial compartment and

here be converted to nitrite. Acid-catalysed nitrosation within the stomach is well recognised and discussed in the literature.

This is, therefore, an interesting study in mapping potential nitrosative stress in the upper gastrointestinal tract. The proximal stomach is the site of maximal nitrite and nitrate concentrations. The study also shows that after a nitrate meal there is an increase in nitrosative stress, and this is most marked in the proximal stomach. While it is possible that some of the increased stress is not NO mediated, the fact that, in these healthy volunteers, the proximal stomach is the site of maximal concentration of nitrite and of the increase in nitrite after a nitrate meal, makes it likely that NO has a significant role to play in nitrosative stress after a nitrate meal.

Chapter 10: Rat model: Nitrite as a marker of nitrosative stress

10.1 Introduction

It has previously been found that nitrite is a marker of nitrosative stress in an *in vitro* model simulating the chemistry occurring at the gastroesophageal junction (44). Nitrite is, therefore, an attractive candidate as a marker of nitrosative stress in biological tissue of the upper gastrointestinal tract. Studies performed using rat stomach tissue were devised to evaluate the efficacy of the cleaning of biological tissue required prior to quantification of intracellular nitrite and nitrate, and later, to assess the effect of NO on epithelial samples to determine if nitrite was a measurable marker of NO in biological tissue.

The availability of human tissue is dependent on biopsies taken at time of endoscopy. Rat tissue is more freely available and can be obtained in large quantities, as the entire stomach can be removed for tissue analysis. Although the rat stomach is not anatomically or physiologically identical to the human stomach, as it is more easily obtained, it allows multiple experiments to be perfomed to assess whether the measurement of biopsy nitrite and nitrate, related to NO, is possible. There is a difference in the thickness of the mucus in the rat stomach compared to the human. The mucus layer between the acidic gastric lumen and the epithelial surface is thicker in human samples than in rat (315). As the whole rat

stomach can be dissected out, an appropriate sized piece of tissue can be placed in specifically designed experimental models.

The rat stomach is divided into two parts, the forestomach and corpus (figure 10.1 and 10.2): **Forestomach**: thin-walled, non-glandular section that receives the oesophagus and serves as a holding chamber for food. Its walls are similar to those of the oesophagus. **Corpus**: thick-walled, glandular section. Its walls have secretory glands that produce digestive enzymes and mucus. Digestion begins in the corpus. The pyloric sphincter controls the movement of food from the corpus to the intestines and specifically the duodenum.

The forestomach and the corpus are separated by a low fold of tissue called the limiting ridge (*margo plicatus*). The limiting ridge extends circumferentially from the large curvature of the stomach to the small curvature, just below the oesophagus. At the oesophagus, the course of the limiting ridge bends into a U-shape and almost surrounds the oesophageal opening (316).

In the following studies in this chapter, the rat forestomach was used to represent the human oesophageal tissue, while the rat stomach corpus tissue was used to represent the human stomach tissue.

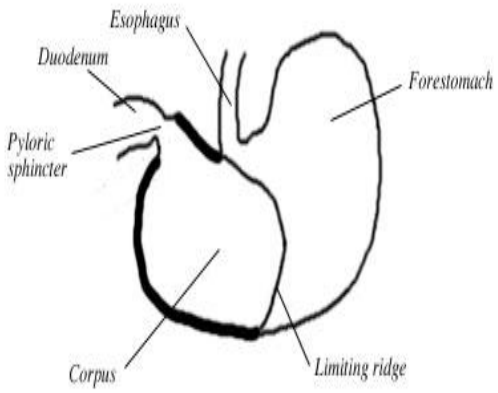


Figure 10.1: A schematic diagram of the rat stomach, showing the forestomach, limiting ridge (margo plicatus) and corpus regions of the stomach. The oesophagus empties into the forestomach. The corpus empties into the duodenum through the pyloric sphincter. The limiting ridge separates the forestomach from the corpus.

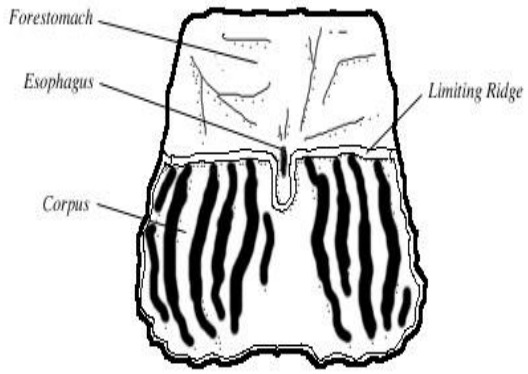


Figure 10.2: The rat stomach can be seen to be divided into the forestomach and the corpus, by the limiting ridge which extends circumferentially from the large curvature of the stomach to the small curvature, just below the oesophagus.

10.2 Methods and materials

10.2.1 Rats

All rats used were female Wistar rats greater than 250g in weight. The large weight was used to ensure that the size of the stomach was adequate for our experiments. The rats were fasted for 24 hours prior to dissection.

Dissection of the rats took place in the animal house after being sacrificed using 50% CO_2 and O_2 causing asphyxiation. The dissection consisted of making a vertical incision the length of the rat, from vagina to sternum. The incision went through to the abdominal cavity where the stomach was located underneath the liver. The stomach was separated from the peritoneum and remainder of the gastrointestinal tract, by several small incisions.

10.2.2 Storage and preparation of tissue

The stomach was immediately placed in chilled Krebs solution and kept on ice after dissection. The stomachs were then transferred to the laboratory, where the experiments were performed within 2 hours of the dissection.

Once in the laboratory, the stomach was cut along the greater curvature to enable visualisation of the margo plicatus. The whole stomach was then briefly

rinsed in dH_2O to remove remaining food residue. Tissue from both the corpus and forestomach was used in the experimental models. It was technically difficult to obtain, solely, the epithelial layer. In these experiments the mucosa was removed by injecting 1 mL saline solution to create a bleb into the submucosa, thereby lifting the mucosa from the underlying layers. This area of mucosa was then carefully dissected from the rest of the tissue, using a 0.5mm scalpel blade. Once tissue for analysis was obtained, it was cleaned anplaced in PBS (80 µL) before being snap frozen in liquid nitrogen before storage at -70^{o}C.

10.2.3 Processing of tissue

After thawing the samples required a cleaning process to remove surrounding mucus from the rat tissue. As discussed in Chapter 8.2.2, 3.1% NAC at neutral pH in PBS is a mucolytic agent with the capability of cleaning surrounding mucus from human tissue. Where rat tissue has been cleaned, in the following experiments, 3.1% NAC has been used.

Samples were then snap frozen, homogenised and sonicated to release intracellular compartment constituents, as discussed in Chapter 8.2.4. Initially there was difficulty homogenising rat stomach samples, both in a glass hand held homogenizer, and also in a larger steel shafted serrated head mortar and pestle. Therefore, softening of the samples by alkalisation was performed to aid the homogenisation process. Alkalisation was achieved using sodium hydroxide, by

adding 50 µL of 5 M NaOH to 420 µL of PBS. After this alkalisation the samples were homogenised using a glass hand held homogenizer (Anachem Ltd, Bedforshire, UK), with subsequent centrifugation of the sample at 13000g.

10.2.4 Chemical analysis

Measurement of NO and O_2 was performed using WPI probes and sensors as described in Chapter 2.1.1 and 2.1.2. Methods for protein, nitrite and ^{15}N analysis were as for human samples and are described in Chapter 2.1.3, 2.1.4 and 2.1.6.

The analysis of nitrate was performed with some adjustments from previous methods. Nitrate reductase which converts nitrate to nitrite is a pH dependent enzyme working best at pH 7.5. Samples in these experiments were strongly alkaline after a softening process prior to homogenisation. Although the samples were placed in PBS, the volume of PBS used was small and did not cause the pH of the sample to reach neutrality. It was found that, when the pH was not corrected to a neutral pH, we did not record any nitrate from our samples. This problem was overcome by using a 'super' acidic buffer which was constituted as 0.1 M NaOH and half volume 0.3 M HCl. When plating the samples, 30µL of sample was taken and 30 µL of the 'super' buffer was added to make the final pH neutral. Samples were then analysed for nitrate concentrations as described in Chapter 2.1.5.

10.2.5 Contamination studies to assess cleaning of sample

10.2.5.1 Immersion in 5 mM nitrate

Dissected rat stomach mucosa from both the forestomach and corpus was removed, and subjected to one of the following conditions in a 1 mL plastic Eppendorf test tube: exposed to dH_2O and left unclean, exposed to dH_2O but cleaned in 3.1% NAC for 30 minutes, exposed to 5 mM KNO_3 for 30 minutes then left unclean or exposed to 5 mM KNO_3 for 30 minutes then cleaned in 3.1% NAC for a further 30 minutes.

The stomach from six different rats was used for these experiments and six different samples from each rat were used for each condition to which the mucosa was exposed. Samples were then analysed for protein, nitrate and nitrite.

10.2.5.2 Three layer Delrin model contamination studies with 5 mM nitrite and nitrate

A specially designed Delrin model was constructed as shown in Figure 10.3. The lower chamber in this model was filled with a solution of 5 mM KNO_3 or 5 mM KNO_2 to contaminate the sample. Contamination studies were performed in this model as it is similar to the model used to perform the studies subjecting rat tissue to NO. Tissue from six rats, from both the corpus and forestomach, were placed between the upper and lower layers of this model. In this experiment there

was a control sample, which was placed within the Delrin model containing only dH_2O only before being either cleaned in 3.1% NAC or left unclean. Of the tissue placed within the KNO_3 Delrin model, half of the samples were left unclean, while the other half of the samples were subjected to the cleaning process in 3.1% NAC. For each experimental condition there were six samples in each arm of the experiment per rat stomach.

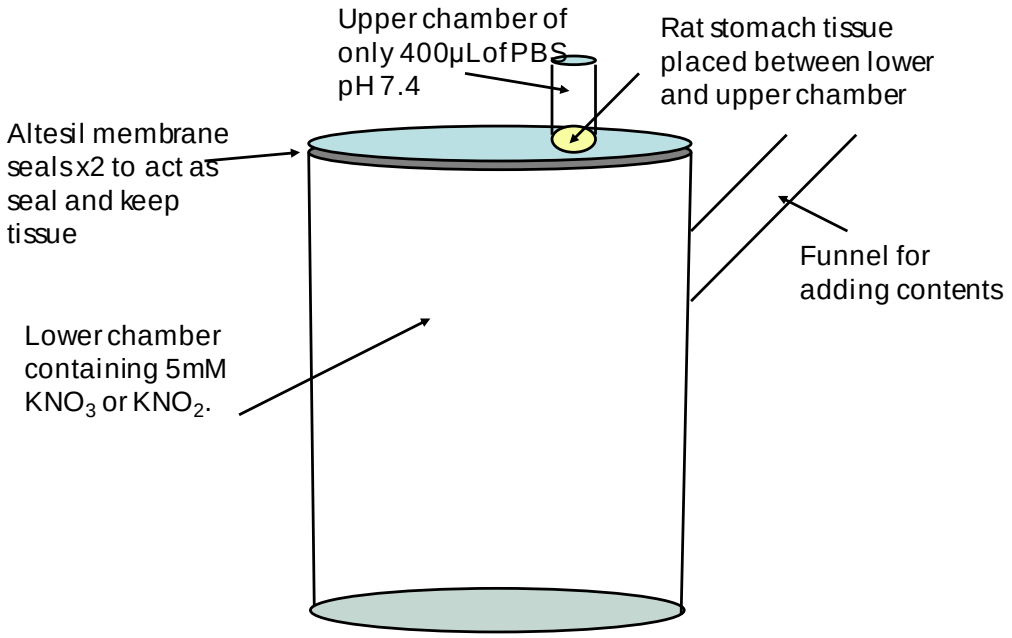


Figure 10.3: Three-phase Delrin model used to subject the epithelial surface of rat stomach mucosa to high concentrations of contaminant KNO_3 or KNO_2. In this three-phase model the rat tissue formed the middle layer. The rat tissue was supported by a hydrophobic altesil membrane (thickness 0.8mm) through which NO and O_2 are known to pass. The tissue was placed between two layers of the membrane. The membrane had a hole cut, in the centre of where the tissue lay, to allow the rat tissue to be the only contact between the upper and lower layers of the model. The lower chamber consisted of 5 mM KNO_3 or KNO_2. The upper chamber was formed by 400 µL PBS at a neutral pH.

10.2.5.3 Three layer Delrin model contamination with ^{15}N nitrite and nitrate

To assess the extent of contamination after cleaning more closely, experiments were perfomed involving a labeled ^{15}N preparation of nitrite and nitrate. This allowed determination of whether nitrogen measured was contamination from the nitrate and nitrite used to spike the samples or whether it was nitrogen already present in the sample. If ^{15}N concentrations were high, then the cleaning was not as efficient as we required for assessing intraepithelial nitrite and nitrate.

The ^{15}N nitrite and nitrate was used in the same Delrin experimental model as shown in figure 10.3. The concentrations used as contaminant solutions were 5 mM $K^{15}NO_2$ and 5 mM $K^{15}NO_3$. After processing in the standard way described in 10.2.3, samples were analysed for protein, nitrite, nitrate and ^{15}N content. Six samples, from each of six different rats, were taken and subjected to experimental conditions.

10.2.6 Model used to assess the effect of NO on rat epithelial cells

Experiments were performed, within a three layer model, with rat stomach tissue being exposed to NO produced from the acidification of nitrite in the presence of ascorbic acid (figure 10.4).

Rat mucosal tissue was obtained as described in 10.2.1. Both the glandular corpus of the rat stomach and the forestomach were used. Once the appropriate tissue was obtained, it was placed between two layers of altesil membrane which had been specially prepared. The membrane was cut with a hole in the middle, this was to allow the rat tissue to be the only layer between the upper chamber and the lower chamber. The tissue was then clamped in place in the Delrin model. HCl (pH 1.5) with ascorbic acid (1 mM) to a volume of 17.9 mL was added to the lower chamber. The experiments were initiated by adding 120 μM nitrite to the lower chamber, except in control experiments where nitrite was replaced with dH_2O.

Experiments were performed to ensure that this system was allowing rat tissue to be exposed to appropriate concentrations of NO. NO levels were measured, using a WPI NO probe, from the upper chamber surface of the rat tissue to ensure that NO was passing through the tissue. We found that it took between 60 and 90 seconds for the NO to diffuse through the tissue, after the addition of the nitrite to the lower layer. After that time there was a steady rise in the NO level to a maximum of between 60 and 70 μM at the end of the twenty minute experiments

(figure 10.5). At no point during the experiment was the upper layer deficient in O_2 as measured with a WPI O_2 probe placed in the upper chamber on the rat surface. At the end of the 20 minute experiment the pH in the upper chamber was still neutral, showing the integrity of the rat stomach tissue to form a middle layer during the experiment.

Thereafter, experiments were performed to determine if we could measure intracellular nitrite/nitrate in the rat stomach tissue by exposing the tissue to generated NO. The experiments were set up as described above. In addition, control experiments, with tissue from the same rat, were set up exactly as above, but on these occasions no nitrite was added to the lower chamber. Instead, in these control experiments there was no generation of NO, as was confirmed by the absence of a rise when the NO level was measured using the NO probe and meter.

A series of experiments to assess the effect of nitrous acid on the samples was also set up, by having the same experimental conditions as in the experiments generating NO except that the lower chamber had no ascorbic acid present. In these experiments there was no recordable NO generation in the upper chamber. All samples were cleaned using 3.1% NAC as described in Chapter 8.2.2.

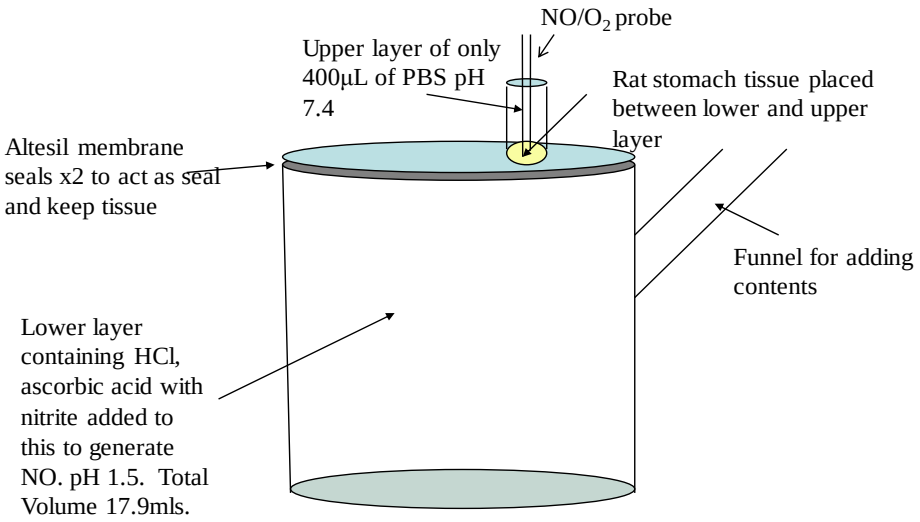


Figure 10.4: Three layer Delrin model to expose rat stomach mucosa to NO generated from acidified nitrite in the presence of ascorbic acid. Rat stomach tissue was placed between two layers of silicone altesil membrane (thickness 0.8mm), with a hole in the membranes so that there was an area of tissue that was not covered by the membrane. This was then clamped between the top of the Delrin lower base unit and the upper Delrin unit. Into the lower unit was added HCl (pH 1.5) and ascorbic acid (1mM). This is to represent the acidic environment of the stomach. The upper layer had 400 µL PBS added to it (pH 7.4). The rat tissue used was the mucosa obtained after a submucosal bleb had lifted the mucosa from underlying tissue. After the addition of

nitrite (120 µM) to the lower layer NO is generated in this layer and the rat tissue that is above is exposed to this NO.

