Proline is not useful as a chemical probe to measure nitrosation in the gastrointestinal tract of patients with gastric disorders characterised by anacidic conditions

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SUMMARY Aspirated fasting gastric juice from patients with lesions of the gastrointestinal tract and from healthy controls was analysed for nitrite before and after (30, 90, and 240 min) oral administration of 200 mg nitrate. Wilcoxon's rank-sum tests showed no significant differences in fasting gastric juice nitrite concentrations between healthy controls and patients after proximal gastric vagotomy or with gastric/duodenal ulcer (median ≤ 0.7 ppm NO_2^-) and only moderate increases after nitrate administration. Chronic atrophic gastritis patients and patients with Billroth I or II gastric resections showed median concentrations of 2 ppm NO_2^- which increased to 20 ppm (up to 200 ppm in one Billroth II patient) after administration of nitrate. Endogenous formation of N-nitrosoproline using the NPRO-test was determined in two groups with low (healthy control and proximal gastric vagotomy patients) and high (Billroth I and II patients) gastric nitrite concentrations. After 12 h fasting, 200 mg nitrate was orally administered, followed 30 min later by 500 mg L-proline. Endogenously formed N-nitrosoproline which is quantitatively excreted in urine was determined in urine over the following 24 hours. In over 80% of the urine samples collected from Billroth I and II patients no detectable NPRO was found whilst in over 85% of the healthy controls and proximal gastric vagotomy patients up to 33.5 μg NPRO was detected. *In vitro* nitrosation kinetics showed that at gastric $\text{pH} > 4$ present in both, patients with Billroth I and II resections and with chronic atrophic gastritis, nitrosation of proline does not occur. As alternative chemical probes for quantifying potential endogenous nitrosation in hypoacidic patients the methyl and ethyl esters of proline were investigated. *In vivo* nitrosation of these two new probes was established in animal experiments using rats and was shown to occur *in vitro* at pH 4-5. During incubation in human gastric juice, however, almost 30% ester cleavage by non-specific gastric esterases occurred within the first five minutes, thus further limiting the use of these compounds in determining endogenous nitrosation in hypoacidic patients.

N-nitroso compounds are well known as potent carcinogens.¹ They are widely distributed in the environment and can be detected in various foods – for example, in nitrite treated meat and in beer,² tobacco products,³ cosmetics,^{4,5} and in drugs.⁶ In

addition to exposure by those exogenous sources, endogenous formation of N-nitroso compounds has been a matter of considerable concern.⁷ In laboratory animals endogenous nitrosation has already been shown more than 15 years ago.⁸ By measuring N-nitrosated amino acids in the urine it has later been shown unequivocally, that endogenous nitrosation of such precursors occurs at substantial rates.^{9,10} The method had been developed by Ohshima and Bartsch⁹ for an assessment of endogenous nitrosation in man.

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Nitrite concentrations in the gastric fluid have been shown to be (directly) correlated with intragastric pH.^{11,12} Under conditions of hypochlorhydria, bacterial contamination of gastric juice is common and nitrate reducing microorganisms are regularly found.^{13,14} The presence of raised nitrite concentrations is considered to favour the intragastric formation of N-nitroso compounds under such conditions.

It has been reported that intragastric concentrations of N-nitroso-compounds are enhanced in the anacidic pH – for example, in patients with Billroth II gastric resection¹⁵ and that a positive correlation between pH and N-nitroso compound concentration was observed.^{11,16} Other authors have, however, not found such a correlation.¹⁷ Analytical problems to be faced with determination of total N-nitroso-compounds in gastric juice may, at least in part, be responsible for these contradictory findings. They have been adequately reviewed by Pignatelli *et al.*¹⁶ In our earlier experiments we had analysed gastric juice before and after gavage of 200 mg nitrate to investigate how various disorders of the upper gastrointestinal tract influence the intragastric concentration of nitrite.¹⁸ Three patient groups were found to have high nitrite formation rates in the gastric juice: gastric resection after Billroth I (BI) or Billroth II (BII) and chronic atrophic gastritis (CAG). These patient groups have now been increased to 12 subjects, and furthermore we have investigated the potential of endogenous formation of N-nitroso compounds using proline as an indicator and monitoring the urinary excretion of nitrosoproline (NPRO). This N-nitroso compound is neither mutagenic nor carcinogenic.^{19,21} The disorders found in our experiments to favour nitrite formation after oral intake of nitrate have been shown to increase the risk for gastric cancer. Gastrointestinal disorders like CAG, BI and BII status being characterised by achlorhydric gastric fluid and bacterial growth in the stomach have been identified as precursor lesions for gastric cancer.^{22–25}

The present investigation wanted to ascertain whether enhanced nitrite concentrations in the gastric fluid of patients having the described gastric disorders are liable to yield enhanced endogenous N-nitrosation rates, as measured by urinary excretion of NPRO.

