Vitamin E and carotenoids in gastric biopsies: the relation to plasma concentrations in patients with and without Helicobacter pylori gastritis1-3

Marion J Sanderson, Kay LM White, Ian M Drake, and Christopher J Schorah

ABSTRACT  The carotenoids—lycopene and α- and β-carotene—and α-tocopherol were measured in plasma and in mucosal biopsies in normal subjects and in those infected with Helicobacter pylori. Two indexes of the presence of the reactive oxygen species malondialdehyde and chemiluminescence were measured in biopsies taken from adjacent sites in the same patient. In general, plasma and mucosal concentrations of all antioxidants correlated well and were of a similar order of magnitude in plasma and mucosa. There was no significant difference between the slope of the regression lines nor an overall difference in the concentrations of these antioxidants between the H. pylori-positive and control groups, indicating an absence of effect of H. pylori infection. However, a marked difference was seen in chemiluminescence and malondialdehyde concentrations in biopsies. For chemiluminescence this was highly significant. These findings confirm the presence of free radicals in the mucosa of H. pylori-infected patients and suggest therefore that the lipid-soluble antioxidants have either no role in protecting mucosal cells from free radical damage or, if they are able to scavenge these species, they are then rapidly regenerated to their original forms by redox and other processes.  Am J Clin Nutr 1997;65:101-6.

KEY WORDS  Lycopene, β-carotene, α-tocopherol, gastric mucosa, Helicobacter pylori, reactive species

INTRODUCTION

Infection of the gastric mucosa with Helicobacter pylori stimulates an inflammatory response (1) with concomitant release of reactive oxygen species (ROS), and it is already known that water-soluble antioxidants in the gastric environment, such as ascorbic acid, become depleted as a result (2, 3). This study aims to discern whether the fat-soluble antioxidants α-tocopherol, lycopene, and β-carotene are also involved in protecting the gastric mucosa from the damaging effects of ROS by comparing plasma and biopsy concentrations in patients with H. pylori–associated gastritis and subjects with normal gastric mucosa.

Generally, plasma concentrations of these compounds are considered an index of tissue vitamin status; however, this is difficult to confirm because of the paucity of information on tissue concentrations as they relate to plasma concentrations, especially in humans. Indeed, it has already been shown that in cirrhosis of the liver plasma concentrations of carotenoids do not necessarily reflect tissue concentrations (4). Existing data on carotenoid concentrations in tissues come from postmortem specimens, for which corresponding plasma samples are not available. Data exist on a few subjects for adipose tissue (5) and skin (6) but no conclusions about the relation of tissue and plasma concentrations can be drawn from these limited studies.

We developed a method to measure the concentrations of carotenoids and vitamin E in small samples of gastric mucosa taken from patients attending an endoscopy clinic. This enabled us to compare plasma and tissue concentrations in patients with H. pylori–associated gastritis and in subjects found to be normal at endoscopy. Two indexes of free radical activity, malondialdehyde concentrations (7) and chemiluminescence (8), were also measured in biopsies obtained simultaneously from adjacent sites to assess the potential for oxidative damage from these reactive species.

SUBJECTS AND METHODS

Patients

The subjects were 44 patients attending an endoscopy clinic for the first time because of symptoms of dyspepsia. Several biopsies were taken from the antrum with a video endoscope (Keymed, Southend-on-Sea, United Kingdom) by using biopsy forceps (Olympus Optical, Hamburg, Germany); those for biochemical analysis were immediately frozen in liquid nitrogen and stored at −70°C. Three of the biopsies were used to determine the presence of gastritis and H. pylori status by histologic criteria using a modified Giemsa stain, by a urease test (CLO/Delta West Pty Ltd, Bentley, Australia), and by microbiological tests involving sensitivity of the resulting culture to various antibiotics. In patients with gastritis, all three criteria had to indicate the presence of H. pylori for patients to