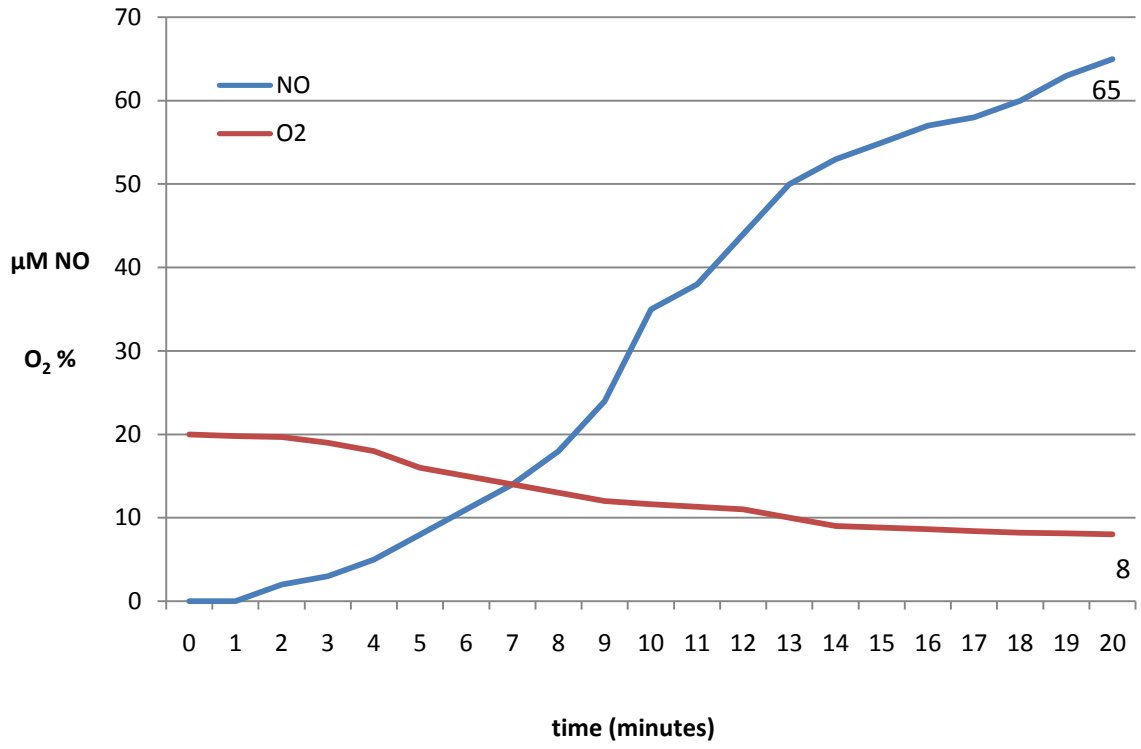


Figure 10.5: NO and O_2 concentrations on the upper surface of rat stomach tissue after the addition of nitrite (120 µM) to an acid environment containing ascorbic acid in the lower chamber of a three-phase Delrin model. Data labels show the mean NO concentration at 15 minutes. n=12

10.3 Results

10.3.1 Contamination studies

10.3.1.1 Immersion studies in 5 mM KNO_3

The immersion form of contamination with 5 mM KNO_3 led to a significant rise in mean nitrate concentration compared to samples which were not subjected to any nitrate (figure 10.6) ($p<0.0001$). In forestomach samples, there was effective cleaning of samples with only 8% more nitrate present after contamination and cleaning as compared to samples that had not been exposed to contaminant. The mean nitrate concentration of samples exposed to nitrate and cleaned was 24.8 (±3.7) µM per µg protein compared to 22.9 (±2.0) µM per µg protein for samples which were not subjected to contaminant nitrate and cleaned (figure 10.6) ($p=0.18$). This represented cleaning of around 96% when compared to the contaminated unclean sample. The rat stomach corpus samples appeared to be more difficult to clean, as the mean nitrate concentration of samples subjected to nitrate and cleaned is 28.6 (±3.2) µM per µg protein compared to 24.5 (±4.1) µM per µg protein when samples were not subjected to nitrate and cleaned ($p=0.03$). This means there was 16% more nitrate on the contaminated samples than the control samples. The cleaning efficiency when contaminated unclean samples were compared to contaminated clean samples was 92%. There was no significant difference in mean

nitrite concentrations for samples subjected to contamination with nitrate ($p=0.64$). There was, however, a trend for the mean nitrite concentration to be lower in samples that were cleaned over those that were not cleaned, but this was not statistically significant ($p=0.29$).

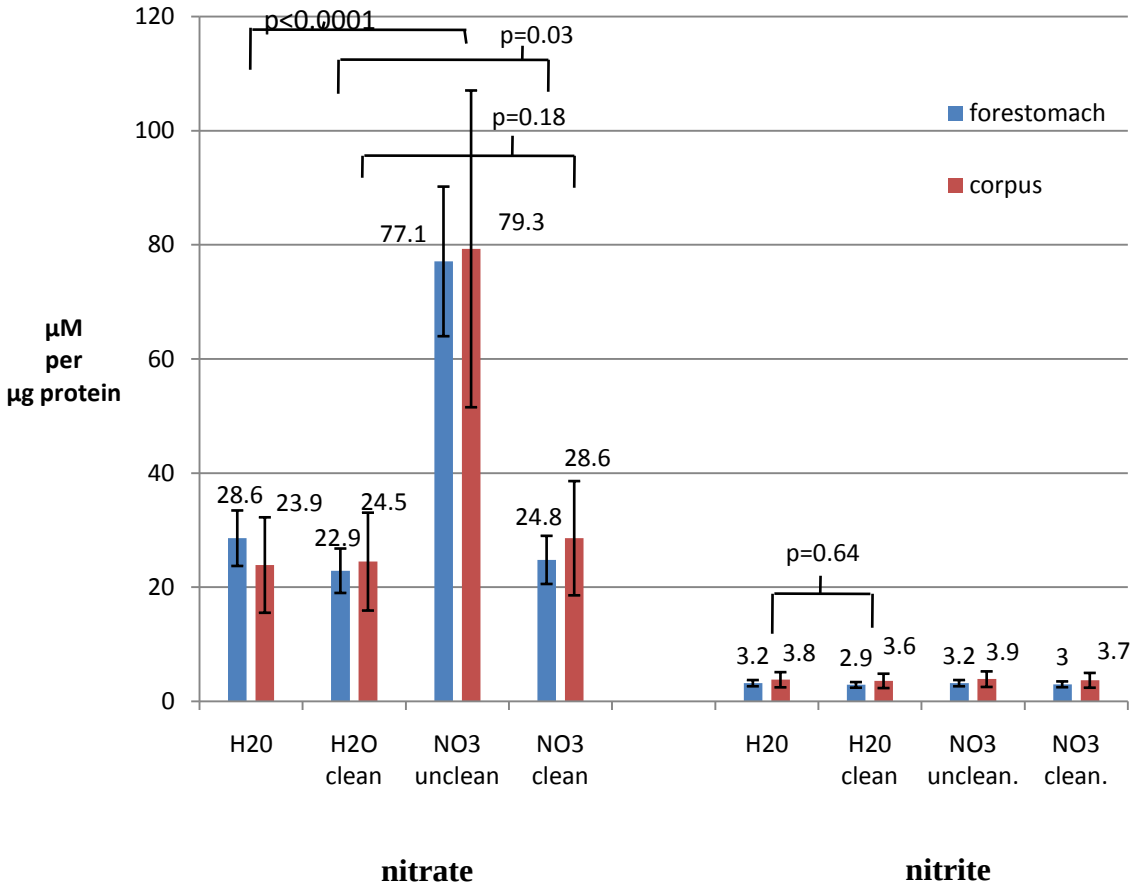


Figure 10.6: The mean nitrate and nitrite concentrations (µM per µg protein) are shown for rat tissue contamination studies. The tissue from either the forestomach (blue bars) or corpus (red bars) were either left unaltered or left immersed in a plastic Eppendorf test tube containing 5 mM KNO_3^- for 30 minutes. Samples were then either analysed without cleaning or subjected to cleaning in 3.1% NAC. The mean concentrations are given to the left for nitrate concentrations and to the right for nitrite concentrations. Data labels show mean values. Error bars represent 1 standard deviation. n=36

10.3.1.2 Delrin model contamination with nitrate and nitrite to assess cleaning

In studies with nitrate as the contaminant: The mean nitrate and nitrite concentrations in the Delrin model contamination studies showed that the cleaning process was effective in cleaning what appeared to be 100% of contaminant KNO_3 from the forestomach samples (figure 10.7). As in the immersion studies, the cleaning process was not as efficient for the corpus samples, although the mean nitrate concentration from contaminated clean samples was only 8% more than uncontaminated cleaned samples ($p=0.28$).

In studies with nitrite as the contaminant: The mean nitrate concentrations, when the contaminant was KNO_2, did not significantly increase compared to control samples exposed to deionised water only ($p=0.08$). There was however a trend for the nitrate concentration to be slightly increased after exposure to KNO_2 with subsequent cleaning, with a mean concentration of 28.0 (±2.7) µM per µg protein for forestomach samples (figure 10.8). The highest concentration of nitrate, in any of the samples, from all four conditions, was found in the corpus samples exposed to KNO_2 and subsequently cleaned, although the difference between this and the cleaned uncontaminated sample was not statistically significant ($p=0.11$). The mean nitrite concentrations were appropriately elevated after exposure and no cleaning. There was however 20% and 27% more nitrite, in forestomach and corpus samples respectively, after contamination and cleaning compared to no contamination and cleaning (figure 10.8).

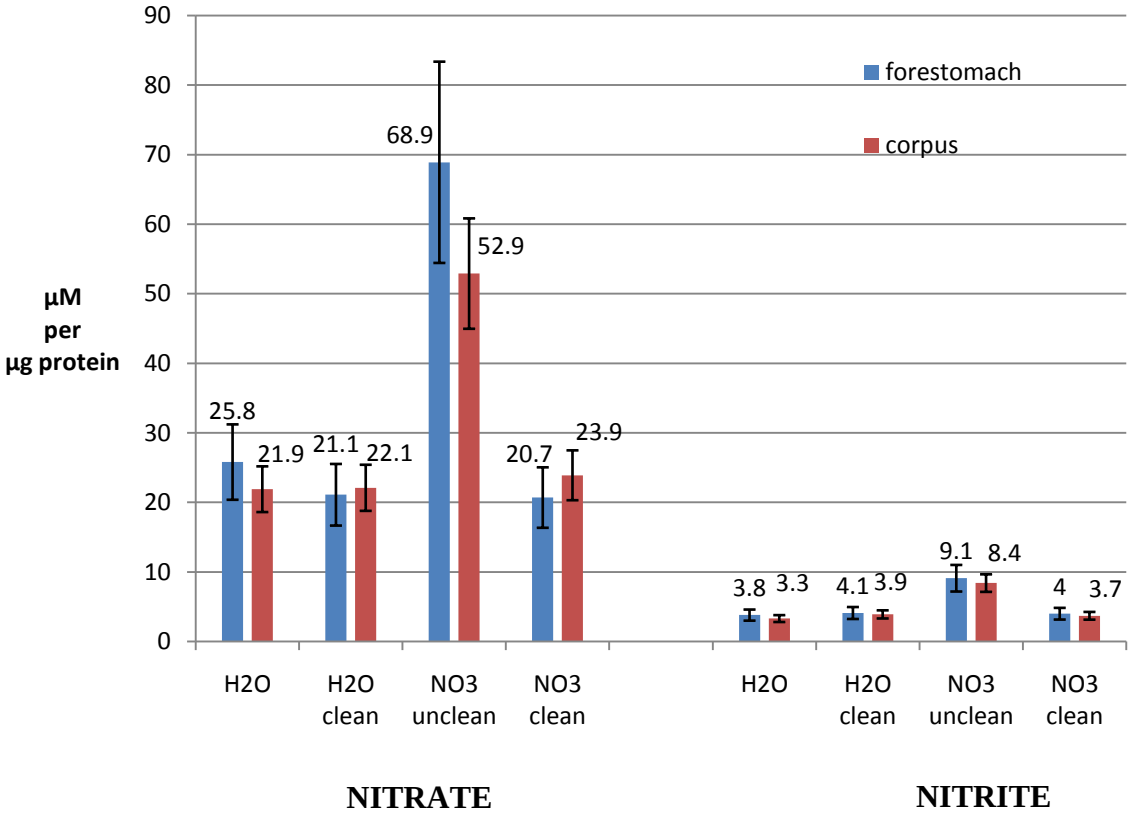


Figure 10.7: Mean nitrate and nitrite concentrations from KNO_3 Delrin model contamination studies. Rat tissue biopsies were either placed in a Delrin model as shown in figure 10.3 and exposed to 5 mM KNO_3 for 30 minutes or were left uncontaminated by placing them in a model containing deionised water only (H_2O). The mean nitrate and nitrite concentrations are shown after either being left unclean or cleaned using 3.1% NAC. The blue columns represent the mean concentrations for tissue from the forestomach while the red column represents the corpus. Mean nitrate concentrations are shown on the left hand side of the chart and mean nitrite concentrations on the right. Data labels show mean values. Error bars show 1 standard deviation. (n=18)

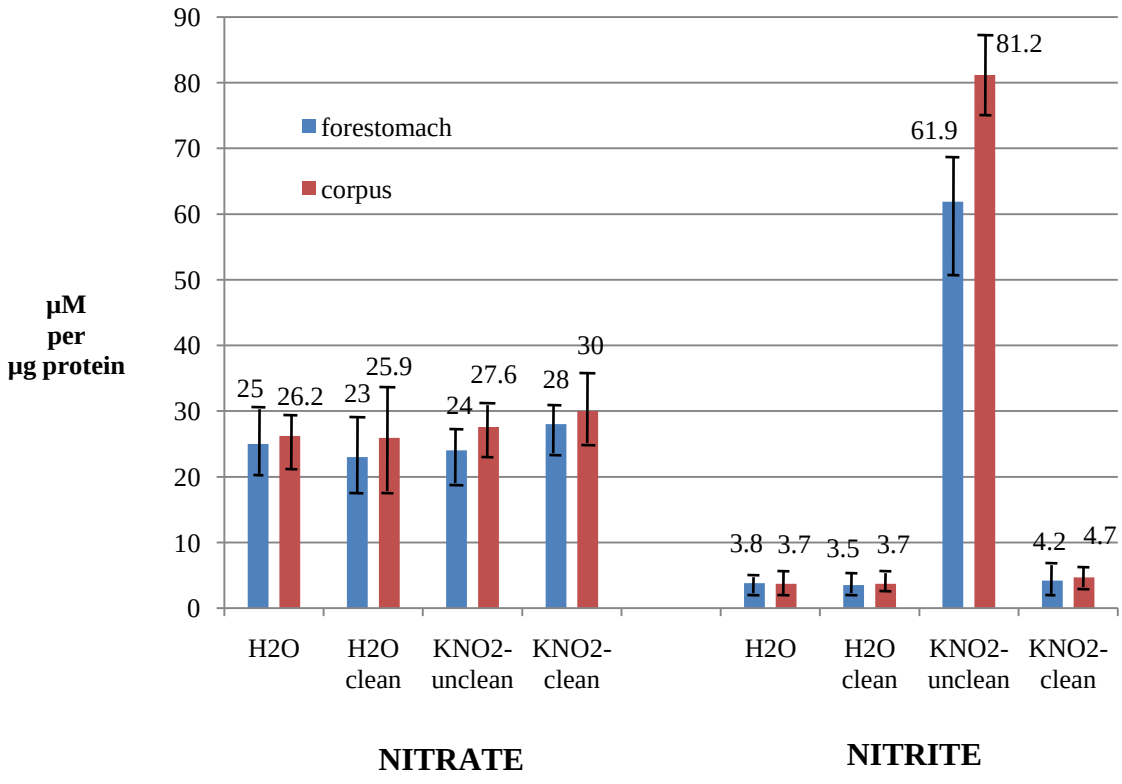


Figure 10.8: Mean nitrate and nitrite concentrations from KNO_2 delrin model contamination rat studies. Rat tissue biopsies were either placed in a Delrin model as shown in Figure 10.3 and exposed to 5 mM KNO_2 for 30 minutes or were left uncontaminated by placing them in a model containing deionised water only (H_2O). The mean nitrate and nitrite concentrations are shown after either being left unclean or cleaned using 3.1% NAC. The blue columns represent the mean concentrations for tissue from the forestomach while the red column represents the corpus. Mean nitrate concentrationsare shown on the left hand side of the chart and mean nitrite concentrations on the right. Data labels show mean values. Error bars represent 1 standard deviation. (n=18)

10.3.1.3 Delrin model with ^{15}N

With ^{15}N Nitrate

The ^{15}N measured after forestomach rat mucosal samples were exposed to contaminant $K^{15}NO_3$ in a Delrin model with subsequent NAC cleaning was no higher than the background ^{15}N measured from samples not exposed to $K^{15}NO_3$ (0.07 ng versus 0.08 ng) ($p=0.7$). There was a slight increase of approximately 20% in the corpus samples ^{15}N content ($p=0.05$), although there appeared to be cleaning effectiveness of over 97% using 3.1% NAC (Figure 10.9).

With ^{15}N Nitrite

The ^{15}N measured in forestomach and corpus cleaned samples exposed to $K^{15}NO_2$ was slightly elevated compared to samples exposed to dH_2O (Figure 10.10). For forestomach samples this difference was not statistically significant ($p=0.23$). However, the difference was statistically different for the more glandular rat corpus samples ($p=0.004$). For both forestomach and corpus samples there was over 98% effective cleaning when the cleaned samples exposed to $K^{15}NO_2$ were compared to unclean $K^{15}NO_2$ against the control samples.

