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N-Nitroso compounds in fresh gastric juice and their relation to intragastric pH and nitrite employing an improved analytical method

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In order to examine further the relationship between intragastric *N*-nitrosation, gastric pH and nitrite, 457 fresh, fasting gastric juice samples were analysed for total *N*-nitroso compounds (NOC) and nitrite concentrations using a recently described improved assay method. Nitrite in log values was linearly related to intragastric pH ($r = 0.887$, $P < 0.01$) with a regression equation log[nitrite] (μmol/l) = $0.489 \times$ pH − 2.209. Significantly higher NOC concentrations were found at intragastric pH ranges of 1.13–2.99 (mean ± SE: 1.45 ± 0.17 μmol/l, $P < 0.05$) and 6.00–8.42 (3.57 ± 0.33 μmol/l, $P < 0.01$) compared with that at pH 3.00–5.99 (1.02 ± 0.12 μmol/l). NOC concentration was significantly related to log nitrite concentration at both the low pH range 1.13–4.99 ($r = 0.169$, $P < 0.01$) and the high pH range 5.00–8.42 ($r = 0.450$, $P < 0.01$). The results in the present study confirm that both acid-catalysed *N*-nitrosation and biologically-catalysed *N*-nitrosation occur in the human stomach. However, great variations in nitrite and NOC concentrations were observed in both low and high pH samples, indicating that, as expected, both the acid-catalysed *N*-nitrosation and biologically-catalysed *N*-nitrosation processes are markedly affected by factors other than intragastric pH and nitrite.

Introduction

In 1975, Correa *et al.* (1) proposed an *N*-nitrosamine hypothesis for human gastric carcinogenesis, postulating that high intragastric pH in hypochlorhydria promotes the growth of a mixed bacterial flora able to reduce dietary nitrate to nitrite and then to catalyse carcinogenic *N*-nitroso compound (NOC*) formation in the stomach. However, another hypothesis, proposed later by Mirvish, emphasised that the formation of carcinogenic NOC in an acidic intragastric environment in high-risk populations begins in early life and that these compounds possibly induce a series of changes in the gastric mucosa leading eventually to gastric cancer (2). The intragastric formation of NOC in man is now well established (3), but published results are contradictory about the relationship between intragastric *N*-nitrosation and intragastric pH, nitrite and bacterial flora. Experimental studies show that *N*-nitrosation can occur at both acidic and neutral conditions, i.e. acid-catalysed *N*-nitrosation (ACN) and biologically-catalysed *N*-nitrosation (BCN) (4–6). By applying two widely-used methods in human studies (7,8), both mechanisms have been demonstrated, but their relative importance has been only partially resolved; ACN appeared to be more important by Ohshima's *N*-nitrosoproline test (9,10), whilst BCN was the major pathway when Walters' group determination procedure was applied to gastric juice samples infected with micro-organisms (11). Various factors, including methodology and study design, have been suggested to explain the above conflicting results (12,13).

Using concentrations of volatile *N*-nitrosamines in gastric juice and nitrosoproline excreted in urine in a well-controlled study, we observed the occurrence of both ACN and BCN in a high-risk population for gastric cancer in China (13). Recently we have developed an improved assay method based on previous procedures for measuring total NOC, as well as nitrite, in fresh gastric juice (14). Applying this method we have observed that some *N*-nitroso derivatives in gastric juice are unstable and that the sulphamic acid usually used as a stabiliser can often dramatically reduce total NOC concentrations in fresh gastric juice. The preliminary results from an analysis of 212 fresh gastric juice samples confirmed the occurrence of both ACN and BCN in the human stomach (15). The aim of the present study was to examine further the relationship between intragastric *N*-nitrosation, gastric pH and nitrite on a larger sample base by applying the improved method.

