Gut, 1977, **18**, 73-77

Nitrite and thiocyanate in the fasting and secreting stomach and in saliva

W. S. J. RUDDELL, L. M. BLENDIS, AND C. L. WALTERS

From the Department of Gastroenterology, Central Middlesex Hospital, London, and the Biochemistry Section, British Food Manufacturing Industries Research Association, Leatherhead, Surrey

SUMMARY The concentrations of nitrite and thiocyanate in fasting and pentagastrin stimulated gastric juice and in saliva have been examined. Nitrite was found in all of 17 samples of fasting gastric juice, mean 4·9 $\pm$ 1·1 μM. Stimulation of gastric secretion with pentagastrin caused no significant change in nitrite concentration. Thiocyanate was detected in all of 21 samples of fasting gastric juice and the difference in concentration between smokers and non-smokers probably reflects similar differences in saliva. In contrast to the nitrite data there was a significant drop in thiocyanate concentration of gastric juice after pentagastrin from 0·9 $\pm$ 0·1 mM to 0·3 $\pm$ 0·04 mM, suggesting a salivary origin for the thiocyanate in gastric juice. Thiocyanate is a powerful catalyst of nitrosation, which, together with small amounts of nitrite and naturally occurring amines could lead to the intragastric formation of carcinogenic nitrosamines and in certain circumstances be a factor in the aetiology of gastric cancer.

The N-nitroso compounds are powerful carcinogens in most animal species tested (Magee and Barnes, 1967) and have been suggested as likely environmental carcinogens in man (Lijinsky and Epstein, 1970). The oral feeding of N-methyl N-nitro N-nitrosoguanidine has been shown by Japanese workers to cause stomach cancer in dogs (Fujita *et al.*, 1974) and rats (Fukushama, 1973). Nitrosamines may be formed in foods and subsequently ingested, and may be produced *in vivo* in the mouth (Tannenbaum *et al.*, 1974) the urinary bladder (Hawksworth and Hill, 1974), and the stomach of rats fed with nitrite and nitrosatable amines (Lijinsky *et al.*, 1973). The nitrosation of secondary amines with nitrite to produce nitrosamines has been shown to occur *in vitro* in human gastric juice (Sen *et al.*, 1969) and this has supported speculation about a possible role for nitrosamines in the aetiology of human gastric cancer.

The rate of nitrosation is influenced by nitrite concentration (Mirvish, 1970), pH (Mirvish, 1970), bacteria (Hawksworth and Hill, 1971) and by certain inorganic ions. Nitrite was reported to be present in 'detectable quantities' in the acid fasting gastric juice of normal subjects who smoked three cigarettes during two hours of continuous gastric aspiration (Schweinsberg, 1974). However, this was not confirmed by the same workers who were able to detect nitrite in neutral gastric juice only (Schweinsberg *et al.*, 1975). However, nitrite is undoubtedly present in saliva, presumably as a result of bacterial reduction of nitrate, as pure parotid duct saliva is free of nitrite (Tannenbaum *et al.*, 1974).

Thiocyanate is a potent catalyst of nitrosation (Boyland and Walker, 1974) and is also normally present in saliva (Langman *et al.*, 1966) and its presence in gastric juice has therefore been assumed (Boyland and Walker, 1974).

This study was initially undertaken to establish whether nitrite and thiocyanate are present in gastric juice, and subsequently extended to measure the concentrations of nitrite and thiocyanate in saliva, as a possible source of that found in gastric juice.

Methods

PATIENTS

The nitrite and hydrogen ion concentrations in the fasting gastric juice of 17 consecutive patients undergoing routine pentagastrin tests in the course of gastrointestinal investigation were measured. Ten patients had chronic duodenal ulcers, three had chronic gastric ulcers, including one with additional duodenal ulcer, and in five patients no evidence of gastroduodenal ulcer was found. No patient had gastric cancer. Of these 17 patients 12 were smokers,

Received for publication 4 August 1976

defined as taking at least 10 cigarettes or the equivalent amount of tobacco per day, and five were non-smokers. Of the smokers eight had duodenal ulcer, two had gastric ulcer, and two were normal subjects—that is, they had no gastroduodenal pathology—and of the non-smokers one had duodenal ulcer, one had both duodenal and gastric ulcer, and three were normal subjects. There were 14 men and three women and the mean age was 47 ± 5 years. The fasting gastric juice was aspirated via a nasogastric tube before a standard pentagastrin test, using 6 μg pentagastrin per kg body weight. The nitrite and hydrogen ion concentrations of 15 minute collections of gastric juice to 90 minutes were measured.