Methods

CHEMICALS

All chemicals were obtained from Merck (Darmstadt, FRG), Fluka (Neu-Ulm, FRG), or Janssen (Beerse, Belgium) and were of analytical grade.

NPRO and NPIC was synthesised according to the method of Hansen *et al.*,²⁶ their purity were checked by established methods.²⁷ Ethereal diazomethane was prepared from N-nitrosomethylurea and was redistilled before use.

PATIENTS

After diagnosis by gastroscopy and histological verification, 67 patients with various complaints of the upper gastrointestinal tract were classified into six groups: Billroth I (BI) or Billroth II (BII), proximal-gastral vagotomy (PGV), chronic atrophic gastritis (CAG), *ulcus ventriculi* and *ulcus duodeni*. With the exception of the CAG group with only seven patients, each group consisted of 12 subjects; healthy, age matched volunteers served as controls.

For the NPRO-test, patients were selected from the BI, BII, and PGV-groups. As CAG patients were not available within this period we restricted our investigations to BI and BII (high nitrite groups) and PGV (low nitrite group).

The patients and healthy controls participating in this study had been given detailed information on the experimental procedure, its aim and its risks and had given their oral consent.

GASTRIC JUICE

After a 12 h fast, samples of gastric juice were collected by gastric tube aspiration before (time zero) and 30, 90, and 240 min after ingestion of 200 mg nitrate in 50 ml water. Immediately after delivery the samples were stabilised with 0.1 ml 10 N NaOH and analysed for nitrite using a continuous flow analyser. Nitrite was detected by diazotation with sulphanilamide and formation of a coloured azo compound by coupling with N-1-naphthylethylenediammoniumdichloride. The concentration range was linear from 0.1 to 50 ppm; $\lambda = 540$ nm.

NITROSOPROLINE TEST

After 12 h overnight fasting, subjects first ingested 200 mg nitrate in 50 ml water and 30 min later 500 mg of L-proline in 50 ml water. No food or beverage was consumed for the next two hours. Urine was collected quantitatively over 24 h in 2 l polyethylene bottles containing 17.6 g ascorbic acid to prevent artifact formation together with 400 mg ethylmercaptobenzoate as a preservative.²⁸ The bottles were stored at 5°C until analysis. Food ingestion was recorded on the day of the experiment and on the day before. Smoked or cured products were not allowed to minimise intake of preformed NPRO.²⁹

NPRO DETERMINATION

The total volume of the 24 h urine was measured.

Aliquots of 15 ml were taken for NPRO determination (double). After the addition of 500 µg NPIC as an internal standard, the sample was deproteinised with 1 ml each of potassium hexacyanoferrate II (106 g K₄Fe(CN)₆/l water) and zinc sulphate (220 g ZnSO₄/l acetic acid, 3%). After filtration through a glass fibre filter, the filtrate was saturated with 9 g ammonium sulphate. Sulphuric acid (1 ml, 50%) was added and the sample was transferred to a column of 13 g silica gel (Extrelut^R, Merck, Darmstadt, FRG) over 2 g anhydrous sodium sulphate. After 20 min equilibration the column was eluted with 60 ml ethyl acetate. The eluate was evaporated in a rotary evaporator at 50°C to a final volume of about 1 ml. The concentrate was transferred to a 20 ml reaction vial and an excess (normally 2 ml) of distilled ethereal diazomethane was added. After 15 min standing at room temperature the volume was reduced to 1 ml by a gentle stream of nitrogen.

GC-ANALYSIS

Glass column: 1 m×2 mm, packed with 3% Carbowax 20 M, TPA on Volaspher (100–120 mesh)
Carrier gas: helium, 30 ml/min
Oven temperature: 130°C (4 min) and programmed to 210°C at 4°/min
Detector: TEA, model 502 (Thermo Electron, Waltham, MA, USA)
Recovery of NPRO was 79% (SD=6.8, n=6) and of the internal standard NPIC 92% (SD=6.8, n=6); detection limit of NPRO was 0.5 ppb.

IN VITRO NITROSATION OF PROLINE AND PROLINE ESTERS

Solutions of proline or proline ester and nitrite (50 mM each) were incubated at 37°C for 60 min in citrate buffer (20 ml, pH 2–6). Yields of N-nitroso products were determined spectrometrically at 320–400 nm against solvent blanks (Lambda 5, recording spectrophotometer, Perkin Elmer, Überlingen, FRG).

