Nitrite and nitrate levels in ileostomy effluent: effect of dietary change

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1. Nitrite and nitrate levels were measured in samples from ileostomy bags or stomal samples of thirty-one ileostomists (twenty-two ulcerative colitis, nine Crohn's disease), 14–16 h after ingestion of a conventional meal or a meal containing a high content of nitrite and nitrate.

2. Ileostomy samples were decolourized with barium chloride, sodium sulphate and charcoal. Nitrite was determined spectrophotometrically by the Griess reaction and nitrate determined as nitrite after reduction with nitrate reductase (*EC* 1.7.99.4) in the presence of sodium formate. The mean percentage recovery from twenty-six spiked samples was 101·9 (SE 3·5)% for nitrite and 82·9 (SE 3·3)% for nitrate.

3. Ileostomy bag samples were obtained in twenty-nine cases of which ten had measurable nitrite (median 0, range 0–20·7 nmol/g) on a conventional meal compared with twenty-three cases (median 7·2, range 0–31·1 nmol/g) on the test meal ($P < 0.01$). Nitrate levels were measurable in sixteen (median 6·7, range 0–48·2 nmol/g) after a conventional meal compared with twenty-one (median 20·5, range 0–53·2 nmol/g) after the test meal ($P < 0.01$).

4. Stomal fresh-catch samples were obtained in twenty-four cases: combined nitrate and nitrite was higher in eighteen, lower in four and unchanged in two subjects after the test meal ($P < 0.05$).

5. The type of foodstuff ingested can significantly alter measurable levels of nitrite–nitrate in the distal ileum and is one factor determining nitrite-nitrate input into the proximal colon.

The content of nitrite and nitrate in foods (White, 1975; Walker, 1975) has received considerable scientific attention (Ministry of Agriculture, Fisheries and Food, 1987) due to concern that these anions may generate *n*-nitroso compounds which have a carcinogenic potential in the gastrointestinal tract (Macdonald *et al.* 1984; Forman *et al.* 1985). Lately nitrite anions have been shown to interact metabolically with colonic mucosal cells (Roediger *et al.* 1986*c*) and enhance ion absorption and secretion in the colon (Roediger *et al.* 1986*a*; Radcliffe *et al.* 1987). These functions can be regarded as beneficial and suggest that the turnover of nitrite and nitrate is subject to metabolic mechanisms in the gastrointestinal tract which are as yet poorly understood.

Nitrite and nitrate enter the colon from the ileum (Tannenbaum *et al.* 1978; Saul *et al.* 1981; Bartholomew & Hill, 1984), transmurally from the circulation (Thayer *et al.* 1982) or through synthesis of nitrite by inflammatory cells in the lamina propria (Stuehr & Marletta, 1985; Iyengar *et al.* 1987). Ambient levels in the colon depend on a balance between absorption and secretion, as well as metabolism of nitrite by anaerobic bacteria (Hall, 1978) and colonic epithelial cells (Roediger & Radcliffe, 1988). The precise quantities of nitrate and nitrite ions reaching the colon from the upper gastrointestinal tract are controversial. Tannenbaum & Green (1981) reported ileal nitrite concentrations ranging from 350 to 1540 μmol/kg, while Saul *et al.* (1981) reported lower ileal concentrations of nitrite (0–15 μmol/kg) and nitrate (0–7 μmol/kg). There is no standard method for determining nitrite and nitrate in ileal effluent, and wide variations in measured levels observed in the distal gastrointestinal tract have been attributed to the differing methods employed for measuring nitrite and nitrate (Ministry of Agriculture, Fisheries and Food, 1987).

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Table 1. *Composition of test meal and estimated content of nitrate and nitrite derived from food tables (White, 1975; Walker, 1975)*

	Amount (g)	Estimated amount (nmol)	
Foodstuff		NO_3^-	NO_2^-
Frankfurter (two)	120	0·49	0·26
Bacon (three slices)	150	0·42	0·43
Spinach	100	3·06	0·006
Beetroot	50	2·26	0·006
Salad			
Lettuce	150	2·06	0·002
Radishes (five)	50	1·19	
Celery	50	1·89	0·017
Salad dressing as desired	—	—	—
Squash drink	100	0·66	0·002

The present investigation was undertaken to establish a simple method of measuring nitrite and nitrate in ileostomy effluent, and to establish whether dietary change influenced the content of nitrite and nitrate in samples from ileostomies. Such an analysis had not been previously undertaken, and was also carried out to provide further information concerning nitrite and nitrate levels in the distal ileum in health.