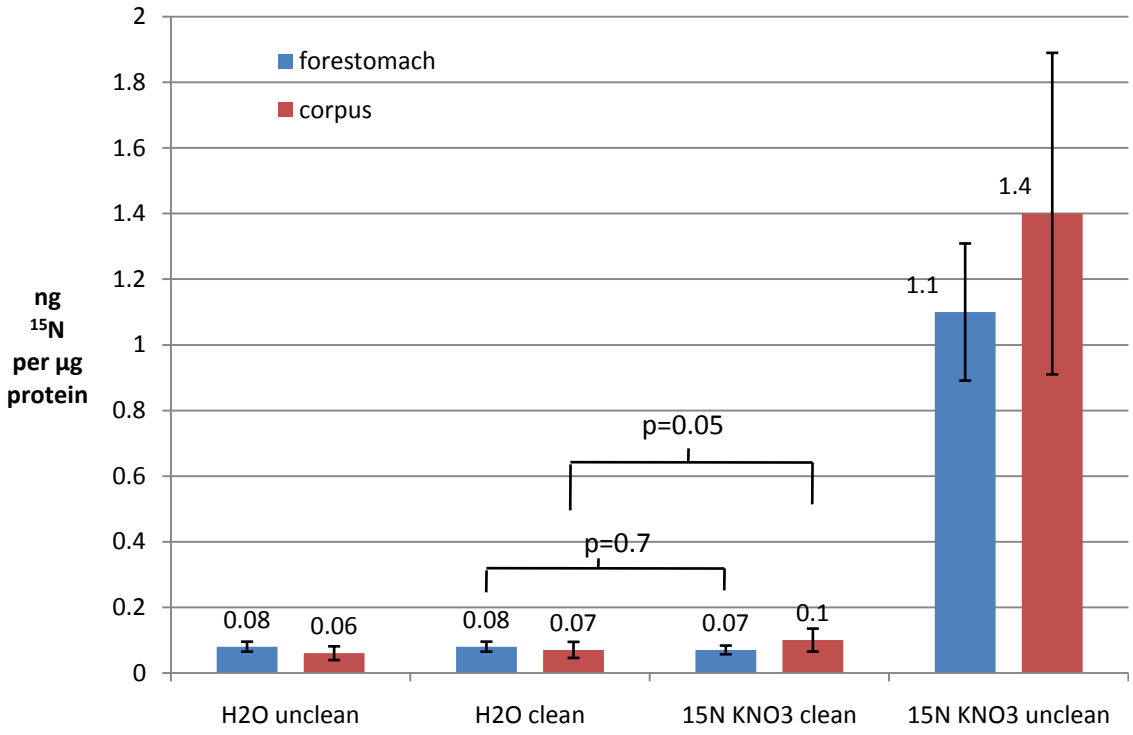


Figure 10.9: The mean ^{15}N content (ng ^{15}N per µg protein) from forestomach (blue column) and corpus (red column) rat stomach tissue samples exposed to either dH_2O or 5mM $K^{15}NO_3$ in a delrin model. The samples were then either cleaned in 3.1% NAC or left unclean. Data labels show mean values. Error bars represent 1 standard deviation. (n=18)

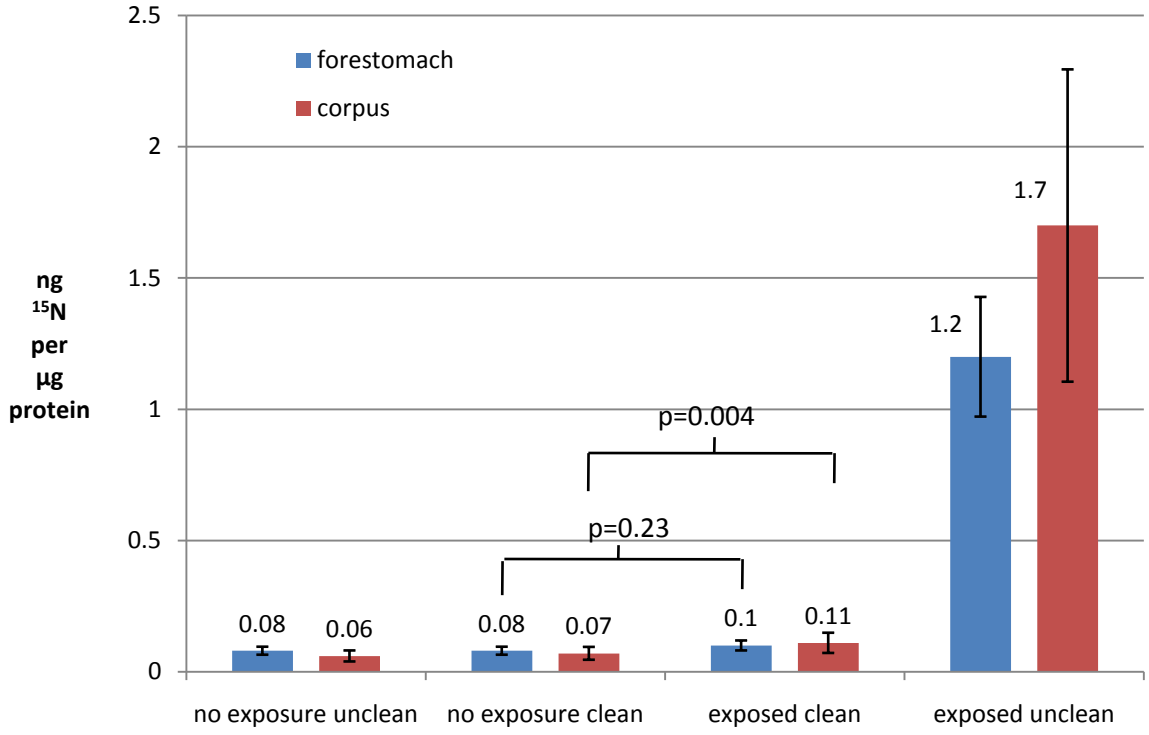


Figure 10.10: The mean ^{15}N content (ng ^{15}N per µg protein) from forestomach (blue column) and corpus (red column) rat stomach tissue samples exposed to either dH_2O or 5mM $K^{15}NO_2$ in a delrin model. The samples were then either cleaned in 3.1% NAC or left unclean. Data labels show mean values. Error bars represent 1 standard deviation. (n=18)

10.3.2 Studies in three-phase Delrin model to assess the effect of NO and nitrous acid on rat epithelial cell nitrate and nitrite

There were significant differences in the mean nitrate and nitrite concentrations of both corpus and forestomach rat samples, when comparing tissue exposed to NO against tissue exposed to deionised water only. There were increases of 50% (corpus) ($p=0.0003$) and 67% (forestomach) ($p=0.00009$) in measurable nitrite and approximately 30% (both corpus and forestomach) increase in mean nitrate concentrations after NO exposure for both forestomach ($p=0.002$) and corpus samples ($p=0.001$) (Figure 10.11).

There was no statistical difference in mean nitrate and nitrite concentrations when samples exposed to nitrous acid were compared to samples exposed to water only for both forestomach (nitrite $p=0.91$, nitrate $p=0.86$) and corpus (nitrite $p=0.96$, nitrate $p=0.79$)) samples (Figure 10.11).

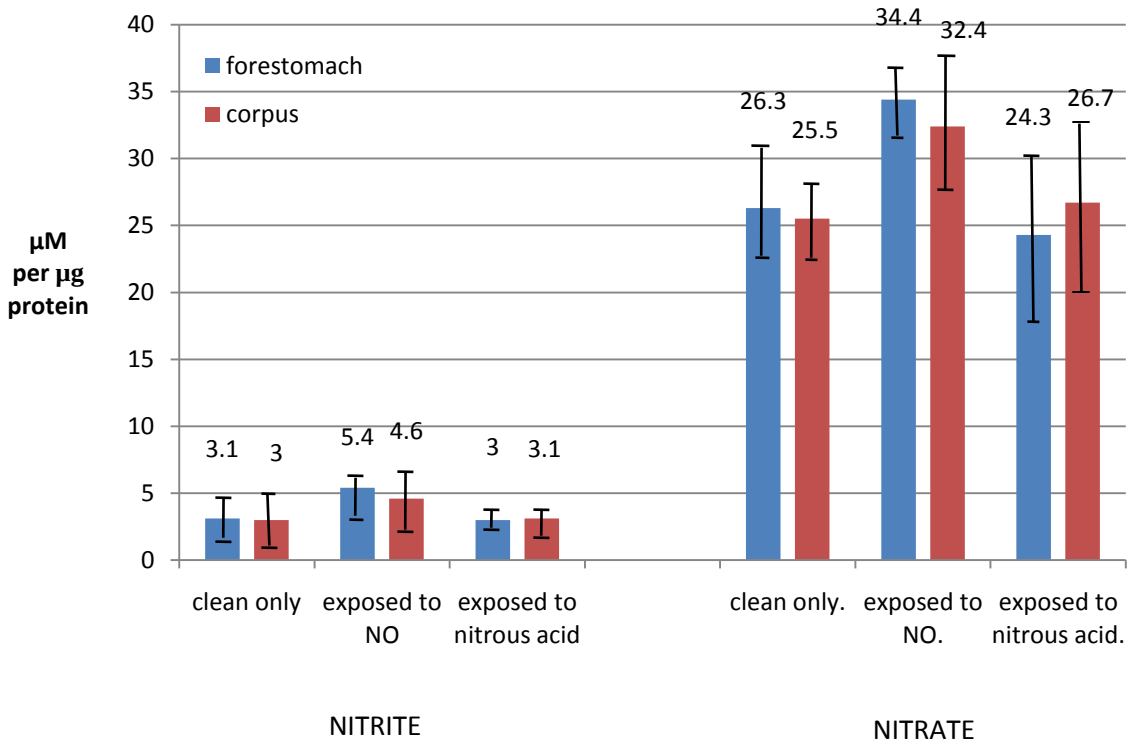


Figure 10.11: Mean concentrations of nitrite and nitrate for both corpus (red column) and forestomach (blue column) rat mucosal samples after exposure to NO or nitrous acid in a three-phase Delrin model. There were three different conditions within the Delrin model shown in figure 10.4 that the samples were exposed to: deionised water and cleaned, exposed to NO and then cleaned, exposed to nitrous acid and then cleaned. Mean nitrate concentrations are shown on the right hand side of the chart and mean nitrite concentrations on the left. Data labels show mean values. Error bars represent 1 standard deviation. (n=18)

10.4 Discussion

The studies described in this chapter had the primary aims of effectively cleaning rat stomach biopsy samples of adherent mucus, and to determine if changes in intracellular nitrite and nitrate were recordable after exposure of this tissue to high concentrations of NO. Both of these primary aims have been achieved. The ability to measure changes in intracellular nitrite and nitrate is important as we move towards determining a marker of nitrosative stress in human subjects.

The samples from the upper gastrointestinal tract are known to have an adherent mucus lining on the surface (317). The rat stomach is divided in two by the margo plicatus, into the more glandular corpus in the more distal portion of stomach, and the more proximal forestomach which in cell type more resembles the human oesophagus. The glandular part of the stomach can be expected to have more adherent mucus and this is consistent with the findings in this study, that the corpus tissue was more difficult to clean when tissue samples were subjected to nitrate and nitrite contaminants. Although the corpus was more difficult to clean, more than 97% of contaminant nitrate and nitrite was effectively washed from samples after being subjected to a cleaning process in NAC, as evidenced in ^{15}N studies. It is not surprising to find that rat mucosa is easier to clean with NAC than human tissue as it has been shown that the mucus layer is more adherent in human stomach tissue compared to rat tissue. This, therefore, allows further work with rat stomach samples to determine intramucosal nitrite and nitrate concentrations.

However, it should be noted that there is no independent marker of cleaning of these biopsy samples and the effectiveness of the cleaning process cannot be considered perfect. This is important as the concentrations of nitrite and nitrate measured were small and any contamination from the mucus could be significant when measuring intracellular concentrations. An independent marker of cleaning is also important as nitrogen containing compounds may have been generated during the cleaning process, thus underestimating the effectiveness of the cleaning. It may be possible in the future to use a protein marker from within the mucus layer as a means of determining the effectiveness of the cleaning process, although this was not performed within these studies.

It was interesting to note that there was considerably more nitrate than nitrite within the biopsy sample. It is likely that this was due to the conversion of nitrite to nitrate. This occurs due to oxidation of nitrate in the presence of haemoglobin, as NO can react with oxyhaemoglobin to form methaemoglobin and nitrate directly (117). This is the likely mechanism through which the nitrate concentrations were found to be increased in our studies where rat tissue was subjected to NO. Nitrate concentrations were increased in samples that had been deliberately exposed to contaminant nitrite in very high concentrations. The concentrations of nitrate found in these experiments with nitrite were higher than those found when the contaminant had been the same concentration of nitrate. This may be explained by the generation of low concentrations of NO during the cleaning process with NAC. NAC is itself an antioxidant, similar to glutathione (310,311), and therefore in

reaction with nitrite NAC can be expected to form NO (313,314). NO could then cross over into the cells and react with either oxyhaemoglobin (117) or superoxide, forming nitrate. The reaction of NO with superoxide generates peroxynitrite which decomposes to nitrate, nitrite and O_2 (294). The reaction of nitrite with NAC is more likely to occur in an acidic environment, therefore in the neutral environment of the mucus layer (317) this reaction if likely to liberate only a small concentration of NO. In contamination studies the concentrations of nitrate and nitrite used were deliberately excessive to ensure the subsequent cleaning process was adequate. This would explain why small concentrations could be found. The effect of NAC on uncontaminated samples is unlikely to be having a significant artefactual effect on nitrite and nitrate concentrations, as evidenced by the fact that samples not contaminated but rather just cleaned in NAC showed no recordable difference in nitrate and nitrite concentrations. Unfortunately, in our experiments we did not examine for the reaction of NAC with nitrite.

It would appear that we are able to measure intramucosal nitrate and nitrite concentrations, and the change in these concentrations after exposure to NO. In studies where rat tissue was exposed to nitrite in the absence of ascorbic acid, so that only nitrous acid rather than NO would be present, there was no increase in nitrate and nitrite concentrations. This shows that it is the presence of acidified nitrite with ascorbic acid present generating NO that is important for the increased intramucosal concentrations of nitrite and nitrate. This not only shows that a flux in intraepithelial nitrite and nitrate concentrations is measurable, but also that the NO

generated does have direct effects on the tissue in terms of nitrosative stress. In these studies we have used nitrite as a marker of nitrosative stress. The concentration of nitrite used in these experiments was not excessive, being 120μM, while the concentrations found *in vivo* have been 5-200μM after a nitrate rich meal (45,143). This means that the concentrations found *in vivo* are significant enough to cause a measurable flux in tissue nitrate and nitrite concentrations, albeit in rat tissue. This would, therefore, allow for damage to occur either through the generation of *N*-nitrosocompounds or by NO directly affecting the cell. The carcinogenic effects of *N*-nitrosocompounds and NO are well established, as discussed in the introduction to this thesis

A recent study by Asanuma et al looked at a different marker of nitrosative stress due to NO in human upper gastrointestinal tract tissue. This study made use of the high affinity of NO for Fe-S cluster proteins, and the fact that measurable DNIC (dinitrosyl dithiolato iron complex) is a biomarker for the interaction between Fe-S proteins and NO. After the ingestion of nitrate drink, the appearance of DNIC was found in biopsies from the gastroesophageal junction in a significant number of human volunteers. This, therefore, provides complimentary evidence of NO generated within the lumen of the upper gastrointestinal tract having a direct effect within the cells in the mucosa (285). In another recent study by Clemons et al, acidification of salivary nitrite, resulting in the generation of NO, was shown to produce double strand DNA breaks in transformed and primary Barrett's oesophageal cells, as well as adenocarcinoma cells (238). These studies show that

cells of the upper gastrointestinal tract have determinable, detrimental changes related to NO generated from salivary nitrite, albeit in the setting of *in vitro* studies.

This chapter adds to the current research on nitrosative stress in the upper gastrointestinal tract by showing that rat tissue can be adequately cleaned of mucus, and that NO flux into the cells is measurable as intramucosal nitrite and nitrate. It would also appear that luminal nitrite requires the conversion to NO to be able to have significant effects, as without ascorbic acid there was no measurable difference in nitrite and nitrate concentrations in experiments where rat stomach tissue was exposed to nitrous acid in three layer model. As well as nitrite being a marker for this nitrosative stress, we have shown that nitrate is also important to measure in the *in vivo* setting.

Chapter 11 Concluding discussion

The studies described within this thesis add to the growing body of evidence in support of a novel mechanism of nitrosation within the upper gastrointestinal tract (43,45,144,221,222,280,285). Nitrosation within the stomach is well described in the literature. This is known to occur both from acid-catalysed nitrosation through decreased ascorbic acid production, and also from colonisation by denitrifying bacteria in those who are achlorhydric. The potential for a third mechanism, but in this case occurring in the healthy acid secreting stomach, in the presence of ascorbic acid, has been explored within the work presented in the preceding chapters. Bench top experiments have been performed and then transferred into *in vivo* conditions to show the importance of this mechanism of nitrosative stress in human subjects.

The major source of this nitrosative stress is dietary nitrate. Through bacterial conversion of nitrate to nitrite, after enterosalivary recirculation of nitrate, swallowed saliva contains high concentrations of nitrite (45). When this saliva meets acidic gastric juice, in the presence of ascorbic acid, NO is produced (44). Ascorbic acid is therefore acting as a luminal antioxidant and, in this compartment, is preventing nitrosative stress by limiting the production of nitrosating species such as NOSCN and N_2O_3 (240,267,318,319).

However, within the upper gastrointestinal tract there is not only a luminal but also a lipid compartment. Lipid is found in two main forms in this region. There is fat provided in the diet which floats within the more proximal stomach, and in

particular in the fundus, post prandially (244). Perhaps providing the greatest surface area of lipid is the epithelial cell bilayer membranes. The lipid bilayer forms a barrier between the luminal and epithelial compartments. Within cells of intestinal metaplasia type there is particularly high lipid content (243). Work presented in chapter 3 showed that when lipid is added to an aqueous phase the aqueous antioxidant (ascorbic acid) can change from being antioxidant to pro-oxidant. This occurs due to the reaction product of nitrite with ascorbic being the highly lipophilic molecule NO. NO then diffuses into the lipid, where it can rapidly react with O_2 to form nitrosating species such as N_2O_3 (251). In support of ascorbic acid being pro carcinogenic in certain situations, a previous study has shown that it can potentiate the growth inhibitory effects of NO in SK-N-MC human neuroblastoma (320). Ascorbic acid was not the first antioxidant to have been shown to have carcinogenic potential. The synthetic antioxidant BHA was demonstrated, in rat forestomach epithelium, to be carcinogenic as early as 1983 (262). One of the limitations of the studies in Chapter 3 was the measurement of lipid nitrosamine concentrations. A robust lipid nitrosamine analysis has subsequently been developed, and results from these experiments confirm the results presented in this thesis, showing that nitrosamines are present within the lipid phase in high concentrations within our model (255).