Table 1 Nitrite concentration (ppm) in fasting gastric juice of various patient groups and healthy controls; median with 95% confidence interval, minimum and maximum values

Group	Median	95% Confrange	Min	Max
Controls	0.4	ND–0.7	ND	4.9
BI	1.5	0.5–1.9	0.1	3.4
BII	2.0	0.8–6.0	0.8	18.3
CAG	1.3	0.3–4.4	0.8	4.4
PGV	0.7	0.2–2.1	ND	9.0
Ulc ventr	0.2	0.1–0.5	ND	0.5
Ulc duod	0.3	0.1–0.4	ND	0.5

t=0, fasting gastric juice

Table 2 Nitrite concentration (ppm) in gastric juice of various patient groups and healthy controls; 30 min after oral intake of 200 mg nitrate, median with 95% confidence interval, minimum and maximum values

Group	Median	95% Confrange	Min	Max
Controls	1.4	0.2–3.5	0.2	9.1
BI	7.5	3.0–19.0	1.3	30.0
BII	14.1	4.6–24.2	3.8	93.4
CAG	10.6	0.4–86.1	0.4	86.1
PGV	2.8	0.8–7.1	0.2	21.2
Ulc ventr	0.9	0.7–2.7	ND	11.3
Ulc duod	0.4	0.2–1.2	0.1	2.2

t=30 min.

CLEAVAGE OF PROLINE METHYLESTER IN GASTRIC JUICE

Proline methyl ester (100 µM) was incubated at 37°C for 5, 10, 30, or 60 min in 1 ml human gastric juice (pH 1.5). After nitrosation with sodium nitrite (10 mg/ml water) the solution was saturated with ammonium sulphate and extracted at pH 8–9 (NaOH, 1 N) with 3×1 ml ethyl acetate to isolate NPRO-methyl ester. The aqueous phase was acidified to pH 1–2 (HCl, 1 N) and extracted with 3×1 ml ethyl acetate to isolate NPRO. After methylation with diazomethane, samples were analysed as described for urine samples.

CLEAVAGE OF NITROSATED PROLINE ESTERS WITH NON-SPECIFIC ESTERASES

To measure cleavage by non-specific esterases, NPRO methyl or -ethyl ester (5 ml of a 10 or 100 µg/ml solution in phosphate buffer) in first experiments were incubated with esterase (10 µl) from porcine liver (suspension: 3 mg/ml; Boehringer, Mannheim, FRG). Furthermore NPRO methyl or -ethyl ester (0.2 ml of a 0.5 mg/ml solution in phosphate buffer) were incubated in rat serum (9.8 ml, male SD rats, protein: 52 mg/ml). Aliquots (1 ml) were taken at

Table 3 Nitrite concentration (ppm) in gastric juice of various patient groups and healthy controls; 90 min after oral intake of 200 mg nitrate, median with 95% confidence interval, minimum and maximum values

Group	Median	95% Confrange	Min	Max
Controls	1.6	0.7–2.2	0.5	5.8
BI	7.0	3.2–35.0	2.8	50.0
BII	20.9	13.3–36.7	8.8	157.7
CAG	12.6	1.0–29.2	1.0	29.2
PGV	1.3	0.6–8.3	0.3	38.0
Ulc ventr	3.1	1.1–4.6	1.0	27.5
Ulc duod	0.3	0.1–0.6	ND	3.0

t=90 min.

Table 4 Nitrite concentration (ppm) in gastric juice of various patient groups and healthy controls; 240 min after oral intake of 200 mg nitrate, median with 95% confidence interval, minimum and maximum values

Group	Median	95% Confrange	Min	Max
Controls	0.7	0.2-3.1	0.2	3.2
BI	5.9	5.0-9.2	4.1	24.2
BII	13.0	8.3-32.7	0.5	200.2
CAG	9.7	1.5-40.0	1.5	40.0
PGV	0.9	0.6-10.4	0.4	30.0
Ulc ventr	1.8	0.5-11.6	0.3	16.1
Ulc duod	0.3	0.1-2.9	ND	4.3

t=240 min.

specific time points up to 15 min, extracted with dichloromethane (3×1 ml) and analysed by GC-TEA as described.

ANIMAL EXPERIMENTS

Male SD rats, 200–250 g (Charles River Wiga, Sulzfeld, FRG), were kept in a 12 h light/dark cycle. They received standard pelleted diet (Altromin, Lage, FRG) and tap water *ad libitum*. Food was withdrawn 12 h before start of the experiment.

