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*Gut* 2007;56;1678-1684; originally published online 4 Sep 2007;  
doi:10.1136/gut.2007.128587

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## GASTRIC CANCER

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

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*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).<sup>1–4</sup> The cancers at the GOJ usually occur in healthy acid-secreting stomachs.<sup>5, 6</sup> 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  $N_2O_3$ ,  $NO^+$ .<sup>7, 8</sup> The latter reacts with thiocyanate which is also present in gastric juice to form the particularly potent nitrosating species NOSCN.<sup>9–11</sup> 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.<sup>8, 12–16</sup> The nitrosating species  $N_2O_3$  is itself mutagenic as it can directly deaminate certain DNA bases and inactivate important DNA repair enzymes.<sup>17–21</sup> 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  $\mu$ M under fasting conditions) is derived from the enterosalivary recirculation of dietary nitrate and its reduction to nitrite by buccal bacteria.<sup>22–27</sup> 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.<sup>28</sup>

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.<sup>29–33</sup> Ascorbic acid is actively secreted in gastric juice and effectively competes with secondary amines and amides for reaction with the nitrosating species.<sup>32, 34–38</sup> In this reaction, the nitrosating species are reduced to nitric oxide and the ascorbic acid oxidised to dehydroascorbic acid.<sup>39</sup> 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.<sup>40</sup>

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.<sup>18, 41, 42</sup> 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.<sup>43, 44</sup> 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.<sup>41</sup> 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.<sup>41</sup> 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.<sup>28–40</sup> 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.<sup>45</sup> 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<sub>2</sub> 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  $\mu$ l) were added to deionised water (900  $\mu$ l). An aliquot of each diluted sample (500  $\mu$ l) was added to MS solution (500  $\mu$ l, 2% metaphosphoric acid/0.5% sulphamic acid, 1:1 v/v), while the remainder (500  $\mu$ l) was added to dithiothreitol/MS solution (500  $\mu$ l, 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<sup>46</sup> based upon the method described by Sanderson and Schorah.<sup>47</sup>

### 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).

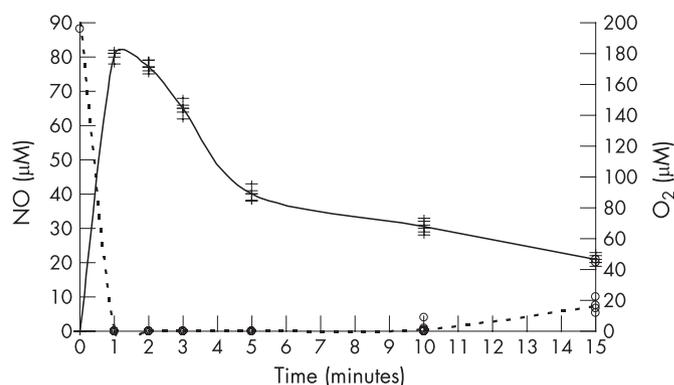
### Statistical analysis

Results are presented as mean values  $\pm$  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 ( $\mu$ M) in single and dual-phase experiments 15 min after addition of nitrite (100  $\mu$ M) 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
<b>Aqueous phase</b>				
NDMA	0.02 $\pm$ 0.01*	0.004 $\pm$ 0.003*	0.07 $\pm$ 0.02*	1.19 $\pm$ 0.13**
NDEA	0.001 $\pm$ <0.001*	ND	0.01 $\pm$ 0.003*	0.56 $\pm$ 0.07**
NMOR	3.43 $\pm$ 0.12*	0.003 $\pm$ 0.003**	3.37 $\pm$ 0.07*	0.75 $\pm$ 0.1***
NPIP	0.002 $\pm$ 0.001*	ND	0.04 $\pm$ 0.002*	2.78 $\pm$ 0.92**
<b>Lipid phase</b>				
NDMA			1.16 $\pm$ 0.27*	4.77 $\pm$ 0.4**
NDEA			ND	1.18 $\pm$ 0.55**
NMOR			ND	4.9 $\pm$ 0.41**
NPIP			0.06 $\pm$ 0.01*	29.3 $\pm$ 7.09**

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

**Figure 1** Nitric oxide (continuous line) and oxygen (dashed line) concentrations in the aqueous phase after the addition of nitrite (100  $\mu$ M) 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