The thiocyanate concentrations were measured in fasting and pentagastrin stimulated gastric juice of 21 patients, comprising the initial 17 patients and four others in whom there was insufficient gastric juice for the nitrite assay. Of these 21 patients there were 16 smokers, comprising nine with duodenal ulcer and three normal subjects; and five were non-smokers, of whom three were normal subjects, one had duodenal ulcer, and one had both duodenal and gastric ulcer. There were 17 men and four women and the mean age was 43 ± 7 years.

No analysis of the results in terms of age and sex was attempted in view of the small number of females, and major diagnostic differences in different age groups.

After detecting both nitrite and thiocyanate in gastric juice we proceeded to examine specimens of saliva as a possible source. The nitrite concentration in saliva was measured in 19 subjects, consisting of nine of the patients undergoing pentagastrin tests and 10 normal volunteers. The thiocyanate concentration in saliva was measured in 18 subjects, consisting of nine of the patients undergoing pentagastrin tests and nine normal volunteers. In five subjects the salivary nitrite concentration before and after pentagastrin was measured, and in four subjects the salivary thiocyanate before and after pentagastrin was measured.

PROCEDURE

Approximately 10 ml of saliva was collected by expectoration into a sterile tube after overnight fasting. For the pre- and post-pentagastrin studies saliva was collected in the same way for approximately 15 minutes before the pentagastrin, and for approximately one hour after the injection. All specimens were kept at −20°C until assayed.

For the nitrite assay a 4 ml sample of gastric juice, or 2 ml of saliva, was buffered to pH 9·4 with 5% borax buffer in deionised water. The sample was deproteinised using zinc sulphate in dilute acetic acid, and potassium ferrocyanide in deionised water (Adriaanse and Robbers, 1969). The diazotisation of sulphonilamide was measured by coupling with 0·1% NED (N-(1-napthyl)-ethylenediamine hydrochloride) (Shinn, 1941). Great care was taken to ensure freedom from contamination with nitrite of each batch of reagents and diluents. The coefficient of variation of the differences between duplicate assays of 52 specimens of gastric juice was 15%.

For the thiocyanate assay 2 ml 0·1 M ferric alum was added to 1 ml saliva, or 6 ml 0·1 M ferric alum to 3 ml gastric juice (Johnson, 1916). The solutions were filtered through glass fibre paper and read on the Unicam SP 500 spectrophotometer at 450 nm against a blank of 1 ml distilled water with 2 ml Ferric alum. A standard curve was prepared using 0·5 to 2 mM potassium thiocyanate. Hydrogen ion was measured by titration to pH 7·4. All determinations were performed in duplicate and the mean value taken. Student's t test was used to calculate the significance of differences between groups of measurements.

Results

STUDIES ON GASTRIC JUICE

Nitrite was present in all 17 samples of fasting gastric juice and the mean concentration was 4·9 ± 1·1 μM (mean ±SEM). The mean nitrite concentration in 12 smokers, 4·4 ± 1·6 μM did not differ significantly from five non-smokers 5·2 ± 1·5 μM. After pentagastrin stimulation there was the anticipated increase in volume and hydrogen ion concentration of gastric juice. In these 17 patients the mean hydrogen ion concentration increased significantly from a basal level of 54 ± 6·8 mM to a maximum of 116·2 ± 4·5 mM at 45 minutes ($P < 0·01$). The volume of gastric juice, expressed as millilitres per 15 minutes, increased from a mean basal level of 21·2 ± 2·4 to a maximum of 88·8 ± 10·8 ml at 15-30 minutes ($P < 0·001$). However, there was no significant change from the basal level in the mean nitrite concentration throughout the whole period of stimulation (Fig. 1). The minimal nitrite concentration was 4·5 ± 1·0 μM, at 45-60 minutes, and the maximal nitrite concentration was 5·8 ± 1·3 μM at 75-90 minutes.

