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Antimicrobial Effect of Acidified Nitrite on Gut Pathogens: Importance of Dietary Nitrate in Host Defense

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Dietary intake of nitrate generates salivary nitrite, which is acidified in the stomach, leading to a number of reactive intermediates of nitrogen, among which are the potentially carcinogenic *N*-nitrosamines. Acidified nitrite, however, also has antimicrobial activity which coincides with the formation of nitric oxide. The present study examines the antimicrobial effect in vitro of acidified nitrite on *Salmonella enteritidis*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Shigella sonnei*, and *Escherichia coli* O157. First-order regression plots showed a linear inverse relationship of log-transformed proton and nitrite concentrations with MICs and MBCs after 30 min, 2 h, and 24 h of exposure ($P < 0.001$ for all antibacterial activities). Susceptibility to the acidified nitrate solutions ranked as follows: *Y. enterocolitica* > *S. enteritidis* > *S. typhimurium* = *Shigella sonnei* > *E. coli* O157 ($P < 0.05$). Addition of SCN^-, but not that of Cl^-, increased the antibacterial activity (paired *t* testing, $P < 0.001$). Generation of salivary nitrite from dietary nitrate may provide significant protection against gut pathogens in humans.

Nitrogen oxides and nitrous acid are recognized in organic chemistry as noxious compounds and atmospheric pollutants and represent a significant population health risk (21). In humans, ingested nitrate (NO_3^-) is absorbed from the gastrointestinal tract into the bloodstream and concentrated in the salivary glands by an active transport system shared with iodide and thiocyanate, increasing concentrations up to 10 times that in plasma (23, 25). Salivary nitrate is then rapidly reduced to nitrite (NO_2^-) by nitrate reductase expressed by microorganisms in the mouth (11). *N*-Nitrosamines are formed from nitrite and secondary amines in the stomach (15, 20), and concerns about the endogenous formation of these potentially carcinogenic compounds has led to calls for restriction of nitrate and nitrite in food products and drinking water (24).

We have recently suggested that the production of salivary nitrite serves a useful purpose as a host defense mechanism against swallowed pathogens via the formation of bacteriocidal compounds in the stomach (1). It has been shown that expelled stomach air contains a high concentration of the antimicrobial gas nitric oxide ($NO^{\cdot}$) which is enhanced by dietary nitrate intake (16). We proposed that the salivary generation of nitrite is accomplished by a symbiotic relationship involving nitrate-reducing bacteria on the tongue surface, which is designed to provide a host defense against microbial pathogens in the mouth and lower gut via chemical $NO^{\cdot}$ production (6).

Patients with infective gastroenteritis have increased plasma nitrate levels compared with those in healthy controls (7), septicemic patients (19), and patients with inflammatory bowel disease (7a). During infective gastroenteritis, salivary generation of nitrite might be greatly enhanced, resulting in increased gastric $NO^{\cdot}$ production. To investigate the role of salivary nitrite in the bacteriocidal function of the stomach, we studied the effect of acidified nitrite on microorganisms involved in the etiology of infective gastroenteritis. Five microorganisms were tested by using acidification with hydrochloric acid and various concentrations of nitrite characteristic of concentrations found in saliva. We also studied the effects of other anions, including thiocyanate (which is also concentrated in saliva) and chloride (which is secreted into the gastric lumen) in combination with nitrite solutions acidified with sulfuric acid.

MATERIALS AND METHODS

Production of standardized bacterial inocula. Patient isolates of *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, *Yersinia enterocolitica*, and *Escherichia coli* O157 were tested. All experiments used early-log-phase cultures. Flasks (125 ml) containing 75 ml of nutrient broth (Oxoid CM1) were inoculated and incubated on a shaker (New Brunswick Scientific Co., Edison, N.J.) for 18 h at 37°C. The optical density was adjusted by dilution with fresh nutrient broth to produce a density of 2×10^7 CFU ml^{-1}.