The importance of lipid antioxidants has been recognised for many years. A-tocopherol is the most abundant lipid antioxidant in living organisms. Our studies have shown that this has an important inhibitory effect on nitrosation, in models of

the gastroesophageal junction. The effect of α-tocopherol was greater than β-carotene, which was in turn greater than the synthetic antioxidant BHT. This is in keeping with *in vitro* experiments assessing the effectiveness of lipid chain breaking antioxidants (270), and also their biological activities *in vivo* (256). Other studies have shown that β-carotene has the greatest inhibitory effect on bacterial mutagenicity from NO (125). Nonetheless, all three antioxidants inhibited nitrosation within our model. The surprising result from this set of experiments was the low concentration of α-tocopherol required to prevent nitrosation. This is due to the *in vitro* recycling of α-tocopherol by ascorbic acid as shown by experiments in chapter 5. Whether this process occurs *in vivo* is an area of controversy with some experiments suggesting such a process (162,321), while others have shown no recycling (174). There have also been studies to show synergy between β-carotene and ascorbic acid (266). Further experiments using different combinations of antioxidants could be undertaken in our models of the upper gastrointestinal tract. Within the studies presented in this thesis it would also have been worthwhile to determine the reaction products of the reaction between α-tocopherol and nitrite, NO and nitrosating species. This may include measurement of reaction products such as α-tocopheroxyl radical and α-tocopherolquinone.

In order to transfer our understanding of gastroesophageal junction chemistry *in vitro* to the *in vivo* setting, a compartmentalised silastic tubing capable of measuring nitrosative stress was produced. In subsequent studies using this tubing, in healthy volunteers, we showed nitrosative stress extending from the gastroesophageal

junction into the proximal stomach. This was increased after a nitrate rich drink was ingested. The proposed mechanism for this is that the increased nitrite swallowed in saliva, after a nitrate rich meal, causes both the generation of NO while ascorbic acid is present, and acid-catalysed nitrosation once gastric ascorbic acid has been overwhelmed in its initial reaction with nitrite with subsequent recycling of this nitrite through NO. The tubing was further developed, *in vitro*, to include lipid antioxidant smeared either inside or outside. Importantly, this showed the generation of NO from the reaction between nitrite and α-tocopherol, whether outside or inside the tube. This modified tubing could be used *in vivo* in due course, although it would be best to standardise the concentrations of α-tocopherol inside the tubing before this is undertaken.

Our *in vivo* studies have not clearly shown whether nitrite is a good biomarker of nitrosative stress in human tissue. This is mainly due to difficulties in cleaning adherent mucus from biopsy specimens. Perhaps a more useful assessment of NO within the cells from the upper gastrointestinal tract would have come from analysis using diaminofluorescence and its diacetate (322). Different markers have been assessed by other groups. In a study by Asanuma et al, Fe-S cluster proteins affinity with NO has been used to assess for NO generation in the upper gastrointestinal tract after a nitrate rich meal. A biomarker of the reaction between Fe-S cluster protein and NO was found in biopsies from the gastroesophageal junction in a significant number of subjects. This, therefore, provides complementary evidence of NO

generated within the lumen of the upper gastrointestinal tract having a direct effect within the cells in the mucosa (285).

It had been hoped that data regarding the measurement the DNA adduct O^6-carboxymethyl-2'-deoxyguanosine and O^6-methyl deoxyguanosine as biomarkers of nitrosative stress would have been presented within this thesis. DNA adducts can be useful markers of stress as they are more frequent abnormalities than cancer itself, they can be recognised early and they predict the onset of disease independently of exposure levels (323). Previous work had shown the DNA adduct O^6-carboxymethyl-2'-deoxyguanosine present in human gastric biopsy tissue which had been exposed to endogenously nitrosated glycine (324). In our studies, we had hoped to measure the DNA adduct O^6-methyl deoxyguanosine and O^6-carboxymethyl-2'-deoxyguanosine before and after a nitrate meal. These studies were performed on healthy volunteers. Unfortunately, however, while waiting for refinement of the assay by collaborative colleagues in Greece, the samples were destroyed by a freezer failure within our department. This is an area of research that could be explored again in the future.

Epidemiological studies do not provide a strong association when assessing the influence of nitrosamine, and related food intake, on oesophageal and gastric cancer (325). However, recent studies carried out in humans have shown that endogenous *N*-nitrosocompounds could be up to 30 fold higher than exposure from dietary

sources, suggesting that only a small part of the human exposure to *N*-nitrosocompounds has been measured (326-328).

The epidemiological evidence for an association between dietary nitrate intake and adenocarcinoma of the upper gastrointestinal tract is admittedly weak. The only study to suggest a positive association was a large Swedish based one in 2001 (25), while many others suggest a negative association with a high fruit and vegetable intake, likely to be due to the antioxidants present within these foodstuffs. The reason for the lack of a positive association may be due to difficulty elucidating the finer details of a subgroup of people who are at risk from this nitrosative stress.

There are a few important considerations to take into account in interpreting the role of dietary nitrate in overall health. It is clear throughout many aspects of biological systems that, while a compound may be protective in some contexts, it can be harmful in others. It would be unreasonable to suggest that nitrate is entirely a harmful substance and should be eliminated from our diet. There is evidence that dietary nitrate may indeed by protective. There has been a recent hypothesis article suggesting a cardioprotective effect from NO derived from dietary nitrate (329). This stems from epidemiological studies suggesting the strongest protection against coronary heart disease was seen in individuals with a high intake of green leafy vegetables which are rich in nitrate (330,331). There is also evidence that dietary vegetables reduce blood pressure (332). As regards the upper gastrointestinal tract, there is some evidence that dietary nitrate has a protective effect. This can be

through increased gastric blood flow, increased mucus thickness and decontamination of the human stomach (333-338).

However, there does appear to be the potential for harmful effects of nitrate in the upper gastrointestinal tract. The specific role has yet to be fully elucidated. It is well recognised that there will be more than one insult required to promote carcinogenesis in the lower oesophagus and proximal stomach (339). Nitrosative stress from dietary nitrate may be most harmful in subjects with acid and bile reflux. It has been shown that nitrosative stress is shifted into the lower oesophagus in subjects with Barrett's oesophagus (286). It is known that acidity has a deleterious effect on the mucosal protective barrier leading to caustic injury of the mucosa, as well as causing DNA damage within oesophageal cells (238). Bile refluxate provides three conditions which would potentiate nitrosative stress within the lower oesophagus. It contains bile acids such as glycocholic and taurocholic acid that are nitrosatable amides. Spermidine, a nitrosatable polyamine, which is carcinogenic, is also present (340). Bile also contains lecithin which is known to promote nitrosation reactions (341). *N*-nitrosamides and related compounds are chemically active under physiological pH conditions and decompose to form the same alkyldiazohydroxide as nitrosamines. Nitrosamides have been shown to produce both local as well as systemic tumours. Conditions within the lower oesophagus, in those individuals with acid and bile reflux, are therefore such that nitrosative stress from nitrate may have an important role in adenocarcinoma development.

The work presented within this thesis shows that dietary nitrate has the potential to generate nitrosative stress within the upper GI tract where salivary nitrite meets acidic gastric juice. This may have relevance to the observed increased incidence of adenocarcinoma in the cardia region of the stomach and the gastroesophageal junction. The combination of molecular tools with biochemistry is likely to provide useful information regarding nitrosative stress in the upper gastrointestinal tract, and in particular the role of dietary nitrate in carcinogenesis of the lower esophagus and proximal stomach, as we search for a mutagen responsible for the increased incidence of adenocarcinoma in this anatomical region.

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Appendix A

Publications and presentations relating to this thesis

Published Papers

1. Winter JW, Paterson S, Scobie G, Wirz A, Preston T, McColl KE. N-nitrosamine generation from ingested nitrate via nitric oxide in subjects with and without gastroesophageal reflux. Gastroenterology 2007;133(1):164-74.

2. Combet E, Paterson S, Iijima K, et al. Fat transforms ascorbic acid from inhibiting to promoting acid-catalysed N-nitrosation. Gut 2007;56(12):1678-84.

Oral Presentations

1. American Gastroenterology Association. Digestive Diseases Week. New Orleans. May 2004. The presence of fat counteracts the ability of vitamin C to prevent acid N-nitrosation. S Paterson, K Iijima, T Preston, G Scobie, KEL McColl.

2. British Society of Gastroenterology. Glasgow. March 2004. The presence of fat counteracts the ability of vitamin C to prevent acid N-nitrosation**.** S Paterson, K Iijima, T Preston, G Scobie, KEL McColl.

3. Glasgow Gastroenterology Club. Glasgow. February 2005. Both lipid and water soluble antioxidants are required to prevent acid N-nitrosation. S Paterson, T Preston, G Scobie, KEL McColl.

4. Glasgow Gastroenterology Club. Glasgow. February 2005. Is measurement of intraepithelial nitrate and nitrite a useful assessment of exposure to nitric oxide within a model of the upper gastrointestinal tract? S Paterson, T Preston, G Scobie, KEL McColl.

Poster Presentations

1. Clinician Scientists in Training. London. May 2004. The preventative effect of ascorbic acid on acid n-nitrosation is counteracted by the presence of fat. S Paterson, K Iijima, T Preston, G Scobie, KEL McColl.

2. British Society of Gastroenterology. Glasgow. March 2004. Rise in oesophageal adenocarcinoma is accompanied by similar rise in oral cancer; evidence for environmental carcinogen. KEL McColl, J Winter, J Manning, S Paterson.

3. Clinician Scientists in Training. London. February 2005. Prevention of Acid N-nitrosation requires both water and fat soluble antioxidants. S Paterson, T Preston, G Scobie, KEL McColl.

4. British Society of Gastroenterology. Birmingham. March 2005. Prevention of Acid N-nitrosation requires both water and fat soluble antioxidants. S Paterson, T Preston, G Scobie, KEL McColl.

Appendix B

GASTRIC CANCER

Fat transforms ascorbic acid from inhibiting to promoting acid-catalysed *N*-nitrosation

E Combet, S Paterson, K Iijima, J Winter, W Mullen, A Crozier, T Preston, K E L McColl

Gut 2007;**56**:1678–1684. doi: 10.1136/gut.2007.128587

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Revised 3 July 2007
Accepted 5 July 2007
Published Online First 4 September 2007

Background: The major potential site of acid nitrosation is the proximal stomach, an anatomical site prone to a rising incidence of metaplasia and adenocarcinoma. Nitrite, a pre-carcinogen present in saliva, can be converted to nitrosating species and *N*-nitroso compounds by acidification at low gastric pH in the presence of thiocyanate.

Aims: To assess the effect of lipid and ascorbic acid on the nitrosative chemistry under conditions simulating the human proximal stomach.

Methods: The nitrosative chemistry was modelled *in vitro* by measuring the nitrosation of four secondary amines under conditions simulating the proximal stomach. The *N*-nitrosamines formed were measured by gas chromatography–ion-trap tandem mass spectrometry, while nitric oxide and oxygen levels were measured amperometrically.

Results: In absence of lipid, nitrosative stress was inhibited by ascorbic acid through conversion of nitrosating species to nitric oxide. Addition of ascorbic acid reduced the amount of *N*-nitrosodimethylamine formed by fivefold, *N*-nitrosomorpholine by >1000-fold, and totally prevented the formation of *N*-nitrosodiethylamine and *N*-nitrosopiperidine. In contrast, when 10% lipid was present, ascorbic acid increased the amount of *N*-nitrosodimethylamine, *N*-nitrosodiethylamine and *N*-nitrosopiperidine formed by approximately 8-, 60- and 140-fold, respectively, compared with absence of ascorbic acid.

Conclusion: The presence of lipid converts ascorbic acid from inhibiting to promoting acid nitrosation. This may be explained by nitric oxide, formed by ascorbic acid in the aqueous phase, being able to regenerate nitrosating species by reacting with oxygen in the lipid phase.

Over the last 20 years, a new pattern of gastric carcinogenesis has emerged in the western world, with a decreasing incidence of distal gastric cancer and an alarming increase in the incidence of adenocarcinomas of the proximal stomach, including cardia and adjacent gastro-oesophageal junction (GOJ).[1–4] The cancers at the GOJ usually occur in healthy acid-secreting stomachs.[5 6] The cause of the increasing incidence of adenocarcinoma of the proximal stomach remains unclear, but the rate of change indicates environmental factors.

For many years, there has been interest in the potential for endogenous generation of carcinogenic *N*-nitroso compounds from nitrite within the human upper gastrointestinal tract. This is due to the fact that the acidic pH of gastric juice converts nitrite to nitrous acid and nitrosating species such as N_2O_3, NO^+.[7 8] The latter reacts with thiocyanate which is also present in gastric juice to form the particularly potent nitrosating species NOSCN.[9–11] These nitrosating species can react with secondary amines and amides to form *N*-nitroso compounds, many of which are carcinogenic and widely used in animal models of cancer.[8 12–16] The nitrosating species N_2O_3 is itself mutagenic as it can directly deaminate certain DNA bases and inactivate important DNA repair enzymes.[17–21] Understanding the factors affecting gastric nitrite chemistry is therefore relevant to our understanding the development of malignancies of the upper stomach.

The main source of nitrite entering the stomach is swallowed saliva. The high level of nitrite in saliva (100 μM under fasting conditions) is derived from the enterosalivary recirculation of dietary nitrate and its reduction to nitrite by buccal bacteria.[22–27] Consequently, the nitrite level in saliva rises several fold for at least 2 h after ingesting nitrate-containing foodstuffs. The nitrite concentration in the distal oesophagus is similar to that in saliva.[28]

A major factor protecting against the generation of *N*-nitroso compounds from salivary nitrite on entering the acidic stomach is ascorbic acid present in gastric juice.[29–33] Ascorbic acid is actively secreted in gastric juice and effectively competes with secondary amines and amides for reaction with the nitrosating species.[32 34–38] In this reaction, the nitrosating species are reduced to nitric oxide and the ascorbic acid oxidised to dehydroascorbic acid.[39] We and others have recently demonstrated that high concentrations of nitric oxide are generated in the human upper gastrointestinal tract following nitrate intake by the above mechanism.[40]

This reduction of acidified nitrite to nitric oxide has been regarded as an effective mechanism for protecting against the generation of *N*-nitroso compounds. However, it has recently been recognised that nitric oxide can generate nitrosating species. This arises from the ability of nitric oxide to react with molecular oxygen to form N_2O_3 – the same species formed by acidification of nitrite.[18 41 42] The rate of the reaction between nitric oxide and oxygen to produce N_2O_3 is proportional to the concentration of oxygen and to the square of nitric oxide concentration.[43 44] Consequently, this reaction is most important at high nitric oxide concentrations.

Recent studies have also demonstrated that the reaction between nitric oxide and oxygen is 300 times faster within lipid than within an aqueous phase.[41] This is due to the fact that nitric oxide is nine times more soluble in lipid, and oxygen is also more soluble in lipid than aqueous solutions, resulting in

Abbreviations: GC-ITMS/MS, gas chromatography–ion-trap tandem mass spectrometry; GOJ, gastro-oesophageal junction; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NMOR, *N*-nitrosomorpholine; NPIP, *N*-nitrosopiperidine; PTV, programmable temperature vaporising; TVC, total vitamin C

both reactants accumulating in the lipid compartment.[41] The potential for generation of nitrosating species from nitric oxide will therefore be greatest when high concentrations of nitric oxide are generated close to lipids.

The above chemistry raises the possibility that inhibition of nitrosation reactions by ascorbic acid in the aqueous phase may promote nitrosation within adjacent lipid compartments. This could occur by diffusion of the nitric oxide produced by the reaction between acidified nitrite and ascorbic acid, into adjacent lipid compartments and their reacting with oxygen to form the nitrosating species N_2O_3. The presence of lipid might therefore have a profound effect on the chemistry occurring between acidified nitrite and ascorbic acid.

We have demonstrated that luminal nitrosative chemistry in the acid-secreting stomach is maximal where swallowed saliva first encounters acidic gastric juice and may therefore be contributing to the high incidence of metaplasia and neoplasia in the proximal stomach and GOJ.[28 40] The aim of this study was to investigate the influence of lipids on this luminal nitrosative chemistry.

METHODS

Assay set-up

Studies were undertaken in a benchtop model representing the GOJ, with and without lipid added to the acidic aqueous solution. The aqueous phase (0.1 M HCl, pH 1.5) contained 1 mM EDTA, 1 mM sodium thiocyanate, and 5 mM of each secondary amine (dimethylamine, diethylamine, morpholine, piperidine), in the presence or absence of ascorbic acid (1 or 2 mM). The lipid phase contained 5 mM of each secondary amine (dimethylamine, diethylamine, morpholine and piperidine). Lipid (5 ml glycerol trioctanoate, glycerol tributyrate or glycerol triacetate) was added to give a ratio of aqueous to lipid of 10:1, as fat comprises 20–30% of solid food ingested, and aqueous drinks along with aqueous gastric juice will further reduce the fat content to approximately 10%. A low pH of 1.5 was used for the aqueous phase, as this has been shown to be the postprandial pH of the cardia region of the stomach.[45] Both phases were combined and placed in a water bath at 37°C and the aqueous phase was stirred with a magnetic stirrer. The reaction was started by adding sodium nitrite (100 μM) to the aqueous phase. The standard assay was carried out for a period of 15 min, after which *N*-nitrosamines were extracted from each phase (aqueous and lipid).

Nitric oxide measurement

Nitric oxide dissolved in the aqueous phase was continuously monitored using a nitric oxide electrode and meter (ISO NO Mark II; World Precision Instruments, Sarasota, Florida, USA). The nitric oxide electrode was calibrated by addition of sodium nitrite at varying concentrations (10, 25, 50, 75 and 100 μM) to simulated gastric juice (0.1 M HCl, pH 1.5, ascorbic acid 2 mM). Under these conditions, 1 mol nitrite yields 1 mol nitric oxide. The nitric oxide meter was interfaced to a computer, converting the electrical signal (pA) to nitric oxide concentrations in μM; the response was linear for the range of concentrations tested.