1 From the Department of Chemical Pathology and Immunology and the Centre for Digestive Diseases, The General Infirmary, Leeds, United Kingdom, and the Division of Clinical Sciences, Chemical Pathology, The University of Leeds, United Kingdom.
2 We thank the Kellogg Company, Manchester, United Kingdom, for financial assistance to cover consumables.
3 Address reprint requests to MJ Sanderson, Department of Chemical Pathology and Immunology, Old Medical School, Leeds LS2 9JT, United Kingdom.
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be selected for the *H. pylori*–positive gastritis group \(n = 23\). Although all patients had come to the clinic with symptoms of dyspepsia, this does not necessarily implicate the stomach. If all three tests were negative, and there was no histologic evidence of gastritis or other stomach disease, they were classified as normal \(n = 21\). Patients with *H. pylori*–negative gastritis, including chemical gastritis and lymphocytic gastritis or other diseases of the stomach, were excluded. Of 21 patients classed as normal, 10 were men and 11 were women, with an age range of 20–57 y (mean: 41 y); 5 were smokers. Of 23 patients who were diagnosed as having *H. pylori* gastritis, 16 were men and 7 were women. They ranged in age from 28 to 67 y (mean: 49 y); six were smokers. All patients had fasted overnight. At the time of endoscopy, blood samples were taken into heparin, the resulting plasma being separated and stored at \(-70^\circ\text{C}\).

### Chemicals

Lycopene, \(\alpha\)-carotene, \(\alpha\)-tocopherol, and the enzymes used for digestion of the biopsies—collagenase from *Clostridium histolyticum* (product no. C7657) and pronase E from *Streptomyces griseus* (product no. P6911)—were obtained from the Sigma Chemical Co, Poole, United Kingdom.

### HPLC equipment

A Waters M6000A pump (Milford, MA) was used in conjunction with two ABI 785A detectors (Advanced Biosystems Ltd, Warrington, United Kingdom), one with a tungsten lamp set at 450 nm for carotenoids and one with a deuterium lamp set at 292 nm for plasma \(\alpha\)-tocopherol or 295 nm for biopsy \(\alpha\)-tocopherol. The chromatography data were analyzed by using the Gilson 715 data handling software (Gilson Medical Electronics, Villiers-le-Bel, France). Sampling of the plasma carotenoid extract was effected by an M660 autosampler (Kontron Instruments, Milan, Italy) fitted with a 40-\(\mu\)L loop, but for the biopsy extract, for which almost the whole sample had to be injected, manual injection (50 \(\mu\)L) was effected by using a Waters U6K injector. The column, an Excel C18 Spherisorb (5 \(\mu\)m; Hichrom Ltd, Reading, United Kingdom), 4.2 mm \(\times\) 25 cm, was used without a guard column and no problems were encountered.

### Plasma carotenoids

Plasma carotenoids and \(\alpha\)-tocopherol were analyzed by using tocopherol acetate as an internal standard as described by Thurnham et al (9). The extraction used was similar to that used by Thurnham et al except that hexane was used instead of heptane to extract the carotenoids from the plasma ethanol mixture, and after the addition of hexane, shaking was effected by a Baxter multitube vortexer (Alpha Laboratories, Eastleigh, United Kingdom). The mobile phase comprised methanol:acetoni- trile:chloroformethane (5:4:1, by vol) containing butylated hydroxytoluene (20 mg/L), ammonium acetate (500 mg/L), and triethylamine (250 \(\mu\)L/L). The flow rate was 0.9 mL/min. Chromatography data were collected by using Gilson 715 software. The baseline was subsequently edited to enable the isomers of lycopene to be measured together. The between-batch CV for \(\alpha\)-tocopherol was 4%, for lycopene 8%, and for \(\beta\)-carotene 5%. When plasma samples were supplemented with \(\alpha\)-tocopherol and \(\beta\)-carotene, recovery was 95–100% and 85–95%, respectively.

### Biopsy carotenoids

The method used for the biopsy carotenoids and \(\alpha\)-tocopherol was based on that of Peng et al (10), but with several modifications to adapt it to the small amounts of tissue available (6 mg) and to overcome the problems associated with the presence of mucus in gastric biopsies.