Determination of the bacteriostatic activity of acidified nitrite. The experiments were carried out on disposable, flat-bottom microwell plates (96 wells of 300 μl). Nitrite solutions to give a final concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1, 2, and 10 μmol of nitrite per ml in the microwells and nutrient broth solutions acidified by hydrochloric acid to give final pHs of 5.4, 4.8, 4.2, 3.7, 3.0, and 2.1 were prepared. The microwells were filled with nitrite solution (60 μl), bacterial suspension (60 μl), and acidified nutrient broth (120 μl). The plates were sealed and incubated for 24 h at 37°C on the shaker. The inhibitory effect of acidified nitrite on bacterial growth was determined by measurement of the optical density (570 nm) of the wells using a microwell plate reader (MRX Microplate Reader; Dynatech Products Ltd., Guernsey, Channel Islands, Great Britain). To determine the effect of Cl^- and SCN^- on the antimicrobial activity of acidified nitrite, the experiment was repeated with *S. enteritidis* using acidification by sulfuric acid (H_2SO_4) with 10 mM NaCl, Na_2SO_4, or NaSCN in the microwells. All experiments were carried out in triplicate.

Determination of the bacteriocidal activity of acidified nitrite. After 30 min, 2 h, and 24 h of exposure to acidified nitrite, 20 μl of the bacterial suspensions was transferred to 180 μl of a recovery medium (nutrient broth; pH = 7.0). From this first transfer, a further 20 μl was transferred to recovery medium to accomplish neutralization of acid, dilution of nitrite concentration, and reduction of the original inoculum size to a final number of 10,000 microorganisms. Recovery media were incubated on the shaker for 24 h at 37°C before assessment of microbial growth with the microwell plate reader.

Interpretation of results and statistical analysis. The MIC of NO_2^- at the different pH settings was defined as the lowest NO_2^- concentration at which no growth of microorganisms had taken place after 24 h. The MBC was defined as the lowest NO_2^- concentration at which no growth was detected after transfer into recovery media. MICs and MBCs of nitrite (in micromoles per milliliter) were log transformed for statistical analysis. Differences in susceptibility of mi-

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TABLE 1. Activity against common gut pathogens of nitrite acidified with HCl at various pH values[a]

Organism	Antimicrobial activity	Exposure time (h)	Antimicrobial nitrite concn (μmol/ml) at pH:					
			2.1	3.0	3.7	4.2	4.8	5.4
Y. enterocolitica	MBC	0.5	0	0.02	1.33	6.67	>10[b]	>10
		2	0	0	0.07	1.67	10.0	>10
		24	0	0	0	0.05	2.0	10.0
	MIC	24	0	0	0	0	0.50	6.67
S. enteritidis	MBC	0.5	0	0.13	1.33	10.0	>10	>10
		2	0	0	0.40	2.0	10.0	>10
		24	0	0	0	0.05	1.0	10.0
	MIC	24	0	0	0	0.02	0.67	6.67
S. typhimurium	MBC	0.5	0	0.83	6.67	>10	>10	>10
		2	0	0.20	1.67	10.0	>10	>10
		24	0	0.02	0.10	0.67	10.0	>10
	MIC	24	0	0	0.05	0.50	5.0	10.0
Shigella sonnei	MBC	0.5	0.20	1.67	10.0	>10	>10	>10
		2	0.07	0.67	3.67	10.0	>10	>10
		24	0	0	0.20	1.0	6.67	>10
	MIC	24	0	0	0	0.40	3.0	10.0
E. coli O157	MBC	0.5	1.0	6.67	>10	>10	>10	>10
		2	0.50	1.33	3.0	10.0	>10	>10
		24	0	0.02	0.30	0.83	6.67	>10
	MIC	24	0	0	0	0	1.0	10.0

[a] All experiments were carried out in triplicate.
[b] No antimicrobial activity at nitrite concentrations of ≤10 μmol/ml.

croorganisms to nitrite acidified with HCl were assessed by analysis of variance and paired *t* testing for means of the MIC, MBC after 30 min exposure time ($MBC_{0.5h}$), MBC_{2h}, and MBC_{24h} at the six pH values applied in the experiments. The same method was used to assess the differences in susceptibility of *S. enteritidis* to nitrite acidified with sulfuric acid with 10 mM Na_2SO_4, NaCl, or NaSCN present in the solution. Paired *t* testing was also applied to compare the mean concentrations of nitrite required to accomplish bacteriostasis, and bacterial killing after 30 min, 2 h, and 24 h of exposure. The slopes of the regression curves of acidified nitrite for the different microorganisms and antibacterial activities were assessed by regression analysis.