Oxygen measurement

Oxygen dissolved in the aqueous phase was continuously monitored with an oxygen electrode and meter (Mark II; World Precision Instruments). Electrode calibration was performed in simulated gastric juice (0.1 M HCl, pH 1.5, ascorbic acid 2 mM) according to the manufacturer's instructions.

Nitrosamines extraction

A 1 ml aqueous phase aliquot was added to 0.5 ml 0.08 M HCl and 5% sulphamic acid solution in saturated NaCl in a glass tube, and mixed prior to the addition of 10 μl internal standard (0.01% deuterated *N*-nitrosodimethylamine (NMDA)-d6 and deuterated *N*-nitrosomorpholine (NMOR)-d8). The sample was mixed well, and 0.5 ml extraction solvent mix (45:55 v/v dichloromethane:diethyl-ether, 0.0025% butylhydroxytoluene) was added. The sample was mixed and the upper layer was transferred to a tapered vial. This step was repeated once more, and the sample was concentrated to 100 μl under a gentle stream of nitrogen, capped and stored at −20°C until analysis.

A 0.5 ml lipid phase aliquot was added in a glass tube to 2 ml 88:12 hexane:ethyl acetate supplemented with 0.025% butylhydroxytoluene. The sample was mixed before addition of 10 μl internal standard (0.01% deuterated NMDA and deuterated NMOR). DSC-Diol solid-phase extraction cartridges (500 mg, 3 ml) were installed on a vacuum manifold (Phenomenex, Macclesfield, UK) and equilibrated with 3 ml methanol, followed by 2 ml 85:15 v/v ethyl acetate:methanol and 3 ml hexane. All fractions were applied dropwise. The cartridges were washed twice with 3 ml hexane, and dried under vacuum for 30 s. *N*-Nitrosamines were eluted into 1.1 ml tapered vials (following the addition of 0.5 ml elution solvents, 85:15 v/v ethyl acetate:methanol), twice. A gentle vacuum was pulled through the cartridges for 30 s while the receiving vials remained in place. The samples were concentrated down to 100 μl under a gentle stream of nitrogen, capped and stored at −20°C until analysis.

Nitrosamines analysis

Nitrosamines analysis was carried out by gas chromatography–ion-trap tandem mass spectrometry (GC-ITMS/MS) on a TRACE GC 2000 Series gas chromatograph interfaced to a Polaris Q ion-trap mass spectrometer equipped with an AS2000 autosampler and a programmable temperature vaporising (PTV) injector (ThermoFinnigan, Hemel Hempsted, UK).

Nitrosamines (NDMA, *N*-nitrosodiethylamine (NDEA), NMOR and *N*-nitrosopiperidine (NPIP)) were separated by chromatography on a ZB-5MS fused-silica capillary column, 30 m×0.25 mm internal diameter, 0.5 μm film thickness (Phenomenex). The oven temperature programme was as follows: 37°C (held for 2 min) to 160°C at 10°C/min, to 250°C at 30°C/min (held for 2 min). Helium was used as a carrier gas at a flow rate of 1 ml/min. The PTV injector was used in splitless mode. The PTV temperature was programmed from 36°C to 115°C (held for 2 min) at 10°C/min and a pressure of 22 psi (151 kPa), prior to a cleaning phase at 325°C (held for 12 min).

Ion-trap tandem mass spectrometry was carried out by positive-electron ionisation at an ionisation energy of 70 eV, with the ion source temperature maintained at 220°C, and the transfer-line at 275°C. Acquisition was performed in three segments, segment 1 for NDMA and NDMA-d6 (start of acquisition at 4.5 min), segment 2 for NDEA (start of acquisition at 7.3 min), and segment 3 for NMOR, NMOR-d8 and NPIP (start of acquisition at 10.9 min) with a trap offset voltage of +100 V on segment 3. Precursor ions, excitation voltage and q values, as well as product ions for each *N*-nitrosamine, are listed table 1.

Acquisition and treatment of GC-ITMS/MS data was performed on Xcalibur version 2. All samples were spiked with 10 μl internal standard (0.01% deuterated NMDA and deuterated NMOR), and GC-MS/MS spectra were calibrated with spiked standard *N*-nitrosamines solutions covering concentrations from 0 to 50 μM. Each calibration sample was extracted according to the protocol used for assay samples, and the response was linear for the concentrations studied.

Analysis of ascorbic acid/total vitamin C/dehydroascorbic acid

The aqueous phase was sampled at t = 0, 1, 2, 5, 10 and 15 min post addition of $NaNO_2$ for ascorbic acid and total vitamin C

Table 1 GC-ITMS/MS ionisation conditions for analysis of selected *N*-nitrosamines

Target compound	Precursor ion (m/z)	Product ion (m/z)	Excitation voltage	Excitation q value
NDMA	74	44	0.85	0.45
NDMA-d6	80	50	0.85	0.45
NDEA	102	85	0.79	0.3
NMOR	116	86	0.63	0.3
NMOR-d8	124	94	0.63	0.3
NPIP	114	97	1	0.45

(TVC) measurements. Sampling for TVC allows us to calculate the amount of dehydroascorbic acid present by subtracting ascorbic acid from TVC. The aqueous phase samples (100 µl) were added to deionised water (900 µl). An aliquot of each diluted sample (500 µl) was added to MS solution (500 µl, 2% metaphosphoric acid/0.5% sulphamic acid, 1:1 v/v), while the remainder (500 µl) was added to dithiothreitol/MS solution (500 µl, 2% metaphosphoric acid/0.5% sulphamic acid, 1:1 v/v, supplemented with 6 mg/ml dithiothreitol). The dithiothreitol regenerates ascorbic acid from any dehydroascorbic acid for ascorbic acid and TVC respectively. Ascorbic acid and TVC levels were measured by high-performance liquid chromatography as previously described[46] based upon the method described by Sanderson and Schorah.[47]

Reagents and chemicals

All solvents and reagents were obtained from Sigma Aldrich (Poole, UK), except for the EDTA (BDH Ltd, Liverpool, UK) and deuterated *N*-nitrosamine standards (Qmx laboratories, Thaxted, UK). Solid-phase extraction cartridges were obtained from Supelco (Poole, UK). Tapered vials were obtained from Chromacol (Welwyn Garden City, UK).

Statistical analysis

Results are presented as mean values ± SE. Statistical analysis of all data was performed by ANOVA or Student's t test. Tukey's HSD test was used for comparison of means within treatments. Mean values were associated to symbols as an indication of significance ($p<0.001$).

Table 2 *N*-nitrosamine concentrations (µM) in single and dual-phase experiments 15 min after addition of nitrite (100 µM) in presence or absence of ascorbic acid (2 mM) (n = 12)

	Aqueous phase only		Aqueous phase + 10% lipid	
	−Ascorbic acid	+Ascorbic acid	−Ascorbic acid	+Ascorbic acid
Aqueous phase				
NDMA	0.02±0.01*	0.004±0.003*	0.07±0.02*	1.19±0.13**
NDEA	0.001±<0.001*	ND	0.01±0.003*	0.56±0.07**
NMOR	3.43±0.12*	0.003±0.003**	3.37±0.07*	0.75±0.1**
NPIP	0.002±0.001*	ND	0.04±0.002*	2.78±0.92**
Lipid phase				
NDMA			1.16±0.27*	4.77±0.4**
NDEA			ND	1.18±0.55**
NMOR			ND	4.9±0.41**
NPIP			0.06±0.01*	29.3±7.09**

For the same *N*-nitrosamine, concentrations on the same row followed by the same number of asterix are not significantly different according to the Tukey's HSD test ($p<0.001$).
ND not detected. Values are means ± SE.

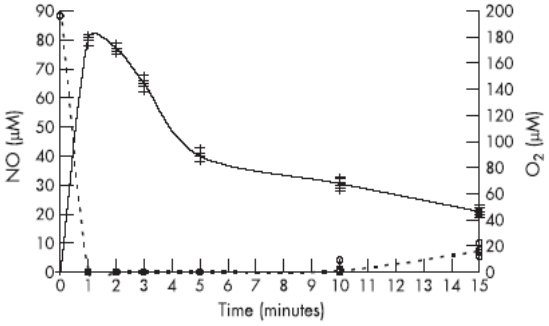

Figure 1 Nitric oxide (continuous line) and oxygen (dashed line) concentrations in the aqueous phase after the addition of nitrite (100 µM) to a dual-phase system containing ascorbic acid (1 mM) (n = 6).

RESULTS

Nitrosation in aqueous solution without lipid present

In the absence of ascorbic acid

When nitrite was added to HCl pH 1.5 containing thiocyanate (1 mM) without ascorbic acid, nitrosation of the secondary amines was observed. After 15 min, 3.43±0.12 µM NMOR was preferentially formed, while only 0.02±0.007 µM NDMA, 0.001±0.000 µM and 0.002±0.001 µM NPIP were detected (table 2).

In these experiments, the addition of nitrite produced only a very low concentration of nitric oxide (approximately 3.5 µM) and no discernible fall in oxygen concentration.

In the presence of ascorbic acid

The presence of ascorbic acid markedly inhibited the nitrosation of the secondary amines. The concentration of NDMA formed was reduced by fivefold, while the concentration of NMOR formed was reduced by more than a 1000-fold. NDEA and NPIP were not detected (table 2). Addition of nitrite produced a rapid increase in nitric oxide concentrations, followed by slow decline. Within 60 s of sodium nitrite addition (100 µM), the dissolved nitric oxide concentration was approximately 80 µM (fig 1). This rapid rise in nitric oxide was accompanied by a rapid fall in the dissolved oxygen concentration (fig 1). Within 60 s of sodium nitrite addition, no dissolved oxygen was detected until 10 min later when the concentration slowly increased to reach 5–10% of its original concentration.

The addition of nitrite (100 µM) triggered a fall in the ascorbic acid concentration, from its original concentration of 970±75 µM to 580±40 µM at 2 min, and then it slowly fell to 480±40 µM at 15 min (fig 2).

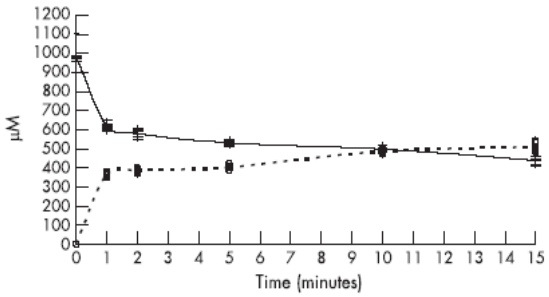

Figure 2 Ascorbic acid (continuous line) and dehydroascorbic acid (dashed line) concentrations in the aqueous phase after the addition of nitrite (100 µM) to a dual-phase system containing ascorbic acid (1 mM) (n = 6).

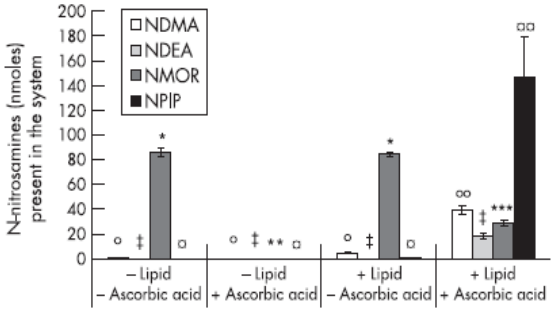

Figure 3 *N*-nitrosamines present in the single and dual-phase systems in the presence and absence of ascorbic acid (2 mM) 15 min after addition of nitrite (100 μM) (n=12).

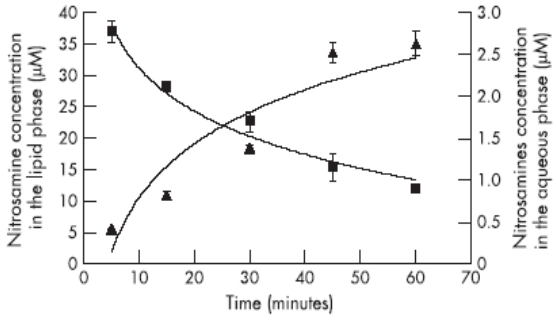

Figure 4 Diffusion of total *N*-nitrosamines in a dual-phase system. *N*-nitrosamine concentration in the lipid phase (square), *N*-nitrosamine concentration in the aqueous phase (triangle) (n=12).

Nitrosation in a dual-phase system containing aqueous solution and 10% lipid

In absence of ascorbic acid

When nitrite was added to HCl pH 1.5 containing thiocyanate (1 mM), no ascorbic acid and 10% lipid, nitrosation of the secondary amines was observed. The concentrations of *N*-nitrosamines formed in the aqueous phase were similar to those observed in the absence of lipid, with NMOR preferentially formed (3.37±0.07 μM) (table 2). Only NDMA (1.16±0.27 μM) and NPIP (0.06±0.01 μM) were detected in the lipid phase (table 2).

In presence of ascorbic acid

In marked contrast to the observations in the absence of lipid, the addition of ascorbic acid did not inhibit the generation of *N*-nitrosamines in the presence of 10% lipid. Indeed, in the presence of lipid and ascorbic acid, substantial concentrations of each of the *N*-nitrosamines were detected in both the aqueous and lipid phases (table 2). After 15 min, the concentrations of each *N*-nitrosamine in the lipid phase, and the concentration of NDMA, NDEA and NPIP in the aqueous phase, were significantly higher than in the absence of ascorbic acid ($p<0.001$). In the aqueous phase, 1.19±0.13 μM NDMA, 0.56±0.07 μM NDEA, 0.74±0.1 μM NMOR and 2.78±0.92 μM NPIP were detected. In the lipid phase, 4.77±0.4 μM NDMA, 1.18±0.55 μM NDEA, 4.90±0.41 μM NMOR and 29.3±7.01 μM NPIP were detected (table 2). In the presence of lipid, the addition of nitrite produced similar changes in the concentrations of nitric oxide, oxygen and ascorbic acid to those seen in the absence of lipid.

Effect of the lipid on the influence of ascorbic acid on acid nitrosation

The presence of lipid transformed the overall effect of ascorbic acid from inhibiting to promoting *N*-nitrosamine formation. In the absence of lipid, ascorbic acid totally inhibited the formation of NDEA and NPIP, and reduced the formation of NDMA by fivefold and NMOR by more than a 1000-fold. The addition of lipid to the system, in a 1:10 ratio, overrode the protective effect of ascorbic acid. In the presence of lipid, the addition of ascorbic acid increased the amount of three of the *N*-nitrosamines in the dual-phase system: NDMA by approximately eightfold, from 4.7±0.77 nmol to 39.19±3.49 nmol; NDEA by approximately 60-fold, from 0.3±0.08 nmol to 18.13±1.98 nmol, and NPIP by approximately 140-fold, from 1.05±0.07 nmol to 147.35±31.59 nmol (fig 3). In the presence of lipid, the addition of ascorbic acid reduced the total amount of NMOR in the system by approximately threefold, from 84.21±1.78 nmol to 28.78±2.59 nmol (fig 3), levels that were nonetheless significantly higher than in the presence of ascorbic acid and absence of lipid (0.08±0.08 nmol) ($p<0.001$). In addition, ascorbic acid increased the concentration of NMOR formed in the lipid from undetectable to 4.9±0.4 μM ($p<0.001$).

Variations in the nature of the lipid phase

The conversion of the inhibiting effect of ascorbic acid on nitrosation to a promoting effect was observed with three different lipid phases (glycerol trioctanoate, a low-density lipid, and glycerol tributyrate and glycerol triacetate, two high-density lipids). While glycerol trioctanoate provided a physiologically accurate representation of a floating lipid layer, glycerol tributyrate and glycerol triacetate were included in the study in order to test the robustness of our benchtop system when lipids are not in direct contact with air. Each of the three lipids generated similar results (data not shown).

Time course study of *N*-nitrosamines diffusion in a dual-phase system

Diffusion of the four *N*-nitrosamines from the lipid phase to the acidic aqueous phase under conditions similar to the previous experiment (0.1 M HCl, pH 1.5, at 37°C, in the presence of EDTA, thiocyanate and ascorbic acid) was monitored by adding 10 μM each *N*-nitrosamine to the lipid phase at t=0, and measuring their concentration in each phase during a 60 min time course. The concentrations of all four *N*-nitrosamines decreased in the lipid phase and increased in the aqueous phase over time (60 min) (table 3). The total *N*-nitrosamine concentration of the lipid phase decreased from 40 μM down to approximately 12 μM, while the total *N*-nitrosamine concentration in the aqueous phase rose to approximately 2.6 μM (fig 4). The diffusion pattern of all four *N*-nitrosamines was comparable, although NPIP tended to remain at higher levels in the lipid phase (table 3).

DISCUSSION

These studies indicate that the presence of lipid profoundly alters acid-catalysed nitrosative chemistry. The lipid phase is able to convert the influence of ascorbic acid from one that protects against nitrosation to one that promotes it. This effect is likely to be due to the ability of the nitric oxide formed by ascorbic acid within the aqueous phase to regenerate nitrosative species by reacting with oxygen within the lipid phase.