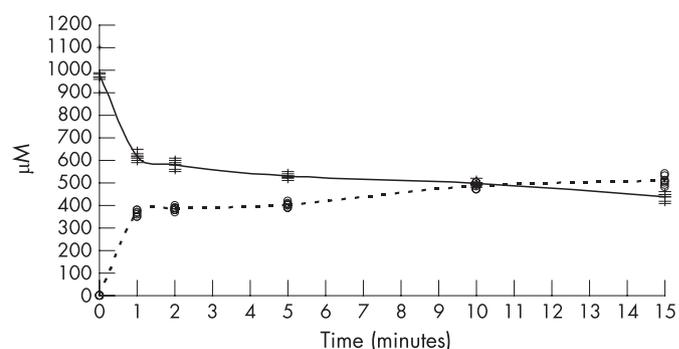
When nitrite was added to HCl pH 1.5 containing thiocyanate (1 mM) without ascorbic acid, nitrosation of the secondary amines was observed. After 15 min,  $3.43 \pm 0.12$   $\mu$ M NMOR was preferentially formed, while only  $0.02 \pm 0.007$   $\mu$ M NDMA,  $0.001 \pm 0.000$   $\mu$ M and  $0.002 \pm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

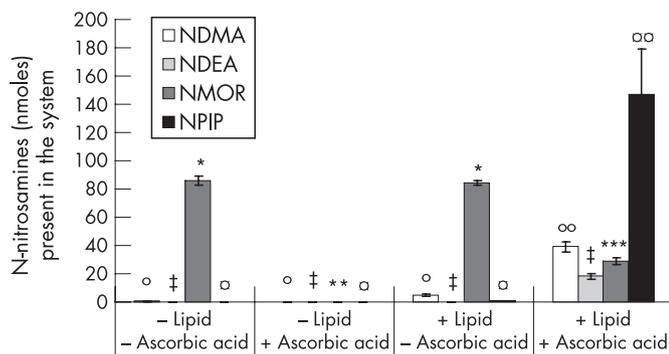
In these experiments, the addition of nitrite produced only a very low concentration of nitric oxide (approximately 3.5  $\mu$ M) and no discernible fall in oxygen concentration.

### 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  $\mu$ M), the dissolved nitric oxide concentration was approximately 80  $\mu$ M (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  $\mu$ M) triggered a fall in the ascorbic acid concentration, from its original concentration of  $970 \pm 75$   $\mu$ M to  $580 \pm 40$   $\mu$ M at 2 min, and then it slowly fell to  $480 \pm 40$   $\mu$ M 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  $\mu$ M) 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  $\mu$ M) (n = 12).

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

#### In absence of ascorbic acid

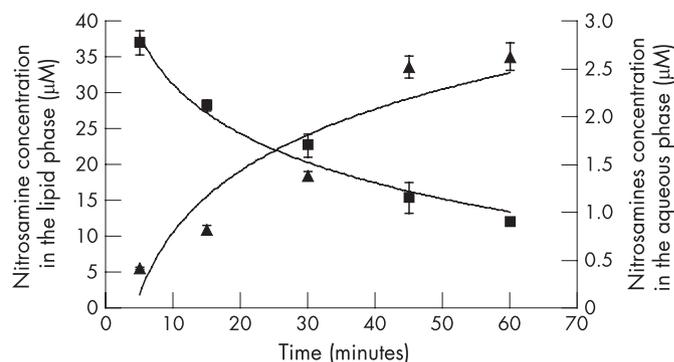
When nitrite was added to HCl pH 1.5 containing thiocyanate (1 mM), no ascorbic acid and 10% lipid, nitrosation of the secondary amines was observed. The concentrations of *N*-nitrosamines formed in the aqueous phase were similar to those observed in the absence of lipid, with NMOR preferentially formed ( $3.37 \pm 0.07 \mu$ M) (table 2). Only NDMA ( $1.16 \pm 0.27 \mu$ M) and NPIP ( $0.06 \pm 0.01 \mu$ M) 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,  $1.19 \pm 0.13 \mu$ M NDMA,  $0.56 \pm 0.07 \mu$ M NDEA,  $0.74 \pm 0.1 \mu$ M NMOR and  $2.78 \pm 0.92 \mu$ M NPIP were detected. In the lipid phase,  $4.77 \pm 0.4 \mu$ M NDMA,  $1.18 \pm 0.55 \mu$ M NDEA,  $4.90 \pm 0.41 \mu$ M NMOR and  $29.3 \pm 7.01 \mu$ M 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  $4.7 \pm 0.77$  nmol to  $39.19 \pm 3.49$  nmol; NDEA by approximately 60-fold, from  $0.3 \pm 0.08$  nmol to  $18.13 \pm 1.98$  nmol, and NPIP by approximately 140-fold, from  $1.05 \pm 0.07$  nmol to  $147.35 \pm 31.59$  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  $84.21 \pm 1.78$  nmol to  $28.78 \pm 2.59$  nmol (fig 3), levels that were nonetheless significantly higher than in the presence of ascorbic acid and absence of



**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).

lipid ( $0.08 \pm 0.08$  nmol) ( $p < 0.001$ ). In addition, ascorbic acid increased the concentration of NMOR formed in the lipid from undetectable to  $4.9 \pm 0.4 \mu$ M ( $p < 0.001$ ).