EXPERIMENTS WITH LABELLED COMPOUNDS

To study excretion rate of NPRO after ingestion of NPRO methyl or ethylesters, [U-¹⁴C] labelled N-nitrosoproline esters (10, 20, or 40 mg/kg; 250 mCi/mM=9.25 GBq/mM) were applied by gavage to rats. A first experiment had shown that exhalation of ¹⁴CO₂ accounted for less than 0.5% of total radioactivity excreted. Therefore normal metabolism cages were used. Animals were placed individually into these cages, urine was collected for 2×24 h and was preserved as described. Radioactivity was deter-

Fig. 1 Nitrite concentration in gastric juice after oral intake of 200 mg nitrate, time course of median with 95% confidence intervals; low nitrite groups.

Fig. 2 Nitrite concentration in gastric juice after oral intake of 200 mg nitrate, time course of median with 95% confidence intervals; high nitrite groups.

mined with a liquid-scintillation-counter (Mark III, Searle, Illinois, USA).

ENDOGENOUS NITROSATION OF PROLINE METHYLESTER IN RATS

Rats received between 5 and 35 μ mol proline methylester in 0.5 ml citrate buffer (pH 3, 0.1 M) by gavage. Immediately thereafter, 0.5 ml sodium nitrite (20 μ M) was applied. Animals were placed individually into metabolism cages, urine was collected for 24 h and was further processed as described.

Results

NITRITE IN GASTRIC JUICE

Median intragastric nitrite concentrations at sampling times specified together with their 95% confidence intervals are shown in Tables 1–4 and Figures 1, 2.

Differences in gastric nitrite concentrations can readily be seen already in fasting gastric juice of the various patient groups. Those known to have low pH values (controls, PGV, *ulcus ventriculi* and *ulcus duodeni*) showed median values up to 0.7 ppm nitrite in their gastric fluids. Billroth and CAG patients however, whose gastric juice has been established to be less acidic to neutral, had 1.3–2.0 ppm nitrite in their fasting gastric fluids.

After ingestion of nitrate, however, differences in the potential to form nitrite become much more

Table 5 Total amounts of NPRO excreted in the 24 h urine of humans after oral gavage of 200 mg of nitrate and 500 mg of proline, arranged according to their distribution in the different range subgroups ($\mu\text{g NPRO}/24 \text{ h urine volume}$)

NPRO-concentration subgroups	Healthy controls		Billroth I		Billroth II		PGV	
	n	$\mu\text{g NPRO}$	n	$\mu\text{g NPRO}$	n	$\mu\text{g NPRO}$	n	$\mu\text{g NPRO}$
(a) ND*-1 μg	3	ND	5	ND	21	ND	1	ND
(b) 1-1-5 μg	3	4-2; 2-4; 2-1	-	-	1	1-7	1	4-9
(c) 5-1-10 μg	10	7-6; 6-3; 6-7; 7-4; 8-0; 8-4; 5-9; 6-2; 6-2; 5-3	-	-	4	7-8; 9-3; 5-4; 5-9	1	7-6
(d) <10 μg	6	11-4; 18-2; 12-3; 33-5; 20-4; 13-9	-	-	-	-	3	15-3; 12-4; 22-9

*ND:<0.5 ppb (detection limit).

evident. Healthy controls had median gastric nitrite concentrations that remained throughout the sampling period in a low concentration range (≤ 1.6 ppm). The same was true for the ulcer- and PGV-patients, who showed nitrite concentrations not significantly different from those of healthy controls (Wilcoxon's rank-sum test). In contrast with these groups, Billroth and CAG patients were found to build up much higher nitrite concentrations. In some cases very high values (up to 200 ppm) were measured.

NITROSATION OF PROLINE IN THE STOMACH

Results are shown in Table 5 and Figure 3.

As can be seen, highest amounts of NPRO were found in the urine of healthy control persons with values up to about 34 $\mu\text{g NPRO}$ (19/22 positives). Likewise, in PGV patients an almost identical frequency distribution was found (five of six positives). In contrast, the urines of BI and BII patients, proven to have high nitrite values in gastric juice were practically devoid of measurable NPRO concentrations (five of five BI and 21/26 BII negatives).

Fig. 3 Frequency distribution of NPRO amounts, excreted in 24 h urine.

IN VITRO NITROSATION KINETICS

Results of *in vitro* nitrosation of proline at different pH-values compared with nitrosation of proline methyl- and ethyl ester are shown in Figure 4. As can be seen, the free amino acid is nitrosated at substantial rates only at pH values <4. In contrast, the esters still undergo appreciable nitrosation at pH 4-5.