METHODS
Subjects and diets

Thirty-one ileostomists were recruited from the Ileostomy Association of South Australia. All but one who had a terminal ileostomy for 6 months, had an ileostomy for at least 2 years. Ileostomies were performed for ulcerative colitis (twelve males and ten females) or Crohn's colitis (two males and seven females). All subjects were free of active disease, had normally functioning ileostomies and were off specific medication. Each subject gave informed consent before participating in the study.

Every subject consumed a self-chosen diet at home before the first visit to donate samples. Wherever possible the test meal was consumed the next evening, the composition of which is shown in Table 1 with approximate values for nitrite and nitrate calculated from analyses of Walker (1975) and White (1975, 1976). Actual nitrite and nitrate intakes were not analysed, but compliance assessed by completion of a questionnaire on the following morning.

Sample collection and processing

Attempts were made to collect two samples at each visit, termed 'bag' and 'stomal' sample. The bag sample was the contents of ileostomy bags present at 09.00 hours and the stomal sample was collected at the same time directly from the ileostomy orifice. Twenty-nine pairs (normal meal and test meal) of bag samples and twenty-four pairs of stomal samples were obtained for analysis.

The ileostomy effluent was collected into preweighed specimen bottles containing 10 ml ice-cold 0·1 M-ammonium chloride buffer, pH 8·2. The filled specimen bottle was weighed again and sufficient NH_4Cl buffer added to give a 1:1 dilution of the effluent. The mixture was homogenized in a Waring blender (three 10 s bursts on high setting) and centrifuged at 12000 g for 30 min at 5° to remove solid debris. The resulting supernatant fraction ranged in colour from pale straw-yellow to green–dark brown. To decolourize the sample 200 mg activated charcoal, 2·0 ml 1·0 M-barium chloride (recrystallized) and 2·0 ml 1·05 M-

sodium sulphate were added to 4·0 ml of the supernatant fraction in centrifuge tubes. The tubes were shaken for 1 h on an end-over-end shaker at room temperature and then centrifuged at 3200 rev./min for 20 min. The supernatant fraction was decanted into clean tubes and re-centrifuged, if necessary, to remove the last traces of charcoal. The resulting decolourized or, on occasion, slightly coloured supernatant was analysed for nitrite and nitrate. A colour blank was run for each sample, and the resulting absorbance used to correct for any residual colour.

It was necessary to recrystallize $BaCl_2$ before use because Reagent Grade $BaCl_2$ contained nitrate (0·01 g/l) which gave unacceptably high background levels at the concentrations found in the ileal effluent. No interference was observed with recrystallized $BaCl_2$ or from Na_2SO_4 or activated charcoal.

Nitrite and nitrate assay

Nitrite was determined spectrophotometrically using the Griess reaction. A portion of 500–1000 μl was brought to 1000 μl with distilled water, and 200 μl sulphanilamide (16 g/l) in 3·6 M-hydrochloric acid added followed by 50 μl N-(1-naphthyl)-ethylenediamine (1 g/l). A colour blank was run for each sample to which 50 μl distilled water was added in place of the N-(1-naphthyl)-ethylenediamine. Absorbance was measured at 540 nm, and the absorbance of each sample corrected by subtracting the absorbance of the appropriate blank.

Nitrate was determined as nitrite after reduction by a formate: nitrate reductase (*EC* 1.7.99.4) prepared from *Escherichia coli B* as previously described (Radcliffe & Nicholas, 1970; Radcliffe *et al.* 1987). Briefly, 500 μl of the extract containing 10–40 nmol nitrite plus nitrate was incubated with 500 μl of an enzyme cocktail containing 0·2 nM-sodium formate, 12·5 mM-Na_2EDTA and 40 μl nitrate reductase/ml in 0·25 M-phosphate buffer (pH 6·0) for 16 h at 37°. Nitrite was determined as described previously. A colour blank was included for each sample, as well as an enzyme blank for each batch of samples. After correction for absorbance contributed by the colour blank, nitrate was calculated as total nitrite measured after incubation minus nitrite originally present in the sample and corrected for nitrate in the enzyme cocktail.

The final pH of the decolourized sample was critical for accurate determination of nitrate by the enzymic reduction to nitrite. The pH must be maintained above 7 to prevent the loss of nitrite that occurs under acid conditions and below 8 to avoid the inhibition or inactivation of nitrate reductase incurred above pH 8 (Davis *et al.* 1985). NH_4Cl buffer (0·1 M) adjusted to pH 8·2 consistently produced a final extract with pH between 7·0 and 8·0. To minimize variability of nitrite estimation due to pH, standard curves were prepared at the same pH as the pH of the sample that was analysed.