Materials and methods

Subjects and collection of gastric juice

Gastric juice samples were obtained randomly at endoscopy from patients with various gastrointestinal conditions, including chronic superficial gastritis, chronic atrophic gastritis, gastric ulcer, gastric carcinoma, pernicious anaemia, oesophagitis and duodenal ulcer, as well as from those with normal gastric histology and patients who had undergone vagotomy or partial gastrectomy. Samples were collected through sterile polythene tubing after an overnight fast of 12 h. Immediately after collection the samples were homogenized using a magnetic stirrer (HI 200, Hanna Instruments) and the pH measured by a glass electrode (Whatman PAH 230). Sulphamic acid (20 mg/ml juice) was added to aliquots of gastric juice samples to scavenge nitrite for measuring thermo- and acid-labile TEA-responsive compounds (TAC) and total NOC concentrations in the gastric juice with added sulphamic acid (SNOC). For samples with pH > 6.0, aliquots (1.0 ml) of the samples were diluted with cold water (2°C, pH 7.4 and 11.0). All samples were kept at 2°C and the analyses completed within 30 min after collection (14).

Analysis of total NOC, SNOC and nitrite in fresh gastric juice

The method for analysis of fresh gastric juice has been reported in detail elsewhere (14). Briefly, two working solutions were first prepared in a refluxing system coupled with a thermal energy analyser (TEA): the HBr solution (HBr mode) contained 120 ml ethyl acetate, 9.5 ml acetic acid, 0.5 ml 35.4% mass/mass HCl and 5 ml 45% mass/vol HBr in glacial acetic acid; the HCl solution (HCl mode) contained 120 ml ethyl acetate, 14.5 ml acetic acid and 0.5 ml 35.4% mass/mass HCl. Immediately after collection at endoscopy, aliquots (0.1 ml) of homogenized fresh samples with or without added sulphamic acid were injected directly into the refluxing ethyl acetate containing HBr/HCl in acetic acid (HBr mode) for determination of TAC + NOC (a) and TAC + NOC + nitrite (b). Aliquots (0.1 ml) of the same samples with or without added sulphamic acid were directly injected into the refluxing ethyl acetate containing 4% (vol/vol) HCl in acetic acid (HCl mode) for determination of TAC (c) and TAC + nitrite (d). The differences b − d, a − c and d − c represent the concentrations of total NOC, SNOC and nitrite, respectively, in the individual samples. A thermal energy analyser (TEA 502, Thermo Electron Corp., USA) was used as the NO chemiluminescence detector, connected to an integrator (HP 3392A, Hewlett Packard Co., USA).

*Abbreviations: ACN, acid-catalysed *N*-nitrosation; BCN, biologically-catalysed *N*-nitrosation; NOC, *N*-nitroso compounds; RSD, relative standard deviation; SNOC, total NOC in gastric juice with added sulphamic acid; TAC, TEA-responsive compounds; TEA, thermal energy analyser.

Table I. Analysis of 457 fresh gastric juice samples for nitrite and *N*-nitroso compounds (NOC) using an improved method

pH	*n*	Nitrite (μmol/l)		NOC (μmol/l)	
		Mean ± SE	Range	Mean ± SE	Range
1.13–	176	0.12 ± 0.02	ND–1.42	1.38 ± 0.21	ND–23.56
2.00–	66	0.17 ± 0.03	ND–1.14	1.63 ± 0.30	ND–11.58
3.00–	26	0.33 ± 0.06	ND–1.13	0.99 ± 0.21	ND–4.61
4.00–	18	2.55 ± 0.53	ND–6.32	0.92 ± 0.18	ND–2.75
5.00–	17	8.75 ± 4.68	0.07–82.10	1.18 ± 0.23	ND–3.43
6.00–	39	25.64 ± 5.78	1.19–190.25	2.85 ± 0.39	ND–10.15
7.00–	100	53.59 ± 8.50	1.78–651.20	3.80 ± 0.46	ND–28.26
8.00–8.42	15	48.49 ± 7.53	12.81–113.26	3.89 ± 1.16	ND–16.07

ND, not detected.
Student's *t*-test: nitrite, all comparisons between any two pH categories, $P < 0.05$ (2.00– versus 3.00–) or $P < 0.01$, except for 1.13– versus 2.00–, 4.00– versus 5.00– and 7.00– versus 8.00–, $P > 0.05$; NOC, comparisons between different pH ranges, 1.13–2.99 (mean ± SE, 1.45 ± 0.17) versus 3.00–5.99 (1.02 ± 0.12), $P < 0.05$; 1.13–2.99 versus 6.00–8.42 (3.57 ± 0.33), $P < 0.01$; 3.00–5.99 versus 6.00–8.52, $P < 0.01$.