CLEAVAGE OF PROLINE METHYL ESTER IN HUMAN GASTRIC JUICE

On incubation of proline methyl ester in gastric juice, 30-40% of the compound decomposed within five minutes (first sampling time). Further incubation, however, did not result in further decomposition.

CLEAVAGE OF NITROSOPROLINE ESTERS BY NON-SPECIFIC ESTERASES

NPRO ethyl ester was hydrolysed about twice as fast as NPRO methyl ester. In rat serum half-life of 6.4 ± 0.8 min for the ethyl and of 13.1 ± 1.5 min for the methyl- ester (each 10 $\mu\text{g ester/ml serum}$) were found. In heat denaturated serum (90°C for 10 min) no decomposition was observed within one hour.

Fig. 4 *In vitro* nitrosation of proline and its methyl- and ethyl ester as a function of pH ($c=50 \text{ mmol}$, $t=60 \text{ min}$, $T=37^\circ\text{C}$).

EXCRETION EXPERIMENTS WITH ^{14}C -LABELLED NPRO ESTERS

Within 24 h, radioactivity was quantitatively excreted as NPRO in the urine. No intact nitrosoproline ester could be detected.

ENDOGENOUS NITROSATION OF PROLINE METHYL ESTER IN RATS

Gavage of 5–35 μmol proline methyl ester together with 10 μmol sodium nitrite led to the excretion of 25–130 nmol NPRO in the 24 h urine. A clear linear correlation between amounts of proline methyl ester applied and amounts of NPRO excreted could not be established.

Discussion

The relevance of endogenously formed N-nitroso compounds for human cancer induction has been controversially discussed over several years.^{30–32} This continuing discussion has clearly shown the need to obtain reliable data on amounts of N-nitroso compounds synthesised under various conditions within man's gastrointestinal tract. We started our experiments analysing human gastric juice for nitrite as the most important precursor compound. Samples were taken from patients with distinct lesions of the stomach or the duodenum, from patients who had undergone gastric surgery (BI, BII, and PGV) and from healthy controls. To study how nitrate (mainly supplied by vegetables) influences the intragastric nitrite level, gastric fluid had been taken immediately before and at three time points after gavage of 200 mg nitrate in 50 ml water.

The results confirmed earlier findings¹⁶ and those of others^{32,33} obtained without previous nitrate loading. Fasting gastric fluids of patients with BI, BII, and CAG status contain significantly higher nitrite concentrations than those from healthy controls. After nitrate ingestion groups with higher basal nitrite values responded with a very much higher nitrite formation in their gastric juice than the groups with low basal nitrite concentrations. Patients with *ulcus ventriculi* or *duodeni* and PGV patients were not significantly different in their nitrite values from healthy controls (Fig. 1).

Gastric fluid from BI-, BII-, and CAG-patients in most cases contains large numbers of micro-organisms, including nitrate-reducing species.^{14,34,35} These conditions in the stomach of BI-, BII-, and CAG-patients are expected to cause an increased synthesis of N-nitroso compounds.¹³ We tried to quantify this endogenous nitrosation potential by means of the NPRO test. The results of this study show that NPRO formation is higher in the low nitrite groups with normacidic pH of gastric fluid than in the

high nitrite groups, characterised by hypoacidic gastric conditions.

Our studies and those of others³⁶ on nitrosation kinetics have shown that proline is nitrosated at measurable rates only at pH values <4. Under conditions approaching neutrality, however, proline is not nitrosated to a measurable extent, in spite of high to very high nitrite concentrations in the stomach. For Billroth-patients, in addition to the high gastric pH the shorter passage time through the gastric stump might also contribute to lower nitrosation yields.

The results clearly show that proline is not a suitable indicator compound for evaluation of endogenous nitrosation potential in the gastrointestinal tract of patients with hypoacidic gastric conditions. The search for more appropriate chemical probes to measure *in vivo* nitrosation is therefore of utmost importance for the assessment of the possible human health risk originating from endogenous nitrosation. Our findings on nitrosation of proline esters show that some shift towards the neutral pH range can be achieved by using proline esters as chemical probes. As appreciable nitrosation occurs only at pH 5, however, this is still not sufficient. The rapid cleavage of proline esters observed in gastric juice also argues against their potential use as nitrosation probes.

Recent data on catalysis of nitrosation by certain bacteria at neutral pH are most relevant.³⁷ These data have shown that in the presence of such bacteria certain secondary amines, especially morpholine, piperidine, and pyrrolidine are nitrosated under near physiological conditions at neutral pH. These data again stress the need to develop chemical probes that are able to detect *in vivo* nitrosation potential in the human gastrointestinal tract at near neutral pH.

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