Thiocyanate was present in the fasting gastric juice of all 21 subjects examined, at a mean concentration of 0·9 ± 0·1 mM. The mean thiocyanate level in 16 smokers, 1·1 ± 0·1 mM was significantly greater than in five non-smokers, 0·4 ± 0·1 mM ($P < 0·02$). After pentagastrin stimulation the mean basal thiocyanate concentration fell significantly from 0·9 ± 0·1 mM to a minimum of 0·3 ± 0·04 mM at 30-45 minutes ($P < 0·01$). At the

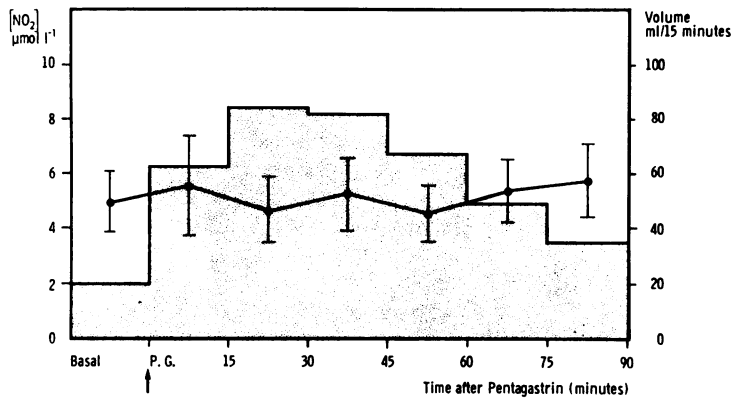

Fig. 1 *Nitrite concentrations, mean ± SEM and volume of gastric aspirate after pentagastrin.*

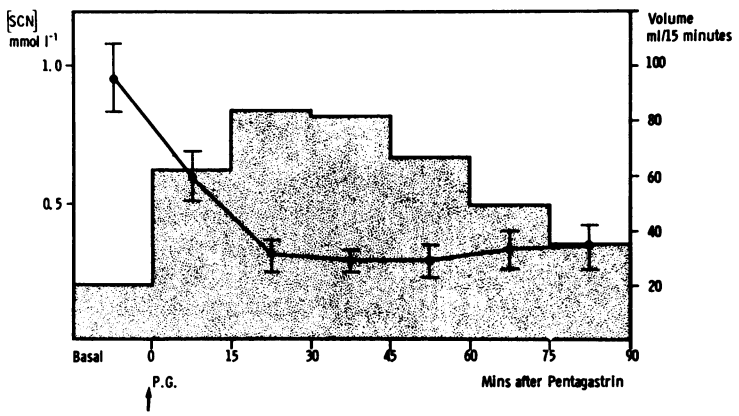

Fig. 2 *Thiocyanate concentrations, mean ± SEM and volume of gastric aspirate after pentagastrin.*

same time the volume of gastric aspirate increased significantly from 20·5 ± 2·2 ml per 15 minutes to a maximum of 86·1 ± 9·8 ml per 15 minutes ($P < 0.01$) (Fig. 2).

STUDIES ON SALIVA

Nitrite was present in the fasting saliva of all 19 subjects examined, mean 100·4 ± 12·4 μM. The salivary nitrite concentration of 10 smokers, 101·2 ± 18·1 μM was not significantly different from that of nine non-smokers, 99·6 ± 18 μM.

In five subjects in whom the pre- and post-pentagastrin salivary nitrite was measured, the mean pre-pentagastrin level 74·5 ± 9·7 μM did not differ significantly from the mean post-pentagastrin level 74·3 ± 13·9 μM.

Thiocyanate was detected in all 18 specimens of saliva examined, mean concentration 1·6 ± 0·3 mM. The salivary thiocyanate of nine smokers 2·3 ± 0·4 mM was significantly greater than that of nine non-smokers, 0·9 ± 0·1 ($P < 0.01$). In four subjects the pre-pentagastrin salivary thiocyanate concentration 0·9 ± 0·2 mM did not differ significantly from the post-pentagastrin concentration 0·9 ± 0·1 mM.

Discussion

Interest in possible nitrosamine carcinogenesis in man has focused on the ingestion of exogenous nitrosamines formed in certain foodstuffs and during cooking, and the theoretical dangers of a high dietary intake of nitrate or nitrite (Hill *et al.*, 1973). This study establishes that small amounts of nitrite, an essential precursor to nitrosamine formation, are present in fasting and stimulated gastric juice, together with thiocyanate, a powerful catalyst of the nitrosation reaction. Potentially nitrosatable amines are also found in normal gastric juice (Heathcote and Washington, 1965), suggesting the possibility of intragastric nitrosamine formation from entirely endogenous substrates.