#### 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 tributryrate and glycerol triacetate, two high-density lipids). While glycerol trioctanoate provided a physiologically accurate representation of a floating lipid layer, glycerol tributryrate 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  $\mu$ M 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  $\mu$ M down to approximately 12  $\mu$ M, while the total *N*-nitrosamine concentration in the aqueous phase rose to approximately 2.6  $\mu$ M (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 NOSC. NOSC reacts with the secondary amine in its unprotonated uncharged state.<sup>48</sup> 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 <i>N</i> -nitrosamine	Concentration ( $\mu\text{M}$ )				
	5 min	15 min	30 min	45 min	60 min
Aqueous phase					
NDMA	0.15 $\pm$ 0.01	0.29 $\pm$ 0.06	0.40 $\pm$ 0.04	0.48 $\pm$ 0.07	0.54 $\pm$ 0.01
NDEA	0.08 $\pm$ 0.01	0.18 $\pm$ 0.02	0.3 $\pm$ 0.01	0.49 $\pm$ 0.15	0.64 $\pm$ 0.02
NMOR	0.09 $\pm$ 0.02	0.25 $\pm$ 0.07	0.33 $\pm$ 0.03	0.89 $\pm$ 0.28	0.70 $\pm$ 0.01
NPIP	0.09 $\pm$ 0.01	0.09 $\pm$ 0.03	0.34 $\pm$ 0.03	0.68 $\pm$ 0.08	0.75 $\pm$ 0.1
Lipid phase					
NDMA	6.64 $\pm$ 0.40	4.03 $\pm$ 0.25	1.97 $\pm$ 0.24	0.86 $\pm$ 0.24	0.39 $\pm$ 0.01
NDEA	6.29 $\pm$ 0.48	5.11 $\pm$ 0.67	5.86 $\pm$ 1.74	4.61 $\pm$ 1.51	2.98 $\pm$ 0.67
NMOR	10.62 $\pm$ 0.61	8.75 $\pm$ 0.51	5.86 $\pm$ 0.24	3.71 $\pm$ 0.12	2.96 $\pm$ 0.20
NPIP	13.34 $\pm$ 0.73	9.75 $\pm$ 0.9	8.95 $\pm$ 0.57	6.49 $\pm$ 0.4	5.69 $\pm$ 0.20

10  $\mu\text{M}$  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  $\pm$  SE.

dissociation constant ( $\text{pK}_a$ ). The  $\text{pK}_a$  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 NOSCEN will therefore be greatest for morpholine and least for piperidine. The differences in  $\text{pK}_a$  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 NOSCEN.<sup>36–38, 49</sup> In the reaction between ascorbic acid and NOSCEN, 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  $\text{N}_2\text{O}_3$ .<sup>43, 44</sup> The  $\text{N}_2\text{O}_3$  formed in this way is a nitrosating species and again preferentially reacts with ascorbic acid and is reduced back to nitric oxide.<sup>39</sup> 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. Stoichiometrically, 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.

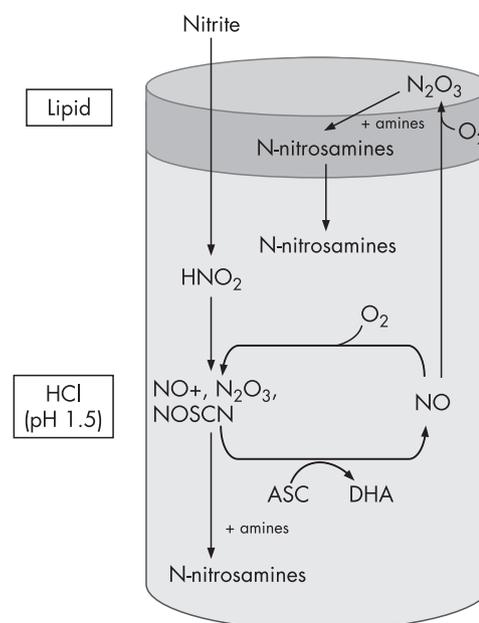
In the presence of the lipid, *N*-nitrosamines were formed despite the presence of ascorbic acid. Indeed, the presence of lipid transformed the effect of the ascorbic acid from effectively inhibiting nitrosation of each amine to powerfully enhancing nitrosation of three of the four amines. (The overall 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  $4.9 \pm 0.4 \mu\text{M}$ .