To test for reproducibility of the method, the relative standard deviation (RSD) for duplicate TEA measurements for analysis of fresh gastric juice was carried out and found to be 1–6%. All samples were analysed in duplicate. The mean recovery of spiked standard *N*-nitrosoproline was 98.7 ± 4.9%. The limit of detection was 1.0 pmol.

Statistics

Statistical analysis was performed using Student's *t*-test, the χ^2 test and linear correlation employing the analysis tools in Microsoft Excel version 4.0a.

Results

Relationship between nitrite and intragastric pH

Mean nitrite concentrations were calculated for each 1-unit pH category, as shown in Table I. Mean nitrite concentrations were very low in samples of low pH range 1.13–4.99, but increased significantly from 0.12 to 2.55 μmol/l ($P < 0.01$) as the pH rose. When the intragastric pH exceeded 5.0, mean nitrite concentrations increased markedly to 8.75–53.59 μmol/l ($P < 0.01$). A linear correlation between log nitrite concentration versus intragastric pH was established ($r = 0.887$, $P < 0.01$), with a regression equation, log[nitrite] (μmol/l) = 0.489 × pH − 2.209 ($P < 0.01$). Significant relationships between log nitrite concentration and intragastric pH were found over both a low pH range 1.13–4.99 ($r = 0.434$, $P < 0.01$) and a high pH range 5.00–8.42 ($r = 0.584$, $P < 0.01$) (Figure 1).

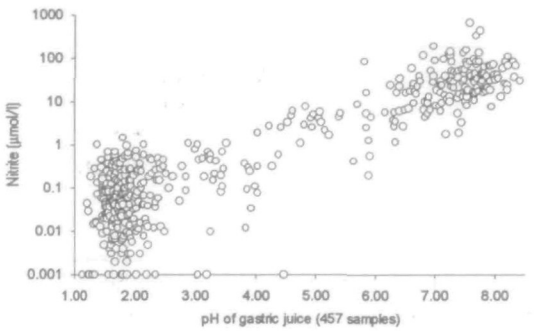

Fig. 1. Relationship between nitrite and intragastric pH in fresh gastric juice samples. Correlation coefficients between nitrite (log values) and pH: for all samples, $r = 0.887$, $P < 0.01$; at pH 1.13–4.99 ($n = 268$), $r = 0.434$, $P < 0.01$; at pH 5.00–8.42 ($n = 171$), $r = 0.584$, $P < 0.01$. Nitrite values for samples with undetectable nitrite levels were put on the scale as 0.001 μmol/l. Regression equation, log[nitrite] (μmol/l) = 0.489 × pH − 2.209, $P < 0.01$.

Relationship between NOC and intragastric pH

Mean NOC concentrations calculated for each 1-unit pH category are shown in Table I and those for each individual sample in Figure 2. A significant relationship between NOC and intragastric pH was found in the high pH range 5.00–8.42 ($r = 0.218$, $P < 0.01$). However, in the low pH range 1.13–4.99 there was no significant relationship between NOC and intragastric pH ($r = -0.067$, $P > 0.05$). Increased NOC concentrations were observed in samples at both low and high pH ranges. Mean NOC concentrations in gastric juice samples at pH 1.13–2.99 (mean ± SE, 1.45 ± 0.17) and 6.00–8.42 (3.57 ± 0.33) were significantly higher than those at pH 3.00–5.99 (1.02 ± 0.12, $P < 0.05$ and $P < 0.01$), and those at pH 6.00–8.42 were also significantly higher than the NOC levels at pH 1.13–2.99 ($P < 0.01$).