In the studies performed without lipid, and without ascorbic acid, the addition of nitrite to the HCl pH 1.5 containing thiocyanate resulted in nitrosation of the secondary amines. The main nitrosating species formed under these conditions is NOSCN.[9–11] NOSCN reacts with the secondary amine in its unprotonated uncharged state.[48] The amount of the secondary amine in its nitrosatable form depends upon its −log

Table 3 Time course study of standard *N*-nitrosamine concentration in a dual-phase system (n = 12)

Standard *N*-nitrosamine	Concentration (μM) 5 min	15 min	30 min	45 min	60 min
Aqueous phase					
NDMA	0.15±0.01	0.29±0.06	0.40±0.04	0.48±0.07	0.54±0.01
NDEA	0.08±0.01	0.18±0.02	0.3±0.01	0.49±0.15	0.64±0.02
NMOR	0.09±0.02	0.25±0.07	0.33±0.03	0.89±0.28	0.70±0.01
NPIP	0.09±0.01	0.09±0.03	0.34±0.03	0.68±0.08	0.75±0.1
Lipid phase					
NDMA	6.64±0.40	4.03±0.25	1.97±0.24	0.86±0.24	0.39±0.01
NDEA	6.29±0.48	5.11±0.67	5.86±1.74	4.61±1.51	2.98±0.67
NMOR	10.62±0.61	8.75±0.51	5.86±0.24	3.71±0.12	2.96±0.20
NPIP	13.34±0.73	9.75±0.9	8.95±0.57	6.49±0.4	5.69±0.20

10 μM of each *N*-nitrosamine was added to the lipid phase at time 0 min, and concentrations in each phase were monitored over the subsequent 60 min.
Values are means ± SE.

dissociation constant (pK_a). The pK_a values of the amines studied are 8.33 for morpholine, 11.22 for piperidine, 10.73 for dimethylamine and 11.09 for diethylamine. The amount of the secondary amine in a form available for nitrosation by NOSCN will therefore be greatest for morpholine and least for piperidine. The differences in pK_a can partially explain why different concentrations of the four *N*-nitrosamines were generated.

In the experiments performed in the absence of lipid, the addition of ascorbic acid effectively prevented the nitrosation of the amines. This can be explained by the ascorbic acid competing with the secondary amines for the NOSCN.[36 38 49] In the reaction between ascorbic acid and NOSCN, the latter is reduced to nitric oxide and the former oxidised to dehydroascorbic acid. Consistent with this, we observed a burst of nitric oxide accompanied by a fall in the ascorbic acid and oxygen concentrations. This can be explained by the nitric oxide reacting with dissolved oxygen to form N_2O_3.[43 44] The N_2O_3 formed in this way is a nitrosating species and again preferentially reacts with ascorbic acid and is reduced back to nitric oxide.[39] This recycling continues until either the oxygen or the ascorbic acid is consumed. Under our experimental conditions, the oxygen was the first to be depleted. Stoichemically, 1 mol ascorbic acid can reduce 2 mol nitrite to nitric oxide. The greater consumption of ascorbic acid observed is due to this recycling of nitric oxide.

In the presence of the lipid, *N*-nitrosamines were formed despite the presence of ascorbic acid. Indeed, the presence of lipid transformed the effect of the ascorbic acid from effectively inhibiting nitrosation of each amine to powerfully enhancing nitrosation of three of the four amines. (The overall amounts of NDMA, NDEA and NPIP in the system were increased by 8-, 60- and 140-fold, respectively, in the dual-phase system when ascorbic acid was present versus absent; fig 3.) The overall amount of NMOR formation was inhibited by ascorbic acid in both the absence and presence of lipid, although the inhibitory effect of ascorbic acid was markedly reduced by the presence of lipid, from over 1000-fold in absence of lipid, to only threefold in the presence of lipids. In addition, the presence of ascorbic acid increased the concentration of NMOR in the lipid from undetectable to 4.9±0.4 μM.

What is the explanation for the ability of the lipid to convert the effect of the ascorbic acid from being an inhibitor to a promoter of nitrosation? The effect observed is likely to be mediated by the nitric oxide produced by the reaction between the ascorbic acid and the nitrosating species within the aqueous phase. The nitric oxide will diffuse into to the lipid phase and within it react with oxygen to form N_2O_3.[50] Liu *et al.* demonstrated that the reaction between nitric oxide and oxygen is 300 times faster in lipid than in aqueous solutions, due to the increased solubility of both gases in lipids.[41] As ascorbic acid is not lipophilic, it is unable to enter the lipid and thus the N_2O_3 generated within the lipid will be able to nitrosate the secondary amines within the lipid. The *N*-nitrosamines generated within the lipid in this way will then diffuse out of the lipid and produce the rise in their concentration observed in the aqueous compartment (fig 5).

In the dual-phase studies without ascorbic acid, low concentrations of NDMA and NPIP were detected in the lipid phase, whereas NDEA and NMOR were both undetectable in the lipid phase. NDMA and NPIP are more lipid soluble than NMOR (ClogP 0.01, 0.73 and −0.33, respectively) and are likely to have diffused into the lipid following their generation in the aqueous phase. Low levels of nitric oxide are present in the aqueous phase even in the absence of ascorbic acid and this may also have nitrosated dimethylamine and piperidine within the lipid phase. The dual-phase experiments with ascorbic acid indicated that dimethylamine and piperidine were the amines most nitrosated by nitric oxide in the lipid phase. Piperidine showed the greatest degree of nitrosation in the dual phase; this may be due to its lipophilicity (ClogP 0.52) and thus it is the one most available for nitrosation within the lipid compartment.[51]

In the dual-phase system, the concentrations of three of the *N*-nitrosamines detected in the aqueous phase were significantly greater in the presence versus absence of ascorbic acid. The one exception was NMOR and a number of factors may explain this. First, the amount of NMOR formed in the aqueous phase in the absence of ascorbic acid is considerably greater than the amount of other *N*-nitrosamines, due to its lower pK_a and thus higher proportion in the nitrosatable unprotonated form.[52] Second, the aqueous concentrations of each *N*-nitrosamine were similar in presence of both ascorbic acid and lipid compared to aqueous concentrations in the absence of ascorbic acid (table 2). This is because the *N*-nitrosamines detected in the aqueous phase in presence of both ascorbic acid and lipid

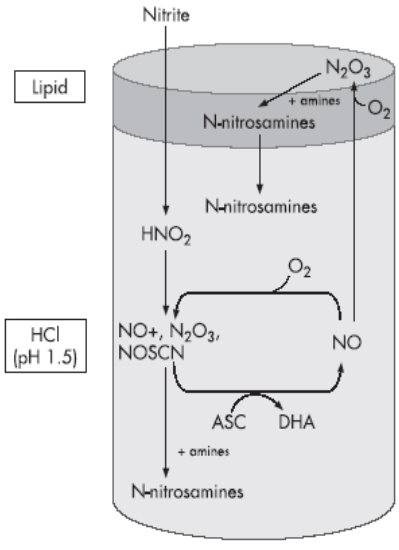

Figure 5 Proposed mechanism of *N*-nitrosamine formation in a dual-phase system with ascorbic acid present in the aqueous phase. Nitrite forms nitrous acid and nitrosating species in the acidic aqueous environment. In the presence of ascorbic acid (ASC), the nitrosating species are reduced to nitric oxide which diffuses into the lipid where it reacts with oxygen reforming nitrosating species such as N_2O_3. Ascorbic acid is reduced to dehydroascorbic acid. Secondary amines present within the lipid are nitrosated by N_2O_3 to form *N*-nitrosamines. The latter then diffuse back to the aqueous phase.

will have been formed mainly in the lipid phase, where the pK_a of the amine is not relevant. The decrease of NMOR aqueous concentration in the dual-phase system in presence of ascorbic acid compared to when ascorbic acid is absent is therefore mainly explained by the inhibition of the aqueous nitrosation of morpholine when ascorbic acid is present. In addition, differences in partitioning of the different amines and *N*-nitrosamines between the lipid and aqueous phase may contribute to the different results.

The above studies indicate that the presence of lipid transforms the regulation of nitrosative chemistry in conditions simulating the proximal stomach. The presence of lipid overcomes the protective effect of ascorbic acid and indeed transforms ascorbic acid from an inhibitor to a promoter of nitrosation. The transforming role of lipids is likely to be relevant to the *in vivo* situation as lipid is present in the proximal stomach for a considerable time after eating and is also an important component of the epithelial membranes.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Valerie Fyfe and Mr David Hughes for the technical support provided.

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This study was supported financially by the World Cancer Research Fund, and the Medical Research Council (a Personal Research Fellowship awarded to SP)

Competing interests: None

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Appendix C

N-Nitrosamine Generation From Ingested Nitrate Via Nitric Oxide in Subjects With and Without Gastroesophageal Reflux

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Background & Aims: **Nitrate ingestion leads to high luminal concentrations of nitric oxide being generated where saliva meets gastric acid. Nitric oxide generates *N*-nitrosative stress on reacting with oxygen at neutral pH. We aimed to ascertain if luminal nitric oxide exerts nitrosative stress in the human upper gastrointestinal tract, and to assess the influence of acid reflux on this phenomenon.** ***Methods:*** **A silicone tube, segmented every 15 mm and containing phosphate buffer pH 7.4 and the secondary amine morpholine, was inserted into the upper gastrointestinal tract of 16 healthy volunteers and 16 Barrett's esophagus patients. The tube wall has the same permeability properties as an epithelial lipid membrane, allowing passage of gases such as nitric oxide, but not hydrogen ions. After 2 hours, the tube was removed and the concentrations of nitrite and *N*-nitrosomorpholine in each segment were measured. Healthy volunteers were studied with and without ingestion of ^{15}N-enriched nitrate and Barrett's esophagus patients were studied with and without stimulation of acid reflux.** ***Results:*** **In healthy volunteers, *N*-nitrosomorpholine was generated in the tube sections exposed to gastric acid and increased 2-fold after nitrate. The *N*-nitrosomorpholine was 77% enriched with ^{15}N, confirming its source was the ingested nitrate. In the Barrett's patients, generation of *N*-nitrosomorpholine was shifted proximally with 80% of nitrosative stress occurring within the esophagus during periods of acid reflux.** ***Conclusions:*** **This study demonstrates the in situ formation of *N*-nitrosamine from dietary nitrate via nitric oxide. During acid reflux, nitrosative stress occurs almost entirely within the esophagus and may contribute toward carcinogenesis at this site.**

The incidence of adenocarcinoma of the esophagus and proximal cardia region of the stomach has increased markedly over the past few decades.[1–6] An important risk factor for esophageal adenocarcinoma is gastroesophageal reflux.[7–9] Excessive exposure of the esophagus to acidic gastric refluxate can result in erosive esophagitis. In some patients, excessive reflux leads to columnar metaplasia of the squamous mucosa of the esophagus, producing a phenotype resembling that of the gastric antrum or small or large intestine.[10–12] This metaplasia is termed *Barrett's esophagus* [13] and is associated with a substantially increased risk of progressing to dysplasia and adenocarcinoma. The etiology of adenocarcinoma at the gastric cardia is unclear although recent studies have indicated that a subgroup of this malignancy has similar pathogenesis to esophageal adenocarcinoma.[14]

Mucosa that is subject to recurrent erosions and chronic inflammation may be more sensitive to the effects of luminal carcinogens than uninflamed intact mucosa. For example, laboratory animals with *Helicobacter pylori*–induced gastritis are more sensitive to the carcinogenic effects of *N*-nitroso compounds.[15] Therefore, there is interest in chemical mutagens that may be present within the lumen of the esophagus and gastric cardia and might be contributing to the high incidence of metaplasia and neoplasia at this anatomic site. Nitrite is a potential premutagen in the human upper gastrointestinal tract because it can be converted to potentially mutagenic *N*-nitroso compounds when exposed to acidic gastric pH.[16,17]

The main source of nitrite entering the upper gastrointestinal tract is saliva. Approximately 25% of nitrate absorbed from the intestine or formed from endogenously produced nitric oxide is taken up by the salivary glands and secreted into the mouth.[18–20] Bacteria on the dorsum of the tongue reduce between 5% and 90% of this nitrate to nitrite.[21–23] Consequently, human saliva contains high concentrations of nitrite that increase several-fold after digestion of nitrate-containing foodstuffs.[19,24,25] The main origin of nitrate in the diet is nitrogenous fertilizers.[26]

When saliva is swallowed and encounters the acidic pH at the gastroesophageal junction, it is converted to nitrous acid (HNO_2) and nitrosating species (N_2O_3, NO^+ and NOSCN).[16,17,27,28] The latter can react with secondary amines to form carcinogenic *N*-nitrosamines. The main factor preventing generation of *N*-nitrosamines is ascorbic acid, which is secreted actively in gastric juice.[29,30] The ascorbic acid effectively competes with secondary amines for the nitrosating species, reducing them

0016-5085/07/$32.00
doi:10.1053/j.gastro.2007.04.047

to nitric oxide.[29–32] Recently, interest has focused on potential harmful and mutagenic effects of this nitric oxide. This is because nitric oxide can react with oxygen at neutral pH to form the nitrosating species N_2O_3.[33,34]

We recently produced a laboratory bench-top model to investigate the chemistry occurring under the conditions existing at the human gastroesophageal junction in both the luminal and adjacent epithelial compartment.[35] We used a fine-bore Silastic (Altec, Cornwall, UK) tube containing phosphate buffer pH 7.4 to represent the epithelium. The chemical characteristics of silastic closely resemble those of lipid membranes, allowing the passage of gases such as nitric oxide and oxygen, but not that of hydrogen ions.[36–38] The silastic wall of the tube thus represented the epithelial cell membrane, and the phosphate buffer pH 7.4 within the tube represented the interior of the epithelial cells, which we referred to as the *epithelial compartment*. The exterior surface of the Silastic (Altec) tube was exposed to the chemistry occurring in the lumen of the gastroesophageal junction when saliva meets acidic gastric juice, and this we referred to as the *luminal compartment*. The secondary amine, morpholine, was added to both the luminal and epithelial compartments. When the luminal compartment contained nitrite and hydrochloric acid, there was formation of *N*-nitrosomorpholine only in the luminal compartment. The further addition of ascorbic acid to the luminal compartment transformed the chemistry in both compartments—preventing *N*-nitrosomorpholine generation in the luminal compartment, generating high concentrations of nitric oxide in the luminal compartment, and high concentrations of *N*-nitrosomorpholine in the adjacent epithelial compartment. The findings are explained by the nitric oxide generated by the reaction between acidified nitrite and ascorbic acid diffusing into the epithelial compartment and within it reacting with oxygen to form N_2O_3, which was nitrosating the morpholine (Figure 1). The addition of ascorbic acid or glutathione to the epithelial compartment could only reduce the generation of *N*-nitrosomorpholine by a maximum of 40%. The silastic tube filled with phosphate buffer thus provides a means of measuring in situ *N*-nitrosation arising from luminally generated nitric oxide and within a chemical environment resembling epithelial cells.

We have used this apparatus to determine whether nitric oxide generated within the human upper gastrointestinal tract from dietary nitrate can lead to in situ generation of nitrosating species and *N*-nitroso compounds. By compartmentalizing the tube into 1.5-cm segments, we were able to compare the chemistry at different sites throughout the esophagus and stomach simultaneously and to determine the influence of gastroesophageal reflux on its site of maximal intensity.

The aims of our study were as follows: (1) to investigate if ingestion of nitrate generated *N*-nitrosamines via nitric oxide within our epithelial model situated in the human upper gastrointestinal tract, and (2) to examine the influence of gastroesophageal acid reflux on the location of this in situ nitrosative stress. To address our first aim, we studied healthy volunteers with and without ingestion of ^{15}N-enriched nitrate and to address our second aim we studied subjects with Barrett's esophagus, a group in whom reflux can be readily induced,[39] with and without stimulation of gastroesophageal acid reflux.

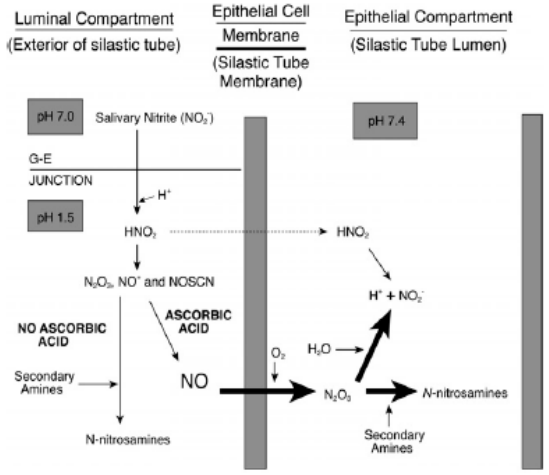

Figure 1. Chemical reactions occurring when nitrite enters acidic gastric juice. Nitrite is converted to nitrous acid and nitrosating species (N_2O_3, NO+, and NOSCN). In the absence of ascorbic acid, these can react with nitrosatable species to form *N*-nitroso compounds in the luminal compartment. Ascorbic acid in the gastric juice prevents this luminal nitrosation by converting the nitrosating species to nitric oxide. However, nitric oxide can diffuse into the epithelial compartment, reacting with oxygen to form the nitrosating species N_2O_3, which exerts nitrosative stress within the epithelial compartment. Nitrite is formed in the epithelial compartment predominantly by the reaction of N_2O_3 with water, but also by the diffusion of nitrous acid (NO_2).

Materials and Methods

Silastic Tube for In Situ Measurement of N-Nitroso Compound Generation

We used Silastic tubing (Altec), with an internal diameter of 2.5 mm and a wall thickness of .5 mm for our in situ epithelial model. A 100-cm section of tubing was filled with the secondary amine morpholine, which was buffered at intracellular pH (7.4) using 2 mmol/L phosphate buffer. The tubing then was partitioned into 21 sections, each 15-mm long, by twisting twice and then tying with 4-0 Vicryl suture (Ethicon Corporation, Somerville, NJ). Every section contained approximately 74 μL. This length of tubing was attached to a customized 4-channel pH catheter (Synectics Medical, Middlesex, UK) so that we could monitor luminal acidity simultaneously. The pH sensors were aligned to the center of the 19th, 14th, 13th, and 8th sections of the Silastic (Altec) tube, respectively, and were attached by tying with Vicryl

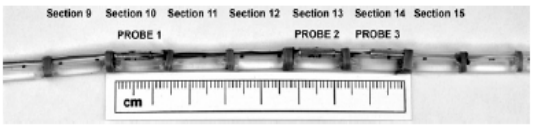

Figure 2. Region of segmented Silastic tube attached to the pH catheter. Each section contains morpholine 25 mmol/L buffered at pH 7.4 and is 15 mm in length. Section 10 is apposed to probe 1 and sections 13 and 14 are apposed to probes 2 and 3, respectively.

suture and securing with Sleek waterproof tape (Smith & Nephew, London, UK) (Figure 2). A 5-cm length of 10F gastric tube (Vygon, Ecouen, France) containing a radio-opaque marker was secured to the distal end of the pH catheter to assist passage.