RESULTS

The means of the nitrite concentrations showing antimicrobial activity at pHs 2.1, 3, 3.7, 4.2, 4.8, and 5.4 are summarized in Table 1. *Y. enterocolitica*, *S. enteritidis*, and *S. typhimurium* were all killed at pH 2.1 after 30 min of exposure. *Shigella sonnei* and *E. coli* O157 survived, unless nitrite was present in the solution (0.20 and 1 μmol/ml, respectively [Table 1]).

A linear relationship between $\log[NO_2^-]$ and pH was present for MIC, $MBC_{0.5h}$, MBC_{2h}, and MBC_{24h} between pHs 2.1 and 4.8 (Fig. 1). The cumulative *R* for the regression lines was significant for all antimicrobial activities ($P < 0.001$). Regression analysis showed a significantly steeper slope for the MIC regression line compared with those for $MBC_{0.5h}$ ($P = 0.007$) and MBC_{2h} ($P = 0.032$).

At a pH of ≥4.8, the nitrite required to achieve bacterial killing after short exposure times ($MBC_{0.5h}$ and MBC_{2h}) was frequently >10 μmol/ml and the linear relationship between $\log[NO_2]$ and pH was lost. There was a significant difference between $MBC_{0.5h}$, MBC_{2h}, MBC_{24h}, and MIC at all pH settings (paired *t* testing for means, $P < 0.001$).

Analysis of variance showed significant differences in susceptibility to acidified nitrite between individual organisms ($P < 0.001$). At pH 2.1, *E. coli* O157 was significantly less susceptible compared with all other microorganisms (paired *t* testing, $P < 0.001$), and throughout the pH range, analysis of regression showed the slope of its regression line to be significantly lower ($P < 0.001$). The susceptibilities of *S. typhimurium* and *Shigella sonnei* were not significantly different. *S. enteritidis* was more susceptible than *S. typhimurium* (paired *t* testing $P < 0.001$), and *Y. enterocolitica* was most susceptible of all microorganisms tested ($P = 0.047$ compared with *S. enteritidis* and $P < 0.001$ compared with all other microorganisms).

Adding 10 mmol of NaSCN per liter to the microwell resulted in a significant reduction of the amount of acidified nitrite required to accomplish activity (Table 2) (paired *t* testing, $P < 0.001$). Addition of NaCl or Na_2SO_4 resulted in identical antibacterial activities.

DISCUSSION

The acidity in the lumen of the human stomach is dependent on physiological variables such as previous food intake, anxiety, age, medication such as antacids, and previous gastric surgery. Under fasting conditions the median of the luminal pH in healthy volunteers is around 2.0, ranging from 1.5 to 5.5 (27). Ingestion of a meal characteristic of the main meal of a Western diet produces an immediate rise in the median gastric pH to about 6.0, which will return over the following 2 to 3 h to premeal values of about 2.0 (14, 22).

Human salivary nitrate and nitrite concentrations are greatly influenced by the amount of nitrate in the diet. It is estimated that some 25% of dietary nitrate is secreted into saliva, and most of this nitrate is converted to nitrite by nitrate reductase (25). The reduction of nitrate to nitrite is enhanced by chewing, which increases salivary contact with the tongue (10). After intake of a nitrate-rich meal, up to 1.5 mmol of nitrite could enter the stomach (25). Therefore, it appears that the concentration of nitrite in saliva varies according to dietary nitrate intake, activity of bacterial nitrate reductase, salivary flow rate, and endogenous production of nitrate. Values between 0.05 and 10 μmol/ml have been reported.

The antibacterial potential of swallowed salivary nitrite at low pH is clearly demonstrated by the data in this study. A highly significant contribution ($P < 0.001$) of nitrite to the MIC, $MBC_{0.5h}$, MBC_{2h}, and MBC_{24h} was observed. The regression plots in Fig. 1 represent the partition between growth and inhibition (MIC) and inhibition and killing ($MBC_{0.5h}$, MBC_{2h}, and MBC_{24h}). Under the conditions prevailing to the