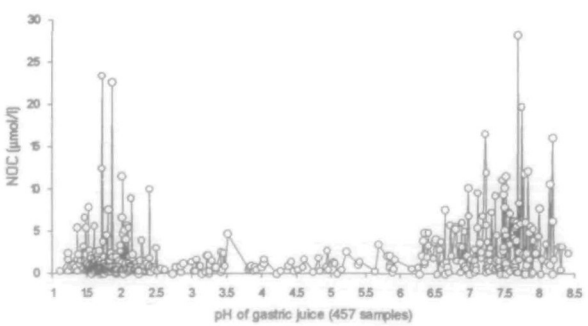

Fig. 2. Relationship between NOC and intragastric pH in fresh gastric juice samples. Correlation coefficients: for samples at pH 1.13–4.99 ($n = 286$), $r = -0.067$, $P > 0.05$; at pH 5.00–8.42 ($n = 171$), $r = 0.218$, $P < 0.01$.

Relationship between NOC and nitrite

NOC was significantly related to log nitrite concentration at both low pH range 1.13–4.99 ($r = 0.169$, $P < 0.01$) and high pH range 5.00–8.42 ($r = 0.450$, $P < 0.01$) (Figure 3).

Individual variations of nitrite and NOC concentrations

There were great individual variations in both nitrite and NOC concentrations (Figures 1 and 2). Relatively smaller variations in nitrite concentrations were seen at a low pH 1.13–2.99 (ND–1.42 μmol/l), with greater variations at pH 6.00–8.42 (1.19–651.20 μmol/l). NOC concentrations varied greatly at both low pH range 1.13–2.99 (ND–23.56 μmol/l) and high pH range 6.00–8.42 (ND–28.26 μmol/l). The percentages of samples with NOC concentrations >1.0 and >3.0 μmol/l for each 1-unit

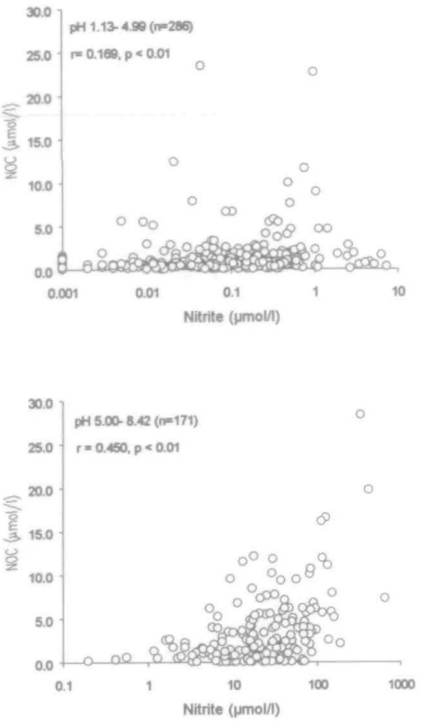

Fig. 3. Relationship between NOC and nitrite in 457 fresh gastric juice samples. Nitrite values for samples with undetectable nitrite levels were put on the scale as 0.001 µmol/l.