We have confirmed the presence of nitrite in saliva, which is thus a possible exogenous source of

the gastric nitrite. However, if this were so, a substantial drop in concentration would be expected when gastric secretion is stimulated by pentagastrin. It can be seen from our results that this does not occur and there was no significant change in the nitrite concentration of aspirated gastric juice throughout the test, despite a greater than fourfold increase in volume secretion. Thus a salivary origin could be supported only if pentagastrin caused a parallel increase in either salivary flow or nitrite concentration. Clendinnen *et al.* (1970) have shown no effect on salivary flow in the dog after intravenous infusion of pentagastrin, and we have shown no change in salivary nitrite concentration after pentagastrin. This suggests that a purely salivary origin for the nitrite in fasting and stimulated gastric juice is unlikely, although it may be a contributory factor. Active gastric secretion of nitrite could explain these results, or the maintenance of a steady low nitrite concentration may represent an equilibrium value that is established in the physiochemical environment of acid gastric juice, by the interaction between nitrite and other reactive substances such as amines and urea.

Thiocyanate was also present in all samples of fasting gastric juice and the overall mean concentration was 0·9 mM. Thiocyanate is a powerful catalyst of nitrosation, and a thiocyanate concentration of 1 mM reduces the optimal pH for the *in vitro* nitrosation of N-methylaniline from pH 3 to 1·5 and accelerates the reaction by 550 times (Boyland and Walker, 1974). Thus if nitrosation is occurring *in vivo* the concentration found in gastric juice will greatly increase the reaction rate.

The thiocyanate level in the gastric juice of smokers was approximately twice that in non-smokers, and the same relationship was found in saliva, presumably due to absorption and metabolism of cyanide compounds in tobacco smoke (Boyland and Walker, 1974). This suggests that the higher concentration of thiocyanate in the gastric juice of smokers is a reflection of salivary differences, and that swallowed saliva is the source of gastric thiocyanate. This hypothesis is supported by secretory data. The mean volume of gastric aspirate increased by a factor of 4·2 after pentagastrin. Assuming that this indicates the increased rate of gastric secretion, then a drop in thiocyanate concentration from the basal level of 0·9 mM to 0·2 mM would be produced by simple dilution, compared with the measured minimum of 0·3 mM. This difference could be accounted for by small amounts of saliva swallowed during the test. These findings suggest that the thiocyanate present in gastric juice is exogenous, and probably salivary in origin.

Cigarette smoke contains oxides of nitrogen which could theoretically contribute to the nitrite content of saliva and gastric juice (Boyland and Walker, 1974). However, we have shown no difference between smokers and non-smokers which suggests that smoking does not increase the nitrite concentration of gastric juice, while epidemiological studies show that smokers do not carry an increased risk for gastric cancer (Zacho *et al.*, 1971).

The significance of small amounts of nitrite in gastric juice is difficult to assess, but the presence of thiocyanate may increase the possibility of nitrosamine formation. The lowest concentration of nitrite so far shown to produce a measurable amount of nitrosamines in body fluids is a salivary concentration of 60 μM (Tannenbaum *et al.*, 1975). There was no correlation between nitrite and hydrogen ion concentrations in the acid pH range we have studied, but a high nitrite concentration has been reported in neutral gastric juice (Schweinsberg, 1975). This may contribute a carcinogenic risk by allowing intragastric nitrosation to occur. The yield of nitrosamines depends on nitrite concentration (Mirvish, 1970) and may be further stimulated in the neutral stomach by bacterial catalysis (Hawksworth and Hill, 1971).

We thank Dr T. D. Kellock and Dr J. J. Misiewicz for allowing us to study their patients, and Dr E. N. Rowlands and Dr H. S. Wiggins for helpful advice and criticism. The assistance and expertise of Staff Nurse Sue Waterman is gratefully acknowledged. Dr. C. L. Walters acknowledges the financial support of the Cancer Research Campaign.