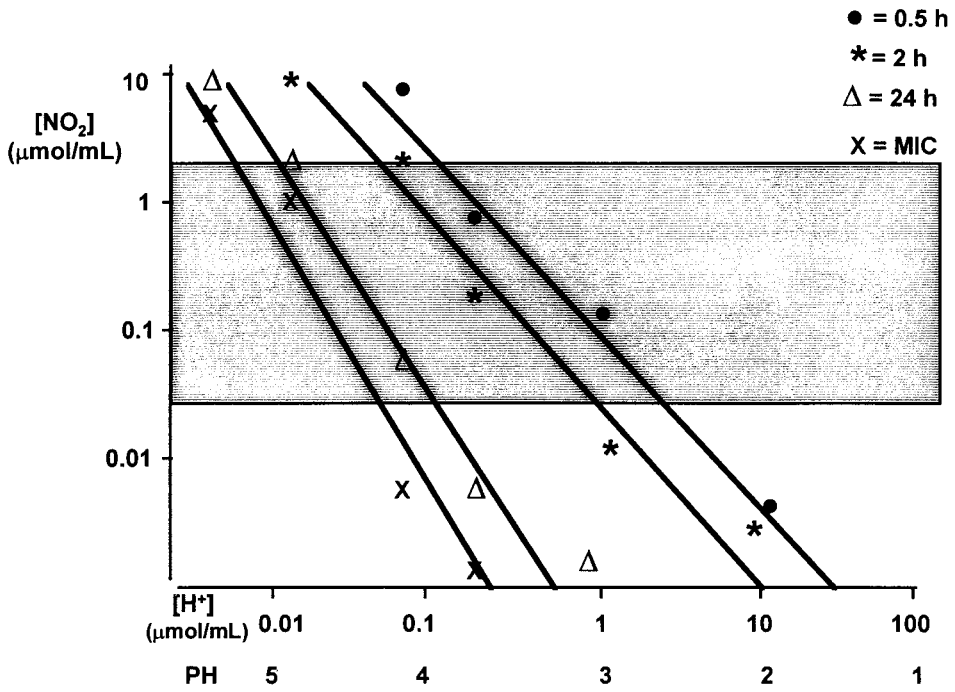

FIG. 1. Antimicrobial activity of acidified nitrite: MIC and MBC after 0.5, 2, and 24 h of exposure. All experiments were carried out in triplicate. The regression plots represent the means of experiments with *Y. enterocolitica*, *S. typhimurium*, *S. enteritidis*, *Shigella sonnei*, and *E. coli* O157. Regression constants: $R_{0.5h} = 0.804$; $R_{2h} = 0.824$; $R_{24h} = 0.861$; $R_{MIC} = 0.825$. The shaded area represents the range of nitrite concentration most commonly observed in human saliva.

left of a regression plot, the microorganisms remain unharmed, while to the right they will be subjected to the antimicrobial activity represented by that particular plot. Thus, at a pH of >3.7, there is no antimicrobial activity unless nitrite is present in the solution. Addition of a small amount of nitrite results in bacteriostasis, and increasing the concentration leads to bacterial killing. More than 5 μmol/ml kills the microorganisms within 30 min. It appears that the antimicrobial activities of nitrite and acid are synergistic within the physiological range of their concentrations in human saliva and gastric juice, respectively (shaded area in Fig. 1). Since we are dealing with organisms that are involved in the etiology of infective gastroenteritis, the bacteriocidal activities are most relevant, as bacteriostasis will allow viable organisms to pass to the small intestine, where the killing mechanism is not active.

TABLE 2. Activity against *S. enteriditis* of nitrite acidified with H_2SO_4 at various pH values with 10 mM Na_2SO_4, NaCl, or NaSCN present in the solution[a]

Additive	Anti-microbial activity	Exposure time (h)	Antimicrobial nitrite concn (μmol/ml) at pH:					
			2.1	3.0	3.7	4.2	4.8	5.4
Na_2SO_4	MBC	0.5	0	0.20	3.0	10.0	>10[b]	>10
		2	0	0	0.07	1.67	10.0	>10
		24	0	0	0.07	1.67	10.0	>10
	MIC	24	0	0	0	0.03	3.0	4.0
NaCl	MBC	0.5	0	0.47	1.67	10.0	>10	>10
		2	0	0	0.50	2.0	10.0	>10
		24	0	0	0	0.03	2.0	10.0
	MIC	24	0	0	0	0	0.50	5.67
NaSCN[c]	MBC	0.5	0	0	0	3.0	>10	>10
		2	0	0	0	0.07	10.0	>10
		24	0	0	0	0	2.0	10.0
	MIC	24	0	0	0	0	0.50	5.67

[a] All experiments were carried out in triplicate.
[b] No antimicrobial activity at nitrite concentrations of ≤10 μmol/ml.
[c] Antimicrobial activity is significantly increased compared with that with Na_2SO_4 and NaCl (paired *t* testing for means, $P < 0.001$).