Statistical analysis

Values were subjected to analysis by the Mann-Whitney U test or the paired *t* test as appropriate.

RESULTS

In order to estimate the efficiency with which nitrite and nitrate were recovered from ileostomy effluent, a portion of each freshly collected sample was spiked with a known amount of nitrite or nitrate. The spiked samples were subjected to the standard decolourizing procedure. The mean recovery from twenty-six spiked samples was 101·9 (SE 3·5)% for nitrite and 82·9 (SE 3·3)% for nitrate. The coefficients of variation for recoveries of nitrite and nitrate respectively were 3·4 and 4·0%.

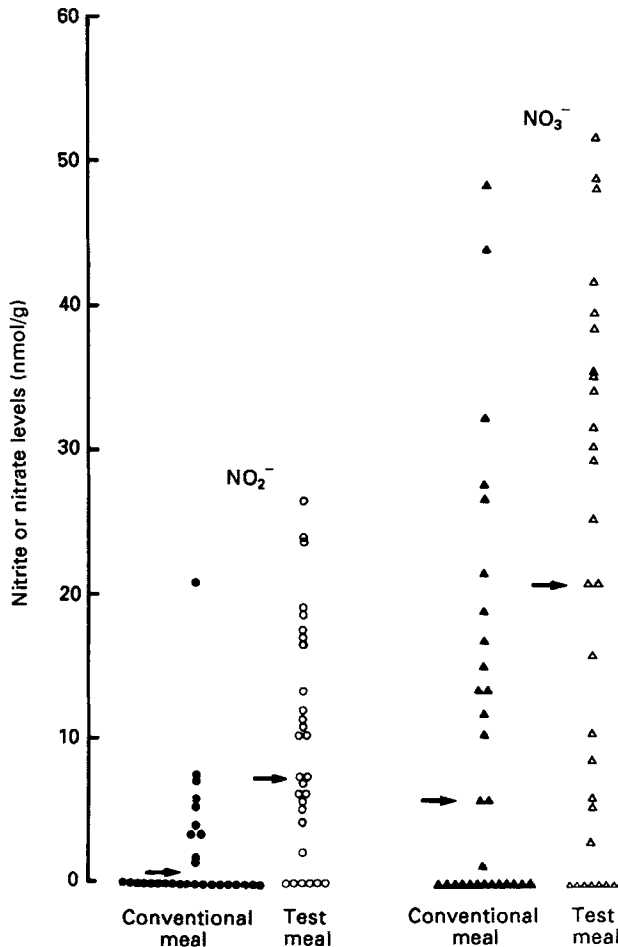Nitrite and nitrate concentrations in ileal effluent were not significantly different with

Fig. 1. Nitrite (O, ●) and nitrate (△, ▲) levels of ilesotomy effluent (bag samples) after a conventional meal (●, ▲) or test meal (O, △), expressed as nmol/g wet weight. →, Median value. For details of meals, see Table 1 and p. 324.

respect to ulcerative colitis or Crohn's colitis groups after either the conventional meal or test meal. The results were, therefore, analysed as a single group without reference to the reason for performing the ileostomy. Compliance with the experimental diet was good, each subject consuming at least 80% of the amount listed in Table 1.

In the bag samples detectable nitrite was found in ten of the twenty-nine samples after the conventional meal compared with twenty-three of the twenty-nine samples after the test meal (Figs. 1 and 2). The median concentration increased from 0 (range 0–20·7) nmol/g after the conventional meal to 7 nmol/g after the test meal. For nitrate, positive samples increased from sixteen of the twenty-nine samples after the conventional meal to twenty-one following the test meal. The median nitrate concentration increased from 6·7 (range 0–48·2) to 20·5 (range 0–53·2) nmol/g for conventional and test meals respectively. Both these increases were statistically significant ($P < 0.01$, Mann–Whitney U test).