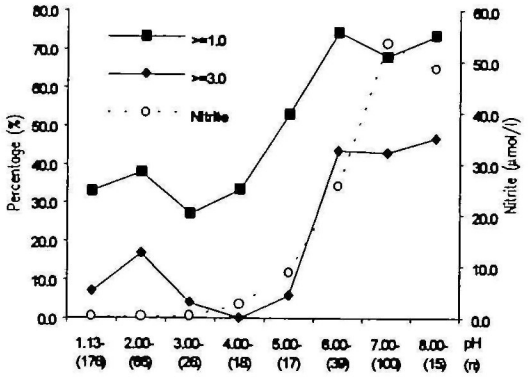

Fig. 4. Percentage of samples with NOC concentration >1.0 and >3.0 (µmol/l) and their relation to nitrite and intragastric pH. Comparison of percentage of samples with NOC concentrations >3.0 µmol/l between pH ranges 1.13−1.99 (12/176) (a), 2.00−2.99 (11/66) (b), 3.00−5.99 (2/61) (c) and 6.00−8.42 (67/154) (d): a versus b, χ^2 =5.413, $P < 0.05$; a versus d, χ^2 = 60.716, $P < 0.01$; b versus c, χ^2 = 6.184, $P < 0.05$; b versus d, χ^2 = 14.543, $P < 0.01$; c versus d, χ^2 = 32.444, $P < 0.01$. Comparison of percentage of samples with NOC concentrations >1.0 µmol/l between pH ranges 1.13−4.99 (106/303) and 6.00−8.42 (108/154), χ^2 = 31.985, $P < 0.01$. Correlation coefficients: between percentage of samples with NOC concentration >3.0 µmol/l and mean nitrite concentration at pH 4.00−8.42, r = 0.895, $P < 0.05$; between percentage of samples with NOC concentration >1.0 µmol/l and mean nitrite concentration at pH 3.00−8.42, r = 0.838, $P < 0.05$.

pH category were also calculated (Figure 4). A significantly higher percentage of samples with a NOC concentration >3.0 µmol/l was observed at pH 2.00−2.99 (16.7%) than at pH 1.13−1.99 (6.8%, $P < 0.05$) or at pH 3.00−5.99 (3.3%, $P < 0.01$). However, when the intragastric pH exceeded 6.0 and nitrite concentrations increased to 25.64−53.59 µmol/l the percentages of samples with a NOC concentration >3.0 µmol/l rose to 43.0−46.7% ($P < 0.01$) and were significantly higher than at pH 2.00−2.99 ($P < 0.01$). The percentage of samples with NOC concentration >1.0 µmol/l at pH 6.00−8.42 (70.1%) was significantly higher than at pH 1.13−4.99 (35.0%, $P < 0.01$). There were significant correlations between the percentage of samples with NOC concentration >3.0 µmol/l and mean nitrite concentration at pH 4.00−8.42 (r = 0.895, $P < 0.05$) and between the percentage of samples with NOC concentration >1.0 µmol/l and mean nitrite concentration at pH 3.00−8.42 (r = 0.838, $P < 0.05$).

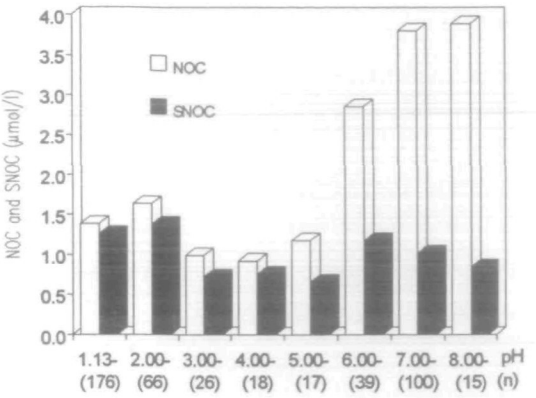

Fig. 5. Effect of the addition of sulphamic acid (2%) on NOC concentration in fresh gastric juice samples. Student's t-test: SNOC between different pH ranges: 1.13−2.99 (mean ± SE, 1.29 ± 0.16) versus 3.00−5.99 (0.71 ± 0.08), $P < 0.01$; 3.00−5.99 versus 6.00−7.99 (1.06 ± 0.16), $P < 0.05$. NOC and SNOC (paired t-test): all comparisons between each pH category, $P < 0.02$ (3.00−, 5.00−) or $P < 0.01$ except for 4.00−, $P > 0.05$.

Effect of sulphamic acid on NOC concentration

Mean NOC concentration in fresh gastric juice was reduced significantly by the addition of 2% sulphamic acid from 2.11 ± 0.15 to 1.13 ± 0.10 µmol/l (SNOC, $P < 0.01$) (Figure 5). Although mean NOC concentrations were significantly reduced at all pH levels ($P < 0.02$ or $P < 0.01$) except at 4.00−4.99 ($P > 0.05$), a greater reduction was seen at the high pH range 6.00−8.42. Significantly higher SNOC concentrations were observed in gastric juice samples of low pH 1.13−2.99 (1.29 ± 0.09, $P < 0.01$) and high pH 6.00−7.99 (1.06 ± 0.16, $P < 0.05$) compared with pH 3.00−5.99 (0.71 ± 0.08).