Biopsies were removed from the freezer just before analysis and kept on ice as each one was in turn blotted, weighed, and reduced in size with a scalpel if it weighed \(>7.0\) mg. Each biopsy was placed in a 5-mL glass screw-capped tube, and a few crystals of butylated hydroxytoluene were added plus 20 \(\mu\)L of a freshly made collagen solution (20 g/L containing 5 mmol CaCl\(_2\)/L). The tubes were capped and incubated in a water bath at 45 °C for 15 min. On removal from the bath 20 \(\mu\)L of a freshly made pronase E solution (30 g/L in 0.02 mol phosphate buffer/L, pH 7.4) was added to each tube, which was then placed in ice. In turn, each softened biopsy, together with enzymes and butylated hydroxytoluene crystals, was transferred to a hand-held glass microhomogenizer (catalog no. TKW-300-030T; Fisons, Middlewich, United Kingdom), and was homogenized for 90 s. The homogenate was then returned to its original tube by using a glass Pasteur pipette. The homogenizer was washed twice by adding first 40 \(\mu\)L 5 mmol CaCl\(_2\)/L and plunging the pestle a few times; the procedure was then repeated with 40 \(\mu\)L water, each time returning the washing to the original homogenate. The tubes were finally incubated in a water bath at 45 °C for 15 min. At the end of this time, without delay, the carotenoids and \(\alpha\)-tocopherol were extracted as follows. A freshly prepared solution (0.12 mL) of 95% ethanol containing 800 mg ascorbic acid/L, 0.55 g sodium dodecyl sulfate/L, and 125 mg tocopherol acetate/L was added to each tube. This solution was added and immediately vortex mixed for exactly 4 s before proceeding to the next tube. Then 0.7 mL hexane was added to each tube. After the tubes were capped they were shaken in a multivortexer for 2 min on setting 4. They were then centrifuged at 200 \(\times\) 3 min at 20 °C, and the upper hexane layer was transferred to a clean 5-mL tube.

The residue was extracted a second time with a further 0.7 mL hexane, this time shaking for only 30 s. The tubes were centrifuged as before and the supernate added to the first hexane extract. The solvent was blown off under a stream of nitrogen in a Dri-block (Techno, Cambridge, United Kingdom) at 40 °C. The residue was reconstituted in 60 \(\mu\)L ethanol and transferred to 0.3-mL glass autosampler vials, which were capped and stored overnight at \(-70\) °C. All these procedures were carried out in subdued light. The following day the samples were injected onto the HPLC column with standards every six samples by using the same mobile phase as for plasma samples. Quality-control material comprised 6-mg portions of a sheet of mucosa stripped from a normal area of a resected human stomach.

The lycopene isomers were measured as one peak and \(\alpha\)- and \(\beta\)-carotene peaks were also measured. The early part of the biopsy chromatogram was difficult to interpret, and peaks eluting before lycopene were not measured, hence the flow rate could be speeded up to 1.3 mL/min as these samples were manually injected. Peaks were identified by comparison with
standards. The between-batch CV for α-tocopherol was 15%, for β-carotene was 7%, and for lycopene was 20%. To determine recoveries, some large biopsies were cut in half and aliquots (10 μL) of ethanolic solutions of α-tocopherol, lycopene, and β-carotene were added to one-half of the biopsy at the pronase stage of the assay. Recoveries of α-tocopherol, β-carotene, and lycopene were 95–100%, 70–75%, and 60–75%, respectively. Malondialdehyde was measured by the method of Yagi (7), adapted for tissues. Chemiluminescence in biopsies was measured by the method of Davies et al (8). Plasma cholesterol was measured with a Hitachi 747 (Boehringer Mannheim GmbH, Mannheim, Germany), using the standard Boehringer Mannheim cholesterol method.

**Statistics**

Plasma and biopsy values for carotenoids and α-tocopherol were compared by using Pearson correlations. The mean values for malondialdehyde, chemiluminescence, the carotenoids, and α-tocopherol in both biopsies and plasma were compared in the two groups by using the Wilcoxon Mann-Whitney test. To test whether the regression lines for α-tocopherol and the carotenoids in the control and patient groups were significantly different, a test for equality of the slopes was performed. DDU software (University of Leeds, United Kingdom) was used for the statistical analyses.

**RESULTS**

A comparison of a chromatogram of plasma carotenoids with a chromatogram of carotenoids extracted from a gastric biopsy from the same patient is shown in Figure 1. A similar pattern was observed in both samples, and this resemblance was typical. However, even if α- and β-cryptoxanthin were present in

![Figure 1](https://academic.oup.com/ajcn/article-abstract/65/1/101/4655415)
plasma, they were rarely observed in the corresponding biopsy except in minimal concentrations. The peaks in the early part of the chromatogram were poorly characterized and hence only the values for lycopene and β-carotene are reported.