Studies in Human Subjects

Healthy volunteers. Seventeen healthy volunteers (11 men all older than 18 y; median age 27 y) with no history of reflux disease were studied on 2 separate days after an overnight fast. All tested negative for *Helicobacter pylori* by ^{14}C urea breath test. Subjects attended the gastrointestinal investigation unit on 2 separate days after an overnight fast. On each study day, the tube was passed nasogastrically or orogastrically. The tube was positioned such that sections 1–13 lay within the esophagus and sections 14–21 lay within the stomach. This was achieved by monitoring the pH step-up and was confirmed by radiograph (Figure 3). The tube tip lay 12 cm within the stomach. Thirty minutes after insertion of the tube, the subjects were administered the study drink. On one day, the subjects drank 60 mL of water containing 2 mmoles potassium nitrate and on the other day subjects drank 60 mL of water without the nitrate. Eight of the subjects received nitrate which was 99% enriched with the heavy, stable isotope ^{15}N (Sercon Ltd., Nantwich, UK). The order in which the different drinks were administered was random.

The tube remained in situ for 2 hours after the drink; the pH was monitored at each site every 4 seconds. Immediately before the drink, and at 15-minute intervals after it, 10-mL blood and 1-mL saliva samples were collected for measurement of serum nitrate and salivary nitrite concentrations. The tube then was removed and each section was aspirated carefully using a 100-μL glass syringe (SGE, Milton Keynes, UK). A total of 10 μL was used for measurement of the serum nitrite concentration and 40 μL for analysis of *N*-nitrosomorpholine.

Patients with Barrett's esophagus. Eighteen *H pylori*–negative patients with Barrett's esophagus (13 men all older than 18 y; mean age 61 y) were recruited from our gastroenterology clinic. The median length of the Barrett's mucosa was 6 cm (range, 2–20 cm). Proton pump inhibitors were stopped 7 days before investigation.

Patients attended the gastrointestinal investigation unit on 2 occasions after an overnight fast. At the first visit, gastrointestinal endoscopy was performed either consciously using lignocaine local anesthetic spray or under conscious sedation using intravenous midazolam. The extent of the Barrett's mucosa was inspected and 2 stainless steel mucosal clips (MD-59; Olympus, Southend-on-Sea, UK) were placed at the proximal margin of the gastric folds to mark the anatomic gastroesophageal mucosal junction. Unlike in healthy volunteers in whom the pH step-up point is a reliable indicator of the squamocolumnar junction,[40] in the patients with Barrett's esophagus we required a radiologic marker to show this anatomic site.

One hour after endoscopy the tube was passed nasogastrically or orogastrically. It was positioned such that sections 1–13 were proximal and sections 14–21 were distal to the gastroesophageal junction, as shown by the radio-opaque clips on the lateral chest radiograph. The tube was placed in an identical position on the second study day, again using the radio-opaque clips, which remained attached.

The study days were designed to maximize and minimize acid reflux. On one day, we studied the patients in the sitting position without attempt to stimulate reflux. On the other visit, we attempted to stimulate acid reflux by placing the subjects in the right lateral decubitus position, with or without the head-down position, and infusing a low dose of pentagastrin (.06 μg/kg/h) (Cam-

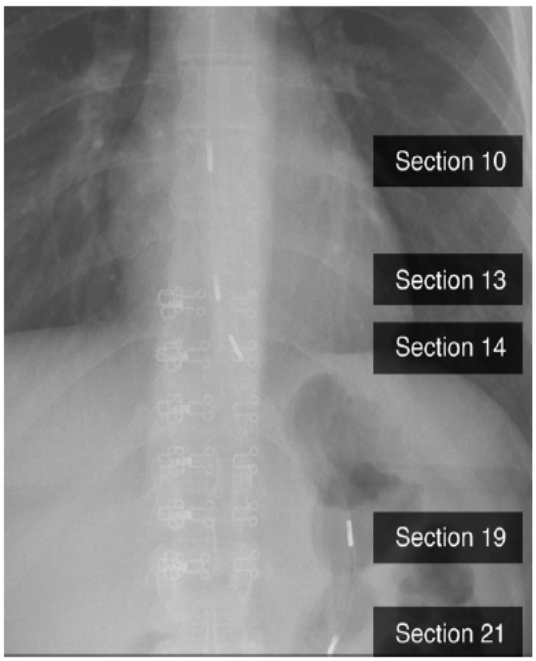

Figure 3. Plain posteroanterior chest radiograph of specialized esophagogastric tube in situ within the upper gastrointestinal tract of a human healthy volunteer. Sections 13 and 14 are apposed to probes 2 and 3 and lie on either side of the pH step-up point. Section 10 is apposed to probe 1 and is positioned 5 cm proximal to the pH step-up point. Section 19 is apposed to probe 4 and section 21 lies 12 cm inside the stomach, apposed to a radio-opaque marker.

bridge Laboratories, Wallsend, UK) intravenously to stimulate physiologic levels of acid secretion. On each study day, once the tube was positioned satisfactorily, subjects drank 60 mL water containing 2 mmoles potassium nitrate. Twelve subjects received nitrate that was 99% enriched with ^{15}N on both study days. The probe remained in situ for 2 hours after the drink, with serum and saliva samples for nitrate and nitrite collected at 15-minute intervals. The position of the tube was confirmed radiologically immediately before its removal. Each section of the tube was aspirated for analysis of nitrite and *N*-nitrosomorpholine concentrations as discussed earlier.

Chemical Analyses

Determination of nitrite and nitrite concentrations. Serum nitrate concentration and salivary and silastic tube nitrite concentrations were analyzed as previously described.[25,31]

Determination of N-nitrosomorpholine concentration. A total of 50 μL of 2 mol/L sodium chloride was added to 1.5-mL polypropylene vials. A total of 40 μL of the Silastic tubing solution was added to each vial along with 10 μL of *N*-nitrosodibutylamine in methanol (as internal standard). A total of 250 μL of extraction solvent mix (45:55 dichloromethane:di-ethylether) then was added to each vial and well mixed to allow transfer of nitrosamines to the upper organic layer. Samples then were centrifuged at 3000 rpm for 3 minutes. The upper (organic solvent) layer then was transferred to a tapered vial, and a further 250 μL of fresh solvent was added to the original vial and the extraction process was repeated. The resulting 300 μL of organic solvent with dissolved nitrosamine then was concentrated to 50 μL by blowing down with a gentle stream of nitrogen gas. The tapered vials then were crimp sealed and stored at −20°C until analysis by gas chromatography:tandem mass spectrometry using the Finnigan Polaris*Q* GC/MS^n Bench top Ion Trap Mass Spectrometer (Thermo Electron Corporation, Austin, TX). Samples were analyzed on the carousel along with extraction samples of known concentrations of *N*-nitrosomorpholine to correct for any error secondary to differential extraction between the 2 nitrosamines. Tandem mass spectrometry allowed us to determine the proportion of *N*-nitrosomorpholine that was enriched with the heavy stable nitrogen isotope ^{15}N.

Statistical Analyses

All data are presented as medians (interquartile range). Paired data were analyzed using a 1-sample Wilcoxon test, and unpaired data were analyzed by the Mann–Whitney *U* test. The Spearman rank test was used to assess correlation when examining the relationship between esophageal acid reflux and nitrosative stress in that region.

Total nitrite and *N*-nitrosomorpholine within the Silastic tube were calculated from the area under the curve on the graph comparing the concentration of each compound by location. The area under the curve was estimated using the trapezoid method, and a volume correction factor (× .074) was applied.

Ethics

The study was approved by the North Glasgow Hospitals Ethics Committee and each subject provided written informed consent.

Results

Studies in Healthy Volunteers

Sixteen of the 17 subjects completed both arms of the study. One subject was achlorhydric (intragastric pH > 4.0), and later was confirmed to be *H pylori* positive by serologic testing after an initial negative breath test.

Serum nitrate. Before administration of the study drink, the median serum nitrate concentration was 11.0 μmol/L (interquartile range, 7.2–13.8 μmol/L) on the day when the control drink was administered, which was not significantly different from 9.1 μmol/L (interquartile range, 7.5–13.9 μmol/L) on the day when the nitrate drink was administered. After administration of 2 mmoles potassium nitrate, there was a large and significant increase in the serum nitrate concentration ($P = .001$), which peaked at 33.1 μmol/L (interquartile range, 21.9–56.5 μmol/L) after 45 minutes and remained at this level for the duration of the study. After administration of the control drink, the serum nitrate concentration did not change from basal, remaining at 11.1 μmol/L (interquartile range, 6.0–15.3 μmol/L) after 45 minutes.

Salivary nitrite. Before administration of the study drink, the median salivary nitrite concentration was 51.4 μmol/L (interquartile range, 24.7–79.4 μmol/L) on the day when the control drink was administered, which was not significantly different from 48.7 μmol/L (interquartile range, 24.8–77.3 μmol/L) on the day when the nitrate drink was administered. After administration of 2 mmoles potassium nitrate, there was a large and significant increase in the salivary nitrite concentration ($P = .001$), which peaked at 331.8 μmol/L (interquartile range, 265.5–575 μmol/L) after 75 minutes and remained at almost this level for the duration of the study. After administration of the control drink, salivary nitrite concentration changed little from basal, remaining at 59 μmol/L (interquartile range, 32–122.3 μmol/L) at 75 minutes.

Silastic tube nitrite. The diffusion of either nitric oxide or nitrous acid through the wall of the tube forms nitrite within the tube. Nitrite was not detected within the tube sections lying within the esophagus. However, nitrite was present in the sections exposed to gastric acid on both study days. The median total nitrite formed within the Silastic tube was 17.3 nmoles (interquartile range, 7.9–28.5 nmoles) on the control day and 62.1

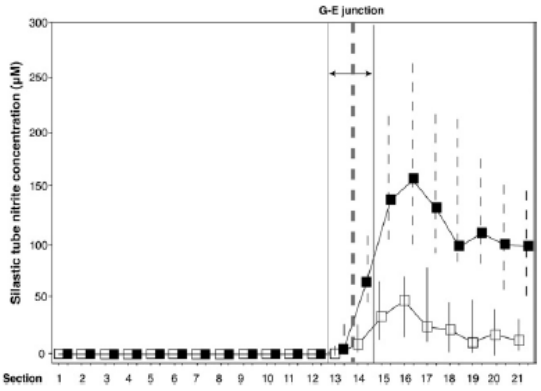

Figure 4. Median Silastic tube nitrite concentration by location for 15 healthy volunteers. Sections 1–13 lie above the pH step-up point and sections 14–21 lie below the pH step-up point. The *grey* broken vertical line between sections 13 and 14 represents the pH step-up point, and the surrounding rectangle represents the range of excursion of the pH step-up point with normal respiration. Results after administration of the control drink are represented by *open boxes*, and after administration of 2 mmoles nitrate by *filled boxes*. Whiskers represent interquartile ranges.

nmoles (interquartile range, 40.8–110.3 nmoles) on the day when potassium nitrate was administered ($P = .001$). The concentration profile along the gastric sections of the tube was similar on both study days, with the highest concentrations being detected in sections 15 and 16 (Figure 4), which are the first sections likely to be exposed constantly to intragastric acidity. By using fluoroscopic screening, we observed that the excursion of the esophagogastric junction with normal respiration is 3–4 cm. The probe at section 13 showed a pH of less than 2 for 7% of the time and the probe at section 14 showed a pH of less than 2 for 63% of the time. Peak concentrations of nitrite were detected in section 16, being 48 μmol/L (interquartile range, 13.8–68.3 μmol/L) after the control drink and increasing more than 3-fold to 159 μmol/L (interquartile range, 93–264 μmol/L) after nitrite. The one subject in our study who was achlorhydric had no detectable nitrite in any of the Silastic tube sections.

Silastic tube *N*-nitrosomorpholine. *N*-nitrosomorpholine was not detected within the esophageal sections of the Silastic tube. However, it was detected in the sections exposed to gastric acid when subjects had taken either the control drink or the nitrate drink. The median total *N*-nitrosomorpholine formed within the Silastic tube was 2.3 nmoles (interquartile range, 0.8–3.2 nmoles) on the control day and 4.5 nmoles (interquartile range, 2.7–7.9 nmoles) on the day potassium nitrate was administered ($P = .008$). On the control day, the peak *N*-nitrosomorpholine concentration was 6.1 μmol/L (interquartile range, 1.7–8.5 μmol/L), measured in section 16. However, after the nitrate the *N*-nitrosomorpholine concentration profile was different, producing a plateau rather than the peak concentration recorded without nitrate (Figure 5). The *N*-nitrosomorpholine concentration in section 16 was 7.3 μmol/L (interquartile range, 5.3–12.7 μmol/L) after nitrate, not much greater than after the control drink. The peak *N*-nitrosomorpholine concentration after nitrate was measured in section 19, where the concentration of 8.9 μmol/L (interquartile range, 4.2–17.7 μmol/L) was more than 3-fold greater than the 2.7 μmol/L (interquartile range, 0–4.5 μmol/L) detected in this section after the control drink. The one subject in this study who was achlorhydric had no detectable *N*-nitrosomorpholine in any of the Silastic tube sections.

Eight of the healthy volunteers were administered nitrate that was 99% enriched with the heavy nitrogen isotope ^{15}N. The *N*-nitrosomorpholine in these volunteers had a median enrichment of 77.4% (interquartile range, 64.4%–87.3%) after the administration of nitrate, compared with no enrichment when the control drink was administered. This confirmed that the excess *N*-nitrosomorpholine formed after administration of 2 mmoles potassium nitrate originated from the ingested nitrate.

Correlation of Silastic tube nitrite versus *N*-nitrosomorpholine. The ratio of *N*-nitrosomorpholine:nitrite within the Silastic tube is an expression of nitric oxide: nitrite in the lumen of the gastrointestinal tract. *N*-nitrosomorpholine can be formed only within the epithelial compartment by the diffusion of nitric oxide, whereas nitrite can be formed via the diffusion of either nitric oxide or nitrous acid into the tube.[35]

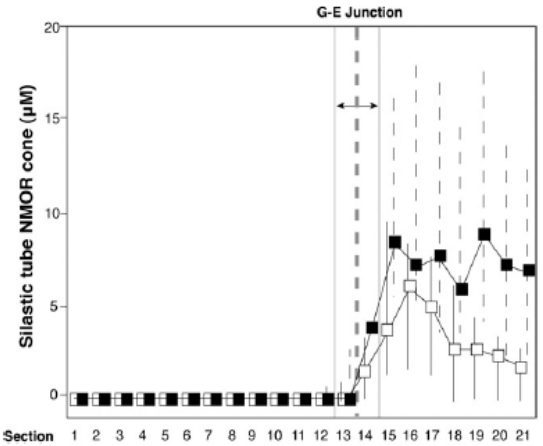

Figure 5. Median Silastic tube *N*-nitrosomorpholine (NMOR) concentration by location for 15 healthy volunteers. Sections 1–13 lie above the pH step-up point and sections 14–21 lie below the pH step-up point. The *grey* broken vertical line between sections 13 and 14 represents the pH step-up point, and the surrounding rectangle represents the range of excursion of the pH step-up point with normal respiration. Results after administration of the control drink are represented by *open boxes*, and after administration of 2 mmoles nitrate by *filled boxes*. Whiskers represent interquartile ranges.

The administration of nitrate resulted in a 3-fold increase in peak Silastic tube nitrite concentrations, but only a 50% increase in peak *N*-nitrosomorpholine concentrations. We calculated the ratio of *N*-nitrosomorpholine:nitrite concentrations for each Silastic tube section in all of the healthy volunteers. The median of all ratios of *N*-nitrosomorpholine:nitrite concentration after nitrate ingestion was .064 (interquartile range, .045–.099), which was significantly lower than .11 (interquartile range, .052–.43) after the control drink ($P = .02$).

Studies in Patients With Barrett's Esophagus

Eighteen patients with Barrett's esophagus were recruited. One patient was unable to tolerate the esophagogastric tube and withdrew from the study. Another patient completed both arms of the study but produced very little saliva. This probably was a result of the patient having psoriatic arthropathy, which has a recognized association with reduced salivary flow.[41] Sixteen patients completed both arms of the study.

Gastroesophageal reflux was defined as a pH of less than 4 detected 5 cm proximal to the clips marking the top of the gastric folds (the anatomic gastroesophageal junction). The pH was less than 4 at this location for a median of 14.5% (interquartile range, 2.6%–48.7%) of 32 study days. On the 16 study days when reflux was stimulated by postural and pharmacologic methods, the pH was less than 4 at this location for a median of 40% of the study time (interquartile range, 14.3%–63.6%). This was significantly higher than the 3.6% (interquartile ranges, 0.1%–19%) when reflux was not stimulated ($P = .003$), which was in turn significantly higher than 0.1% (0%–1.0%) in the healthy volunteers who received nitrate ($P = .03$).

Serum nitrate. Before administration of the study drink, the median serum nitrate concentration was 11.3 μmol/L (interquartile range, 7–13.4 μmol/L) on the day when reflux was not provoked, which was not significantly different from 7.8 μmol/L (interquartile ranges, 5.8–13.4 μmol/L) on the day we provoked reflux. After administration of 2 mmoles of potassium nitrate, the serum nitrate concentrations increased almost 4-fold on both days, peaking after 60 minutes at 43.1 μmol/L (interquartile range, 30.4–50.6 μmol/L) on the day when reflux was not provoked and 37.5 μmol/L (interquartile range, 24–48.6 μmol/L) on the day we provoked reflux. Basal and peak nitrate concentrations in patients with Barrett's esophagus were not significantly different from the healthy volunteers.

Salivary nitrite. Before administration of the study drink, the median salivary nitrite concentration was 38.2 μmol/L (interquartile range, 16.4–70.7 μmol/L) on the day when reflux was not provoked, which was not significantly different from 59.2 μmol/L (interquartile range, 41–90.1 μmol/L) on the day we provoked reflux. After administration of 2 mmoles of potassium nitrate, salivary nitrite concentrations increased significantly on both days, peaking at 578 μmol/L (interquartile range, 271–666 μmol/L) after 90 minutes on the day when reflux was not provoked and at 457.7 μmol/L (interquartile range, 307–572.6 μmol/L) after 60 minutes on the day we provoked reflux. Basal and peak salivary nitrite concentrations in patients with Barrett's esophagus were not significantly different from the healthy volunteers.