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  $\text{N}_2\text{O}_3$ .<sup>50</sup> 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.<sup>41</sup> As ascorbic acid is not lipophilic, it is unable to enter the lipid and thus the  $\text{N}_2\text{O}_3$  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.<sup>51</sup>

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  $\text{pK}_a$  and thus higher proportion in the nitrosatable unprotonated form.<sup>52</sup> 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  $\text{N}_2\text{O}_3$ . Ascorbic acid is reduced to dehydroascorbic acid. Secondary amines present within the lipid are nitrosated by  $\text{N}_2\text{O}_3$  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<sub>a</sub> 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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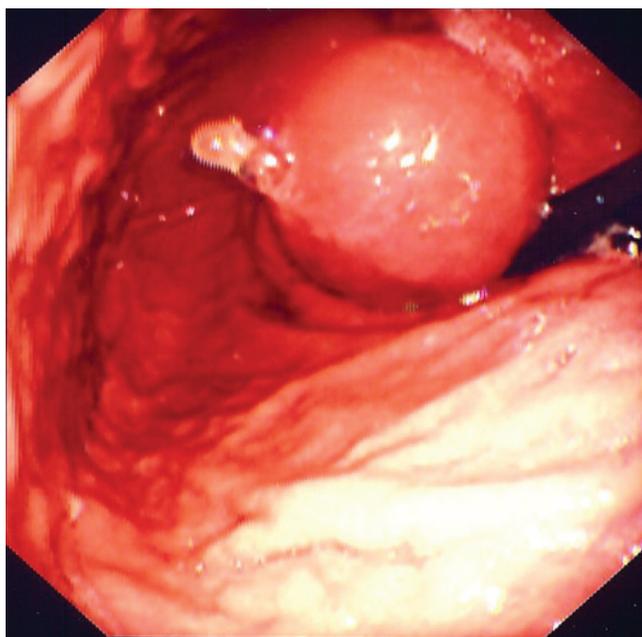
## EDITOR'S QUIZ: GI SNAPSHOT .....

### Haematemesis with mediastinal lymphadenopathy

Robin Spiller, editor

#### Clinical presentation

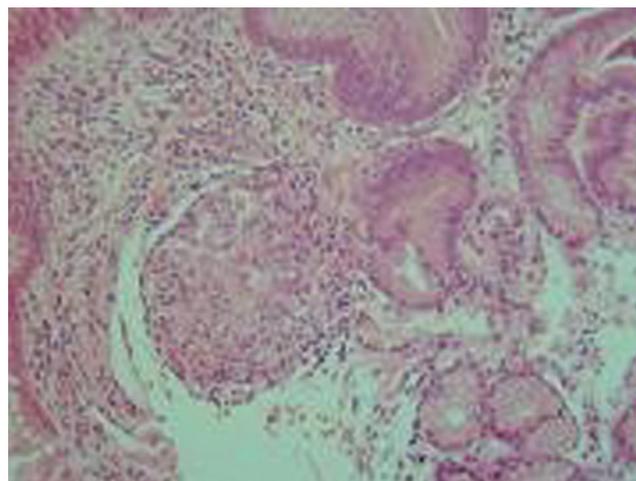
A 33-year-old previously well man presented with sudden haematemesis and mild epigastric tenderness. At endoscopy, bleeding from a "ragged" lesion at the gastro-oesophageal junction was noted and biopsy samples were taken. Follow-up gastroscopy showed a 3 cm submucosal mass (fig 1). A



**Figure 1** Endoscopic appearance of gastro-oesophageal mass.



**Figure 2** CT appearance of gastro-oesophageal mass.



**Figure 3** Microscopic appearance of biopsy samples from the gastro-oesophageal junction.

computed tomography (CT) scan confirmed the presence of a soft tissue mass arising from the gastro-oesophageal junction extending into the gastric lumen and also showed hilar and mediastinal lymphadenopathy and bilateral, predominantly mid-zone, nodular shadowing (fig 2).

The patient later developed epigastric discomfort, nausea, exertional dyspnoea and cervical lymphadenopathy. Figure 3 shows the histopathology of the specimen taken from the gastro-oesophageal junction.

#### Questions

What was the cause of this patient's gastrointestinal bleed?

What is the diagnosis?

*See page 1695 for answers*

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doi: 10.1136/gut.2006.108472