Scatter diagrams of individual biopsy carotenoids and α-to
copherol plotted against their corresponding plasma concentrations are shown in Figure 2. For each analyte the concentrations were of a similar order of magnitude in both plasma and mucosa. In Figure 2A, lycopene in plasma correlated well with lycopene in biopsies (control subjects: \( r = 0.77, P < 0.0001 \); gastritis patients: \( r = 0.79, P < 0.0001 \)). A similar correlation was observed in Figure 2B for β-carotene (control subjects: \( r = 0.8, P < 0.0001 \); gastritis patients: \( r = 0.65, P < 0.0008 \)). However, when the results for lycopene and α- and β-carotene were combined (Figure 2C) the correlation between values for plasma and biopsies was intermediate between that of lycopene

![Figure 2](https://academic.oup.com/ajcn/article-abstract/65/1/101/4655415)

**FIGURE 2.** Individual carotenoid and α-tocopherol concentrations in gastric biopsies plotted against their corresponding plasma concentrations. A: lycopene; B: β-carotene; C: lycopene plus α- and β-carotene; D: α-tocopherol; and E: α-tocopherol corrected for plasma cholesterol. ●, Patients with gastritis that were positive for *Helicobacter pylori*; ○, control subjects.
and β-carotene (control subjects: \( r = 0.79, P < 0.0001 \); gastritis patients: \( r = 0.74, P < 0.0001 \)). For α-tocopherol (Figure 2D) the correlation between plasma and biopsies was significant (\( r = 0.69, P < 0.0003 \)) in gastritis patients; however, the relation in the control group was obscured by three patients having raised plasma concentrations (\( r = 0.31, P = 0.16 \)). Correction of plasma tocopherol concentrations to a standard cholesterol concentration [5.7 nmol/L, European average (11)] only reduced the correlation further (Figure 2E). Exclusion of smokers from both groups increased the correlation but decreased the significance because of the fewer numbers in the analysis. For all three analytes there was no significant difference in the slope of the regression line between control subjects and gastritis patients.

Mean (± SEM) micronutrient values with indexes of free radical activity as measured by malondialdehyde and chemiluminescence in the biopsies from control subjects and \( H. pylori \)-positive patients are shown in Figure 3. Both malondialdehyde and chemiluminescence were significantly higher in the samples from the gastritis patients than in the control subjects (\( P = 0.01 \) and \( P = 0.0001 \), respectively). In contrast, there was little difference between the patient groups for α-tocopherol or β-carotene concentrations in either biopsies or plasma. However lycopene concentrations were greater in \( H. pylori \)-positive samples for both biopsies and plasma, but were only significantly so for the biopsies (\( P = 0.01 \)).

**DISCUSSION**

The execution of this work has highlighted some of the problems associated with extracting carotenoids and α-tocopherol from tissues. They may be protein-bound and esterified, but methods typically used to break these bonds may be too destructive for such easily oxidized compounds. Until recently, saponification was the standard procedure to hydrolyze conjugates before extraction. However, more recent work shows that gentler enzymic methods give higher yields (10). It is unlikely that any one method is completely suitable for all tissues because their matrices may present particular problems, such as high fat content in adipose tissue and high mucus content in samples derived from the gastrointestinal tract. For gastric mucosa, mucus is contained within the cells and cannot be blotted off. However, an increase in the temperature of the enzyme incubation step to 45°C was sufficient to destroy the occluding properties of contained mucus and enabled better enzyme penetration with more reproducible results. The higher temperature was permissible because pronase, which was already in excess, is thermostable in the presence of calcium ions (12).

Further difficulties were encountered when α-tocopherol was analyzed in tissues (where the wavelength of detection was 295 nm) because of the release of unknown compounds, which gave spurious peaks on the chromatograms if the biopsies had been frozen and thawed, or had not been transferred immediately to −70°C on collection. Therefore, great care was taken to avoid an increase in the temperature of the biopsies before analysis.

In general, we found that the pattern of carotenoids on an HPLC trace from a biopsy reflected fairly accurately the pattern shown in the corresponding plasma sample (Figure 1). Gastric mucosal cells have a short half-life (13) and this may be why plasma concentrations reflect tissue concentrations so precisely. However, some isomerism can be seen to have occurred in the biopsy carotenoids, particularly for β-carotene, for which a cis-isomer can be seen eluting as a shoulder on the right of the main peak. A similar effect was found by Stahl et al (14), who used freeze-dried material and did not perform any enzyme incubations, hence this effect was unlikely to be due to degradation during extraction.