TAC concentration in fresh gastric juice

Mean TAC concentrations in samples for each 1-unit pH increment ranged from 0.34 ± 0.05 to 0.69 ± 0.20 µmol/l, but varied between ND and 6.03 µmol/l (data not shown). A significant difference was found only between pH levels 1.13−1.99 (0.34 ± 0.05 µmol/l) and 2.00−2.99 (0.60 ± 0.10 µmol/l, $P < 0.05$). Those at pH 3.00−7.99 were essentially unchanged (0.45−0.57). No relationship was found between TAC and nitrite concentrations (data not shown). During the study we had only two fasting samples (pH 2.76 and 2.42) contaminated with residual food, which had very high TAC concentrations of 12.14 and 24.79 µmol/l, respectively, with corresponding concentrations of nitrite of 0.81 and 12.65 µmol/l and of NOC of 5.56 and 4.18 µmol/l. These results were not included in the database of the present paper.

Discussion

This study confirms the now well-established findings from previous studies (16) that the nitrite concentration in gastric juice

is related closely to intragastric pH and that at a low pH range (<5.0) concentrations are extremely low (Table I, Figure 1). This finding can be explained by two major factors, nitrite source and nitrite stability. The normal acidic stomach is essentially sterile and any intragastric nitrite found is largely of salivary origin. It also has a very short half-life (<10 min), because of its high reactivity and physiological absorption. When gastric acid secretion is impaired by various causes and the resting gastric pH rises consistently above 4, then overgrowth with nitrate-reducing micro-organisms occurs, leading to a substantial increase in intragastric nitrite concentration (16). The pH optimum for bacterial nitrate reductase activity is between 7 and 8 (26). Nitrite is also much more stable in a neutral milieu, as it is predominantly in the ionised form (27).

The present study reveals quite a complicated picture of intragastric *N*-nitrosation. Significantly higher NOC concentrations were observed at both low pH range (1.13−2.99) and high pH range (6.00−8.42) compared to that at pH range 3.00−5.99. Although at the low pH range NOC was not directly related to intragastric pH, it was significantly related to nitrite. The results demonstrate a close similarity between *N*-nitrosation occurring in the acidic stomach and ACN observed *in vitro*. Since *in vitro* kinetic studies show that the reaction rate for nitrosation of amides (including peptides, guanidines, ureas and carbamates) increases about 10-fold for each 1-unit drop in pH from 3 to 1 (2), the results in the present study and those in our earlier study (15) clearly indicate that not only does ACN occur in the human stomach, but probably also that labile *N*-nitrosamides can be formed by this process, though the exact properties of the NOC revealed in this study are unknown. The intragastric ACN level is probably determined by gastric pH, nitrite and nitrosatable precursors available in the stomach and markedly affected by catalysts and inhibitors (2,13). However, nitrite may be the first limiting substrate, because the total level of nitrosatable compounds in gastric juice is in large excess (17). Consequently, the ACN process is likely to be of greater significance in populations with a high nitrate intake, as in high-risk populations for gastric and oesophageal cancers (3,13).

The present study has shown that at a pH > 5.0 intragastric *N*-nitrosation is closely related to intragastric pH and nitrite, confirming the results of our earlier study (11). It has been demonstrated that colonisation with bacterial flora with strong nitrate-reducing activity is responsible for the dramatically increased nitrite concentrations in the achlorhydric stomach (5). The highest NOC concentration observed, at pH 6.0−8.4, was in close agreement with BCN *in vitro* by isolated microorganisms from gastric juice (5,18,19) and *in vivo* (6). Therefore, this study shows that NOC formation in an achlorhydric stomach depends primarily on the activity of intragastric bacterial flora.