Silastic tube nitrite. On the day that reflux was stimulated in the patients with Barrett's esophagus, a median of 33% (interquartile range, 17.13%–54.9%) of the total nitrite formed within the Silastic (Altec) tube was formed within the esophageal sections. This was a significantly greater proportion than the median of 8.1% (interquartile range, 1.5%–31.8%) when reflux was not stimulated ($P = .01$). In the healthy volunteers who received nitrate, the proportion of nitrite present within the esophagus was 0.7% (interquartile range, 0%–2.5%), which was significantly lower than the Barrett's patients in whom reflux was not stimulated ($P = .007$).

The concentration profile of nitrite along the length of the tube in the 32 studies from 16 Barrett's patients correlated with the frequency of reflux. A linear relationship existed between the duration of acid reflux (proportion of the study that pH was <4 at 5 cm above the gastroesophageal junction) and the proportion of the total nitrite that was formed within the esophageal sections (Spearman $\rho = .927$; $P < .01$), with 80% of the nitrite being formed within the esophagus during reflux (Figure 6).

Silastic tube *N*-nitrosomorpholine. On the day that reflux was stimulated in the patients with Barrett's esophagus, a median of 29% (interquartile range, 6.9%–50.5%) of the total *N*-nitrosomorpholine formed within the Silastic tube was formed within the esophageal sections. This was significantly greater than the median of

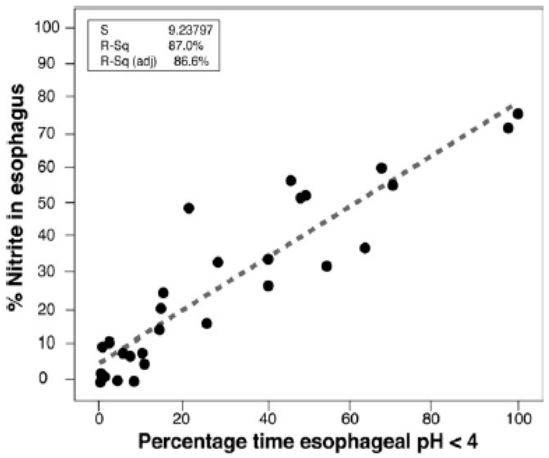

Figure 6. Fitted line plot comparing the percentage of time pH was less than 4, 5 cm above the gastroesophageal junction (marked by the clips at the top of the gastric folds), with the proportion of total nitrite formed within the esophageal sections.

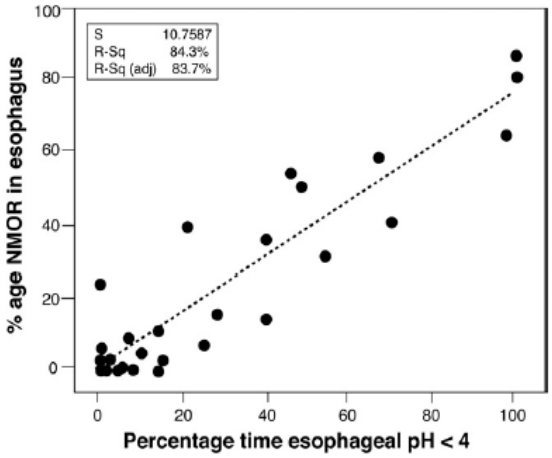

Figure 7. Fitted line plot comparing the percentage of time pH was less than 4, 5 cm above the gastroesophageal junction (marked by the clips at the top of the gastric folds), with the proportion of total *N*-nitrosomorpholine formed within the esophageal sections.

5.1% (interquartile range, 0%–24%) on the day when reflux was not stimulated ($P < .001$). In the healthy volunteers who received nitrate, the proportion of *N*-nitrosomorpholine present within the esophagus was 1.6% (interquartile range, 0%–5.1%), which was lower than in the Barrett's patients in whom reflux was not stimulated.

From 28 studies in 14 Barrett's patients, a linear relationship existed between the duration of acid reflux and the proportion of the total *N*-nitrosomorpholine that was formed within the esophageal sections (Spearman's $\rho = .75$; $P < .01$), with 80% being formed in the esophagus during reflux (Figure 7).

Twelve patients with Barrett's esophagus were administered nitrate that was 99% enriched with the heavy nitrogen isotope ^{15}N on each study day. The median enrichment was 74.6% (interquartile range, 59%–83.1%), confirming that the *N*-nitrosomorpholine formed was derived from the nitrate ingested.

Correlation of Silastic tube nitrite versus *N*-nitrosomorpholine. We calculated the ratio of *N*-nitrosomorpholine:nitrite concentrations for each Silastic tube section in all of the subjects with Barrett's esophagus. The median of all ratios of *N*-nitrosomorpholine:nitrite concentration was 0.053 (interquartile range, 0.026–0.089) for the gastric sections. This was significantly higher than 0.028 (range, 0–.06) for the esophageal sections ($P < .001$), but not significantly different from 0.064 (interquartile range, 0.045–0.099) for the gastric sections of the healthy volunteers who had received nitrate. This finding indicates that a greater proportion of the nitrite measured within the esophagus was generated via nitrous acid compared with the stomach. The median *N*-nitrosomorpholine concentration as a percentage of nitrite concentration by location is depicted in Figure 8.

Discussion

Our studies show a novel mechanism by which dietary nitrate may induce nitrosative stress and generate *N*-nitroso compounds within the upper gastrointestinal tract of subjects with healthy acid-secreting stomachs. This mechanism is via the generation of nitric oxide formed when nitrite in saliva encounters acidic gastric juice containing reducing agents such as ascorbic acid. The nitric oxide formed in this way can diffuse from the lumen into an adjacent compartment of neutral pH and, within it, combine with oxygen to form the nitrosating species N_2O_3.

The potential for generation of *N*-nitroso compounds within the lumen of the acidic stomach has been recognized for many years.[16,17] This is because of the large amounts of nitrite constantly delivered into the stomach in swallowed saliva.[21,42] The nitrite in saliva is derived from the enterosalivary recirculation of dietary nitrate and its reduction to nitrite by buccal bacteria.[19,21,22] When the nitrite encounters the acidic pH of gastric juice at the gastric esophageal junction, it is converted immediately to nitrous acid and nitrosating species such as NO+, NOSCN, and N_2O_3. The latter can react with secondary amines to form potentially carcinogenic *N*-nitros amines. The main factor protecting against this acid-catalyzed *N*-nitrosation is ascorbic acid,[29] which effectively competes with secondary amines for the nitrosating species. In this reaction between the nitrosating species and ascorbic acid, the former is reduced to nitric oxide and the latter is oxidized to dehydroascorbic acid. In this previously recognized pathway of acid-catalyzed nitrosation, the reaction between the nitrosating species and ascorbic acid has been regarded as entirely protective by preventing the generation of *N*-nitroso compounds within the acidic lumen. However, our current studies show that the nitric oxide formed from the reaction between ascorbic acid and acidified nitrite can induce nitrosative stress and the generation of *N*-nitroso compounds within an adjacent compartment of neutral pH.

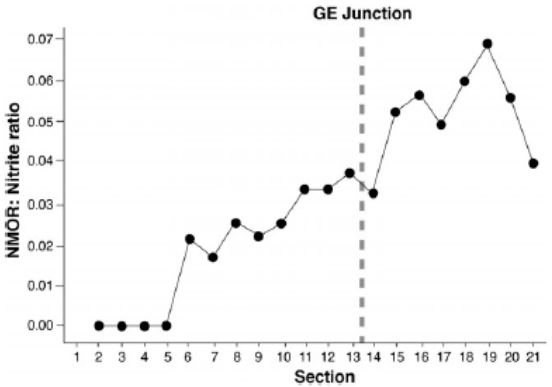

Figure 8. Median ratio of *N*-nitrosomorpholine:nitrite by location in the upper gastrointestinal tract in patients with Barrett's esophagus.

How do we know that the *N*-nitrosomorpholine generated in situ within the epithelial compartment was formed via nitric oxide and not by the direct reaction between morpholine and the nitrosating species formed by the acidifications of salivary nitrite? In our previously published bench-top studies,[35] we examined the generation of *N*-nitrosomorpholine within the Silastic tube when its exterior surface was exposed to the chemical conditions occurring within the lumen of the human proximal stomach. The amount of *N*-nitrosomorpholine formed inside the Silastic tube was directly proportional to the nitric oxide concentration generated in the luminal compartment to which the external surface of the tube was exposed. If the luminal compartment contained only nitrite and hydrochloric acid then the nitrosating species formed in this solution only generated N-nitrosomorpholine in the same luminal compartment and not in the epithelial compartment. However, the further addition of ascorbic acid to the luminal compartment prevented nitrosation in this compartment and produced high concentrations of nitric oxide in the luminal compartment and high concentrations of N-nitrosomorpholine in the epithelial compartment. This, therefore, indicates that the *N*-nitrosomorpholine generated within the Silastic tube is derived from nitric oxide entering the tube and not from acid-catalyzed nitrosating species entering the tube.

How do we know that the *N*-nitrosomorpholine generated within the tube was derived from the administered nitrate? The administration of nitrate increased the concentration of *N*-nitrosomorpholine formed in the Silastic tube in the healthy volunteers. Further confirmation of the origin of this *N*-nitrosomorpholine was obtained by administering ^{15}N nitrate to 8 of our healthy volunteers and 12 of our patients with Barrett's esophagus. In these subjects, we found that 75% of the *N*-nitrosomorpholine formed within the tube was enriched with ^{15}N. This shows that the administered nitrate had been absorbed from the gastrointestinal tract, taken up by the salivary glands and secreted into the mouth, reduced to nitrite by buccal bacteria, swallowed in saliva, converted to nitrous acid and nitrosating species when encountering acidic gastric pH, and converted to nitric oxide by ascorbic acid or other reducing agents within the stomach. The nitric oxide generated then was able to pass through the gas-permeable Silastic wall of the tube and, within that neutral pH environment, react with oxygen to form N_2O_3, which in turn reacted with the secondary amine to form *N*-nitrosomorpholine.

In addition to *N*-nitrosomorpholine, we also detected substantial amounts of nitrite within the Silastic tube exposed to gastric acidity. Our previous studies have indicated 2 mechanisms by which nitrite can accumulate within the Silastic tube exposed to conditions occurring within the lumen of the human upper gastrointestinal tract. The first and major mechanism is by nitric oxide formed from the reaction between nitrosating species and ascorbic acid, diffusing through the wall of the tube and then reacting with O_2 to form the nitrosating species N_2O_3. The latter then can react either with the secondary amines to form *N*-nitrosamines as described earlier or can react with water to form nitrite ($N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+$). The second mechanism by which nitrite may accumulate within the tube at acidic pH is owing to nitrous acid diffusing through the wall of the tube. Nitrous acid is a weak acid ($HNO_2 \leftrightarrow H^+ + NO_2^-$:pKa = 3.34) and at intragastric pH it exists predominantly as the relatively nonpolar molecule HNO_2, which is able to pass through the wall of the Silastic tube. Nitrous acid is a much larger molecule than nitric oxide and thus passes through the Silastic tube wall much more slowly. When the nitrous acid passes through the wall of the Silastic tube and encounters the solution of neutral pH within the Silastic tube, it immediately is converted to nitrite. Unlike nitric oxide, nitrous acid does not form N_2O_3 or other nitrosating species required for the formation of *N*-nitroso compounds within the neutral pH environment of the tube. The ratio of *N*-nitrosomorpholine to nitrite formed within the tube indicates the extent to which the nitrite added to the acidic luminal compartment has been converted to nitrous acid and nitrosating species vs nitric oxide. When ascorbic acid is present in abundance in the luminal compartment, causing all the added nitrite to be converted to nitric oxide, then the ratio is .1–.15. In contrast, when there is no ascorbic acid in the surrounding medium and any nitrite added is in the form of nitrous acid or nitrosating species, then the ratio is less than .01 because almost no *N*-nitrosomorpholine is formed.[35]

In the present study, the administration of nitrate to the healthy volunteers not only increased the production of *N*-nitrosomorpholine and nitrite within the tube, but also reduced the ratio of *N*-nitrosomorpholine to nitrite within the tube. This can be explained by the increased amount of nitrite in swallowed saliva not all being converted to nitric oxide in the proximal stomach but some remaining in the form of nitrous acid and nitrosating species. This will happen if the increased nitrite load overwhelms the gastric juice concentrations of ascorbic acid and its ability to reduce all the nitrous acid and nitrosating species to nitric oxide. This means that after the nitrate administration, there were nitrosating species within the lumen of the gastrointestinal tract and thus the potential for the generation of *N*-nitroso compounds by the previously well recognized acid-catalyzed luminal chemistry.

The design of our Silastic tube also enabled us to investigate the anatomic location of the nitrosative chemistry occurring throughout the upper gastrointestinal tract and its correlation with luminal pH. In the healthy volunteers with or without nitrate administration, no nitrite or *N*-nitrosomorpholine was formed in the segments

located in the esophagus and not exposed to gastric acid. Without nitrate administration, both nitrite and *N*-nitrosomorpholine were formed in the segments exposed to the acidic gastric environment and the concentrations of both were highest in the most proximal segments constantly exposed to intragastric acidity. After nitrate administration, the peak nitrite concentration again was evident in the proximal stomach, but the *N*-nitrosomorpholine formed more of the plateau. There was therefore a tendency for the ratio of nitrite to *N*-nitrosomorpholine to be lower in the proximal stomach after the administration of nitrate. This may be explained by the higher amounts of nitrite delivered into the proximal stomach after the nitrate, exhausting the ascorbic acid concentration in the most proximal stomach and allowing the nitrite to enter as nitrous acid rather than as nitric oxide. We previously observed that the ratio of nitrite to ascorbic acid is highest in the most proximal stomach.[31]

One of the healthy volunteers was found to be achlorhydric. In this subject, no nitrite and no *N*-nitrosomorpholine was detected in any of the tube segments. This highlights the key role of acid in forming nitrosating species within the lumen and subsequently in generating nitric oxide, which may form *N*-nitrosomorpholine within the Silastic tube. There is of course a third mechanism of *N*-nitrosation that may occur in the achlorhydric stomach. This is when the stomach becomes colonized by bacteria that can both reduce nitrate to nitrite and nitrite to nitrosating species and *N*-nitroso compounds. This mechanism that occurs in the achlorhydric stomach is thought to be relevant in patients who develop noncardia gastric cancer secondary to atrophic gastritis.[43,44]

We were able to investigate the effect of acidic gastroesophageal reflux on the location of the nitrosative chemistry. To do this, we studied patients with Barrett's esophagus because they usually have severe reflux disease. In these subjects, we found that nitrite and *N*-nitrosomorpholine were detected in the esophageal, as well as gastric, segments. In addition, the extent to which the chemistry was detected in the esophagus vs the stomach was related directly to the amount of reflux occurring while the probe was in situ. From this correlation, it is apparent that during reflux 80% of the nitrosating chemistry occurred within the esophageal segments. Normally, the nitrosative chemistry occurs as a result of the swallowed saliva encountering acid on entering the stomach and occurs predominantly within the proximal stomach. In contrast, during reflux, the chemistry occurs in the esophagus as a result of the acid entering the esophagus and reacting with the nitrite within the esophageal lumen. The impaired esophageal clearance characteristic of severe reflux disease is likely to contribute to the degree of nitrosative chemistry occurring within the esophagus.

We also were able to study the ratio of nitrite to nitrosomorpholine occurring within the esophageal segments vs the gastric segments. The ratio of nitrite to *N*-nitrosomorpholine observed in the stomach of the reflux patients after nitrate was similar to that observed in the healthy volunteers after nitrate. However, the ratio in the esophageal sections of the Silastic tube was significantly lower than in the stomach, indicating that in the esophagus a higher proportion of the luminal nitrite was in the form of nitrous acid and nitrosating species vs nitric oxide than in the stomach. A similar trend was observed in the proximal vs the distal stomach of the healthy volunteers after nitrate as discussed earlier. This indicates that during acid reflux, nitrate induces nitrosative stress within the esophagus by both the generation of luminal nitrosating species and via nitric oxide. Both mechanisms of nitrosative stress also are apparent in the proximal stomach after nitrate.

Currently available analytic methods do not allow the measurement of *N*-nitroso compounds within endoscopic epithelial biopsy specimens and we therefore studied their generation using an in situ compartment of neutral pH containing the *N*-nitrosatable compound morpholine. Our current studies do not allow an accurate prediction of the extent to which the luminal nitrosative chemistry is generating *N*-nitroso compounds within the actual epithelial cells. However, epithelial cells do contain an abundant and wide range of compounds (secondary amines, polyamines, amino acids, peptides, and amides) that can be *N*-nitrosated to mutagenic products.[45–50] Our use of morpholine within the Silastic tube provides a measure of nitric oxide–mediated nitrosative stress per se, and not just of its ability to form *N*-nitroso compounds. Nitric oxide–induced nitrosative stress also can induce DNA damage by other mechanisms. These include directly deaminating DNA and inactivating DNA repair enzymes such as O_6-alkylguanine DNA alkyl transferase[51–53] and DNA repair proteins such as formamidopyrimidine DNA-glycosylase, which repairs 8-oxodeoxyguanine residues.[54–56]

In summary, our studies indicate that nitric oxide generated by the reaction between salivary nitrite and acidic gastric juice may induce nitrosative stress and the generation of *N*-nitroso compounds within adjacent compartments of neutral pH such as the epithelial cells. Our studies also show that during acid reflux, this potentially mutagenic chemistry occurs predominantly or entirely within the lumen of the esophagus. This chemistry may be particularly harmful to patients with erosive esophagitis whose epithelium will be more sensitive to chemical mutagenesis.[57–59]

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Received December 22, 2006. Accepted April 12, 2007.

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Supported by a grant from the Chief Scientist's Office, Edinburgh, Scotland.