For stomal samples, detectable nitrite increased from eight of the twenty-four samples after the conventional meal to sixteen after the test meal; the median nitrite concentration increased from 0 (range 0–29·9) to 5 (range 0–100·8) nmol/g ($P < 0.01$, Mann–Whitney U
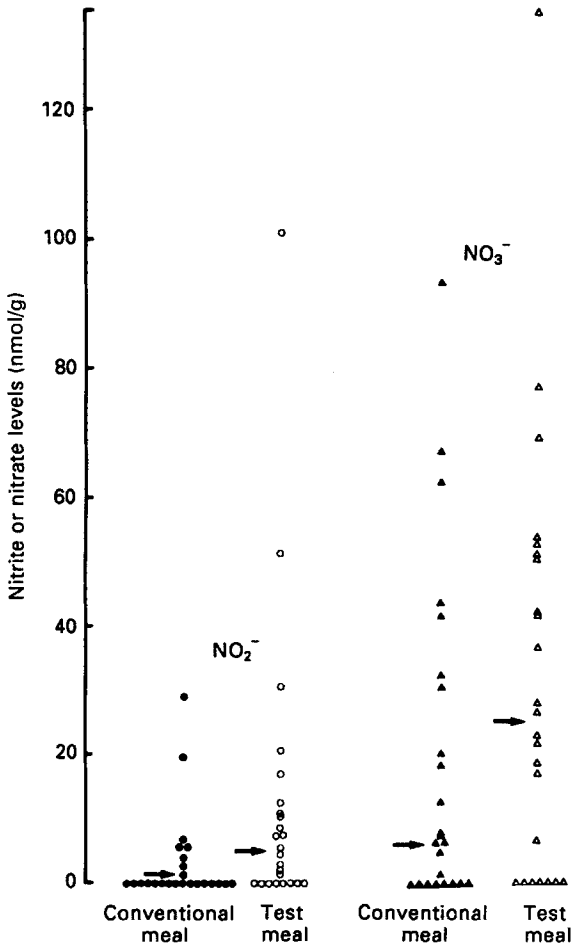
Fig. 2. Nitrite (O, ●) and nitrate (△, ▲) levels of ileostomy effluent (stomal sample) after a conventional meal or test meal, expressed as nmol/g wet weight. →, Median value. For details of meals, see Table 1 and p. 324.
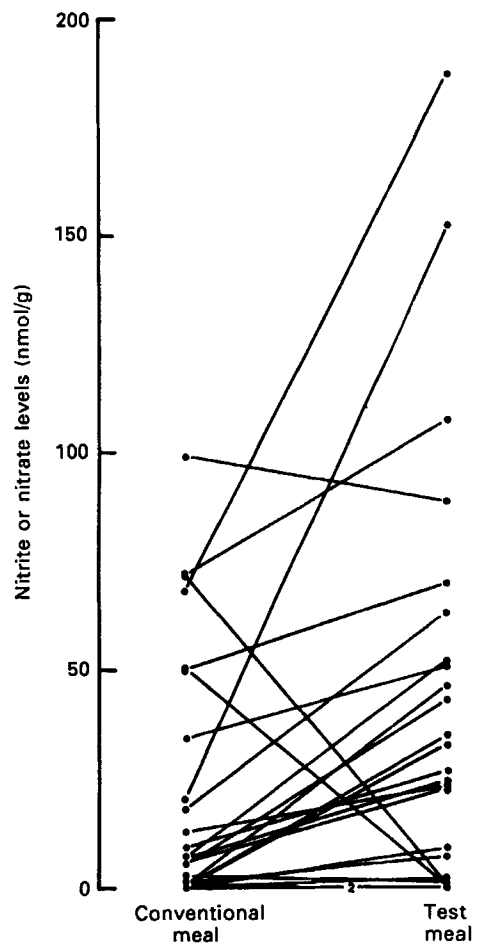
Fig. 3. Comparison of total nitrate and nitrite (expressed as nmol/g wet weight) in stomal samples for each subject after a conventional meal or test meal. For details of meals, see Table 1 and p. 324.

test). Although nitrate also tended to increase after the test meal in these samples (from 6·7 to 25·1 nmol/g), the difference did not reach statistical significance. However, comparing total nitrate plus nitrite in the stomal samples, after the conventional and test meals, eighteen of the twenty-four subjects had a higher total concentration after the test meal than after the conventional meal, four values decreased and two remained the same (Fig. 3). These changes were statistically significant ($P < 0.05$; paired t test).

DISCUSSION

The method of nitrite and nitrate analysis now reported differs from published methods of measuring nitrite and nitrate in biological samples in the manner in which samples were

decolourized. Charcoal with barium chloride and sodium sulphate was used instead of the Carrez reagents (potassium ferrocyanide and zinc sulphate (Ruddell *et al.* 1976; Fox *et al.* 1982) or alumina gel (Stockbrugger *et al.* 1982)) because Carrez reagents may interfere with colour development in the Griess reaction if the ferrocyanide is not completely precipitated, and thus underestimation of the actual level of nitrite may occur (Fox *et al.* 1982). The following method was consequently used: dilution with an alkaline buffer, precipitation of particulate matter followed by adsorption of colour with activated charcoal and recrystallized barium chloride.