The present study confirms that there are pronounced inter-individual variations in intragastric nitrite and NOC concentrations under both acidic and achlorhydric conditions. They are probably contributed to mainly by: (i) the microbial species composition and their metabolic activity in the mouth and stomach (5); (ii) exposures to nitrite, nitrate, nitrosatable compounds and *N*-nitrosation catalysts, such as thiocyanate, as well as inhibitors, such as vitamins C and E and phenols; (iii) other physical conditions. The existence of such great variations highlights two important concepts. First, that intragastric nitrosation is influenced by many factors and that marked intragastric ACN or BCN is not invariable, even when some favourable conditions exist, such as a high nitrite level, low pH or a stomach colonised with appropriate bacterial flora with strong nitrate-reducing activity. This may in part explain why only a small number of people in high-risk areas or with achlorhydria eventually develop gastric cancer. Also, in the field/laboratory study of the aetiological relationship between intragastric *N*-nitrosation and gastric cancer and other human cancers, a sufficiently large sample size, as well as condition control and a reliable quantification method, are needed. In the current study, we used a more reliable analytical method and studied a much larger sample size.

As reported recently (15), sulphamic acid has been shown to reduce total NOC concentration in fresh gastric juice significantly. It has been suggested that those NOC susceptible to sulphamic acid are labile *N*-nitrosamides (15). Even so, we still observed significantly higher NOC formation peaks at both low and high intragastric pH ranges by using the improved method in the present study. In an earlier study, we only observed elevated *N*-nitrosation levels at a high pH range (11). The difference is probably due to: (i) decomposition of unstable NOC during storage of samples with added sulphamic acid and work-up procedure (12,15); (ii) losses of non-extractable NOC, as shown in the previous study and as suggested by Pignatelli *et al.* (17). Much higher TAC concentrations in two fresh samples containing residual food might suggest that a major part of total NOC concentration is contributed by TAC if it is not separated from total NOC determination for such samples in the assay procedure (20), though the sample number was very limited in this study.

From our study we cannot tell whether any of these unidentified NOC are carcinogenic, as the method used here assays total NOC as a group. If intragastric *N*-nitrosation is aetiologically related to gastric cancer, an explanation is required as to why some subjects with a low gastric pH (<2.5) from this low-risk population had markedly higher NOC levels (Figure 2). It has been shown that there is a basal level of endogenous nitrosoproline in all subjects tested (21) and the optimum gastric pH for nitrosoproline formation is 1.5−2.0 (9). This suggests that some at least of the NOC detected in acidic gastric juice are stable and non-carcinogenic. Obviously, one measurement cannot represent an individual's long-term intragastric *N*-nitrosation potential. However, prolonged continuous exposure to high NOC levels from intragastric *N*-nitrosation might suggest an increased risk. It is noteworthy that under achlorhydric conditions the percentage of subjects with higher NOC levels was significantly increased, leading to a significantly higher mean NOC concentration than in the acidic stomach. This is consistent with the observation that achlorhydria resulting from a variety of disease states carries an excess risk of gastric cancer (22,23). Though the total volume of gastric juice is usually reduced in achlorhydria, significantly increased levels of NOC continuously acting locally on an already abnormal gastric mucosa may be regarded as a high-risk situation. It has further been suggested that such an atrophic mucosa may be more sensitive to NOC (28).

The subjects in our study were from a low-risk population for gastric cancer. The average daily individual nitrate intake in the UK has been reported as 95 mg (24), much lower than in high-risk populations, such as in Japan, where the daily intake has been estimated at 218−314 mg (25). Since intragastric *N*-nitrosation, especially ACN, is closely related to nitrate intake, as demonstrated by employing the nitrosoproline test (7), it is very likely that much higher concentrations of intragastric NOC would be found to exist in those high-risk populations if the improved method were used. Confirmation of this in high-risk populations is required. Further research activity should be directed to identifying individual NOC, especially unstable ones, in gastric juice. However, it is probable that owing to the great

variety of NOC present, individual separation of all *N*-nitroso derivatives may not be possible. Nevertheless, even if only a few major unstable NOC with a carcinogenic potential were identified in gastric juice, then confirmation of the hypotheses of gastric carcinogenesis would be much nearer realisation.

Acknowledgements

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