The results shown in Figure 2 enable a comparison to be made, for the first time, between gastric mucosa concentrations of vitamin E and carotenoids and corresponding plasma concentrations. It appears that, in general, the concentrations were of the same order of magnitude in plasma and mucosa, and no mechanism exists for concentrating these compounds similar to that seen in the adrenal gland (15) or in the retina (16). This

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**FIGURE 3.** Micronutrient changes compared with changes in malondialdehyde (MDA) and chemiluminescence in control subjects and in \( H. pylori \)-positive patients. P, plasma; B, biopsy; β-caro, β-carotene; lyco, lycopene; α-toc, α-tocopherol; Chemi, chemiluminescence. (cpm × 10⁻³/mg). \( \bar{x} \pm \) SEM.
finding for fat-soluble antioxidants contrasts with that for vitamin C, a water-soluble antioxidant, which is concentrated in gastric mucosa many times above the corresponding plasma concentration (2, 3). The actual mean (± SD) concentrations of lycopene (226 ± 154 nmol/kg) and β-carotene (172 ± 132 nmol/kg) found in normal mucosa by us were of the same order of magnitude as those found in nonfatty tissues, i.e., heart, thyroid, and kidney, by Kaplan et al. (15), in colon tissue by Nierenberg and Nann (17), and in ovarian and cervical tissue by Peng et al. (10). For α-tocopherol (16.3 ± 6.6 μmol/kg), concentrations found by Peng et al. were similar, but Nierenberg and Nann reported concentrations in colon tissue that were five times higher than the concentrations we found in gastric mucosa.

The correlation between plasma and biopsy concentrations of carotenoids was strong in each patient group. However, the correlation between α-tocopherol concentration in mucosa and biopsy was masked by the presence of a few outliers. This situation was not improved when plasma α-tocopherol results were corrected for cholesterol concentrations. However, exclusion of smokers from the set did improve the correlation but reduced the significance. Larger numbers are required to establish whether smoking is a confounding factor. Gastric mucosal biopsies sometimes contain a considerable amount of mucus within the cells, in other cases biopsies include a proportion of the intima, hence a certain amount of variability in the data might be attributable to this. Despite this, a surprisingly good correlation was seen between biopsy and plasma lycopene and β-carotene content.

Measurement of chemiluminescence has been shown to be a nonspecific indicator of ROS generation by neutrophils (8), whereas measurement of malondialdehyde reflects the presence of ROS damage. The patients with H. pylori infection showed a modest increase in biopsy malondialdehyde concentrations and a manifold increase in chemiluminescence. These findings confirm that ROS were present in abundance in biopsies from the infected patients. Therefore, it was surprising to find no difference in the concentrations of α-tocopherol and β-carotene in biopsies from patients and control subjects and even an increase in lycopene concentrations in gastritis patients because these micronutrients were hitherto considered to have a significant effect on the defense of the cell against free radical attack. The plasma concentrations of micronutrients were not depleted, indicating that high concentrations in gastritis patients were not due to more rapid removal from the plasma. We conclude that lipid-soluble antioxidants either have no obvious role in protecting mucosal cells from free radical damage or they do indeed have scavenging potential against free radicals, but they can be rapidly converted back to their original form by redox or other processes. For α-tocopherol it is possible that the ascorbic acid present during extraction could reduce the oxidized tocopheroxyl radical back to the reduced form.

There is considerable evidence linking H. pylori infection with gastric cancer (18), the infection eventually giving rise to cellular atrophy and degeneration. By this means, H. pylori can be said to initiate the sequence of events leading to gastric cancer (19).

Of 19 recent studies on the protective action of fruit and vegetables against gastric cancer, 17 showed positive results (20). It is generally assumed that vitamin E and carotenoids contribute to this effect. However, the present study showed that H. pylori–infected mucosal cells, although showing evidence of active free radical formation, were no more depleted of these antioxidant micronutrients than were normal control subjects. It is possible that tissue concentrations could have been maintained in H. pylori–positive patients by more rapid transfer from plasma, but there is no evidence of depleted plasma concentrations in the H. pylori–positive patients. This study therefore provides no support for supplementation with carotenoids in patients with H. pylori–associated gastritis.

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REFERENCES