References

Adriaanse, A., and Robbers, J. E. (1969). Determination of nitrite and nitrate in some horticultural and meat products, and in samples of soil. *Journal of the Science of Food and Agriculture*, **20**, 321-325.

Boyland, E., and Walker, S. A. (1974). Effect of thiocyanate on nitrosation of amines. *Nature*, **248**, 601-602.

Clendinnen, B. G., Reeder, D. R., Davidson, W. D., and Thompson, J. C. (1970). Effect of gastrointestinal hormones on salivation in the dog. *Archives of Surgery*, **101**, 596-598.

Fujita, M., Taguchi, T., and Takami, M. (1974). Carcinoma and related lesions in dog stomach induced by oral administration of N-methyl N-Nitro N-Nitrosoguanidine. *Gann*, **65**, 207-214.

Fukushima, S. (1973). The combined effects of N-methyl N-Nitro N-Nitrosoguanidine and 20 methylcholanthrene associated with surfactants on the induction of cancer of the glandular stomach in rats. *Nagoya Medical Journal*, **18**, 143-155.

Hawksworth, G., and Hill, M. J. (1971). Bacteria and the N-Nitrosation of secondary amines. *British Journal of Cancer*, **25**, 520-526.

Hawksworth, G., and Hill, M. J. (1974). The in vivo formation of N-Nitrosamines in the rat bladder and their subsequent absorption. *British Journal of Cancer*, **29**, 353-358.

Heathcote, J. G., and Washington, R. J. (1965). Amino acids and peptides in human gastric juice with particular

reference to pernicious anaemia: a review. *Nature*, **207**, 941-944.

Hill, M. J., Hawksworth, G., and Tattersall, G. (1973). Bacteria, nitrosamines, and cancer of the stomach. *British Journal of Cancer*, **28**, 562-567.

Johnson, M. O. (1916). On the determination of small quantities of hydrocyanic acid. *Journal of the American Chemical Society*, **38**, 1230-1235.

Langman, M. J. S., Doll, R., and Wilson, J. (1966). Plasma and salivary thiocyanate in gastric cancer. *Gut*, **7**, 549-552.

Lijinsky, W., and Epstein, S. S. (1970). Nitrosamines as environmental carcinogens. *Nature*, **225**, 21-23.

Lijinsky, W., Taylor, H. W., Snyder, C., and Nettesheim, P. (1973). Malignant tumours of liver and lung in rats fed aminopyrine or heptamethyleneimine together with nitrite. *Nature*, **244**, 176-178.

Magee, P. N., and Barnes, J. M. (1967). Carcinogenic nitroso compounds. *Advances in Cancer Research*, **10**, 163-246.

Mirvish, S. S. (1970). Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. *Journal of the National Cancer Institute*, **44**, 633-639.

Schweinsberg, F. (1974). Catalysis of nitrosamine synthesis. In *N-Nitroso Compounds in the Environment*. Edited by P. Bogovski and E. A. Walker. IARC Scientific Publications No. 9, WHO (Lyon), 80-85.

Schweinsberg, F., Sander, J., Schweinsberg, E., and Kollat, P. (1975). Untersuchungen über die Nitrosierbarkeit von Nicotin und Nornicotin und über die Frage der Bildung von N-Nitrosonornicotin im Magen von Rauchern. *Zeitschrift für Krebsforschung*, **84**, 81-87.

Sen, N. P., Smith, D. C., and Schwinghamer, L. (1969). Formation of N-Nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Food and Cosmetic Toxicology*, **7**, 301-307.

Shinn, M. B. (1941). Colorimetric method for determination of nitrite. *Industrial and Engineering Chemistry (Analytical Edition)*, **13**, 1, 33-35.

Tannenbaum, S. R., Archer, M. C., and Wishnok, T. S. (1976). Nitrosamine formation in saliva. In *Proceedings of the Fourth Meeting on the Analysis and Formation of N-Nitroso Compounds, Tallinn, Estonia, 1975*. (In press).

Tannenbaum, S. R., Sinskey, A. J., Weisman, M., and Bishop, W. (1974). Nitrite in human saliva. Its possible relationship to nitrosamine formation. *Journal of the National Cancer Institute*, **53**, 79-84.

Zacho, A., Neilson, J., and Larsen, V. (1971). Smoking habits of patients with gastric cancer. *Acta Chirurgica Scandinavica*, **137**, 455-458.