Acidification of nitrite will lead to generation of reactive intermediates of nitrogen that have cytotoxic properties (Fig. 2). At a given pH value, the quantity of $NO^{\cdot}$ generated in vitro is dependent on the nitrite concentration (6). In the solutions used during this experiment, a nitrite concentration of 0.01 μmol/ml at pH 3 generated a peak concentration of nitric oxide of 1 ppm in the headspace and a nitrite concentration of 1.2 μmol/ml generated 10 ppm. Within this range of nitrite concentrations at pH 3, a bacteriocidal effect within 2 h of exposure was accomplished for all microrganisms (Table 1), suggesting that gastric contents generating 1- to 10-ppm $NO^{\cdot}$ would have a bacteriocidal effect on these gut pathogens within the transit time of a food bolus through the stomach. In vivo measurements of $NO^{\cdot}$ production in the human stomach have shown values between 1 and 180 ppm, depending on dietary nitrate intake (5, 16).

Nitric oxide inhibits respiratory chain enzymes through inactivation of iron-sulfur complexes (9) and disrupts DNA replication by inhibiting ribonucleotide reductase (17). Its toxicity has been demonstrated for a rapidly expanding list of microorganisms (3) as well as for tumor cells (18). However, experiments with $NO^{\cdot}$ donor compounds have shown little antibac-

$$NO_2^- + H^+ \Leftrightarrow HNO_2 \quad (pK_a = 3.42)$$

$$2HNO_2 \Leftrightarrow H_2O + N_2O_3$$

$$N_2O_3 \Leftrightarrow NO\cdot + NO_2$$

FIG. 2. Acidification of nitrate will lead to generation of reactive intermediates of nitrogen that have cytotoxic properties.

terial activity of $NO^{\cdot}$ itself (4), and its toxic effects are more likely to be accomplished via the formation of peroxynitrite in the presence of superoxide (29), the oxygen-dependent generation of the nitrogen dioxide radical when nitric oxide concentrations are high (2), and/or the formation of still-uncharacterized nitrogen species (28). It seems most likely that the antibacterial activity of acidified nitrite is due to an additive contribution of reactive intermediates of nitrogen (12).

Susceptibilities to the acidified nitrite solutions ranked as follows: *Y. enterocolitica* > *S. enteritidis* > *S. typhimurium* = *Shigella sonnei*. *E. coli* O157 was different in its response to acidified nitrite compared with the other four microorganisms; in the absence of nitrite it was significantly more resistant to acid, but addition of nitrite seemed to abolish this difference (Table 1). In conclusion, addition of nitrite to acidic solutions achieves killing of gut pathogens where acid alone allows growth to continue. Physiological concentrations of nitrite accomplish killing after exposure times that are comparable with the transfer time of a food bolus through the stomach. Addition of thiocyanate, which is also concentrated in saliva, but not of chloride increased antibacterial activity (Table 2).

Generation of salivary nitrite increases greatly after nitrate ingestion, suggesting that ingestion of foods rich in nitrate protects against infective gastroenteritis. The high plasma nitrate levels observed in patients that are suffering from infective gastroenteritis may protect against the fecal-oral route of reinfection via increased generation of salivary nitrite. This mechanism may limit the impact of outbreaks of gastroenteritis, which would be relevant in humans but also would be of particular importance in other mammalian species and animal husbandry.

Health-conscious individuals and government authorities have advocated restriction of dietary nitrates for the last 20 years after ingestion of amines and nitrates had been associated with gastric cancer in animal models. Although the harmful and potentially carcinogenic activity of *N*-nitrosamines cannot be dismissed, epidemiological evidence for this association has been lacking (8, 13). We submit that the mechanism of chemical host defense which seems to take place in symbiosis with nitrate-reducing bacteria on the surface of our tongues may be of fundamental importance. Rather than a potential carcinogen, we postulate that nitrate may be a useful nutrient, particularly when accompanied by ascorbic acid (26), as is the case with vegetables. A conclusive demonstration of the antimicrobial effect of acidified nitrite in vivo would require a major reinterpretation of the role of dietary nitrate in human health and animal husbandry.

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