Enzymic reduction of nitrate has been used in measurements of nitrate in urine, saliva (Phizackerley & Al-Dabbagh, 1983; Bartholomew 1984; Forman *et al.* 1985) and ileostomy effluent (Bartholomew & Hill, 1984) where enzymic reduction was found superior to cadmium column reduction, a method employed previously in an analysis of nitrate in ileostomy effluent (Saul *et al.* 1981).

The observed recovery rates of nitrite and nitrate in spiked samples of twenty-six cases compare favourably with recoveries in spiked samples of two cases of ileostomy effluent of Saul *et al.* (1981) who reported recoveries of 82 and 76% for nitrite and nitrate respectively. In two further studies (Tannenbaum *et al.* 1978; Bartholomew & Hill, 1984) in which nitrite and nitrate were estimated in ileostomy fluid, recovery rates for nitrite and nitrate were not reported.

Drawbacks of the current study were the lack of consideration of intestinal transit time in each subject and failure to measure the actual amounts of nitrate–nitrite ingested by each subject. Transit times to the distal ileum may vary from 80% recovery of a marker at 8·7 (SD 3·4) h (Holgate & Read, 1983) to 100% recovery of a marker at 11·3 (SD 1·4) h (Chapman *et al.* 1985). Collection times of samples were kept constant and timed to 14–16 h after the meal eaten the previous night.

The experimental diet was chosen from foods likely to contain high nitrite and nitrate levels (Walker, 1975; White, 1975, 1976). Levels of nitrite and nitrate in vegetables vary with the degree of fertilization, plant age, part of plant used and exposure to sunshine (Breimer, 1982; Nelson, 1984; Greenwood & Hunt, 1986), variables that were not controlled in the study. Despite these limitations it was possible to show that an acute change in diet significantly altered the concentration of nitrite in the distal ileum. Ishiwata *et al.* (1975) reported similar changes in response to dietary change when they measured nitrite content of saliva.

Values of nitrite reported for the first time from ileostomies by Tannenbaum *et al.* (1978) were very high, but subsequently stated to be incorrect in view of methodological difficulties experienced with the original analysis (Tannenbaum & Green, 1981). Further studies (Saul *et al.* 1981; Bartholomew & Hill, 1984) recorded much lower values than Tannenbaum *et al.* (1978). In order to facilitate comparison with the previously mentioned studies we expressed our results per unit wet weight, rather than dry weight, which is more accurate as it minimizes variability due to differences in absorption of water. Present concentrations of nitrite and nitrate were similar to those reported by Saul *et al.* (1981), who found nitrate in three and nitrite in seven of eight samples of ileal effluent collected from two subjects consuming a diet of free choice. They reported a concentration range of 0–15·0 nmol/g for nitrite and 0–7 nmol/g for nitrate. Present concentrations in bag samples after conventional meals ranged from 0–20·7 nmol/g for nitrite and 0–48·2 nmol/g for nitrate. Similarly to Bartholomew & Hill (1984) we found that fresh-catch samples contained a higher concentration of nitrate than samples taken from the bag; differences, however, were not statistically significant. In view of the agreement with previous studies, and the larger number of subjects now studied, it seems likely that the values reported provide a good estimate of nitrate and nitrite reaching the distal ileum.

In general the proportion of ingested nitrite and nitrate reaching the colon via the ileal lumen is very small (Ishiwata *et al.* 1981; Bartholomew & Hill, 1984). While this proportion was not currently estimated, the amount of nitrite–nitrate actually entering the colon in total, especially transmurally from the circulation, is far greater than previously thought possible. Measurements indicate that approximately half the nitrate not removed in the urinary tract is transported from the bloodstream to the lumen of the large intestine and removed through metabolism of enteric bacteria (Thayer *et al.* 1982; Schultz *et al.* 1985). Dietary change may thus alter colonic nitrite and nitrate levels by routes other than the ileal lumen.

The demonstration that nitrite and nitrate reach the terminal ileum, and that the concentration of these anions is dependent on dietary intake, is of importance both to the physiological effect, e.g. absorption and secretion (Roediger *et al.* 1986*a*; Radcliffe *et al.* 1987), and pathological effect, e.g. nitrosamine formation (Suzuki & Mitsuoka, 1981), that these ions may exert in the colon. Further work is required to determine the consequences of prolonged exposure of the lower gastrointestinal tract to nitrite and nitrate both in health and in such diseases as ulcerative colitis where high levels of lumen nitrite have been recorded (Roediger *et al.* 1986*b*).

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