

Does increased endogenous formation of *N*-nitroso compounds in the human colon explain the association between red meat and colon cancer?

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High red meat diets have been linked with risk of sporadic colorectal cancer, but their effects on mutations which occur in this cancer are unknown. G→A transitions in *K-ras* occur in colorectal cancer and are characteristic of the effects of alkylating agents such as *N*-nitroso compounds (NOC). We studied the effect of red meat consumption on faecal NOC levels in eight male volunteers who consumed diets low or high in meat (60 or 600 g/day), as beef, lamb or pork, whilst living in a metabolic suite. Increased intake of red meat induced a significant ($P < 0.024$) 3-fold increase from 40 ± 7 to an average of 113 ± 25 µg/day NOC, a range of exposure in faeces similar to that from tobacco-specific NOC in cigarette smoke. The diets were isoenergetic and contained equal amounts of fat, but concentrations of heterocyclic amines were low. Faecal excretion of the promotor ammonia was significantly increased to 6.5 ± 1.08 mmol/day. When the high red meat diets were supplemented with 20 g phytate-free wheat bran in six volunteers there was no reduction in NOC levels (mean 138 ± 41 µg/day NOC), but faecal weight increased. Higher starch and non-starch polysaccharide intakes reduced intraluminal cross-linking in microcapsules ($r = -0.77$) and reduced faecal pH ($r = -0.64$). In two volunteers there was no effect of 600 g white meat and fish on faecal NOC (mean low white meat diet 68 ± 10 µg/day, high white meat diet 56 ± 6 µg/day) nor on faecal nitrate, nitrite and iron. Faecal nitrite levels increased on changing from a white to red meat diet (mean high white meat diet 46 ± 7 mg/day, high red meat diet mean 80 ± 7 mg/day). Increased endogenous production of NOC and precursors from increased red meat, but not white meat and fish, consumption may be relevant to the aetiology of colorectal cancer.

Introduction

Epidemiological studies suggest that the high rates of colorectal cancer in developed countries are potentially preventable by dietary means. National incidence rates for colon cancer are

***Abbreviations:** NSP, non-starch polysaccharides; NOC, *N*-nitroso compounds; HAA, heterocyclic amines; LM, low meat; HRM, high red meat; HRMHB, high red meat high bran; HWM, high white meat; PEI, polyethylenimine; ROM, radio-opaque markers; CPTS, copper phthalocyanine; MTT, mean transit time; IQ, 2-amino-3-methylimidazo(4,5-*f*)quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine).

strongly correlated ($r = 0.85$) with average consumption levels of meat in 23 countries (1). In some prospective studies individuals consuming higher amounts of red or processed meat, but not white meat or fish, experience a greater risk of developing colon cancer (2,3). Vegetarians are known to be at low risk of cancer, including cancer of the large bowel, but it is not clear which aspects of vegetarianism are protective (4,5). Vegetables, starch and non-starch polysaccharides (NSP*), increased stool weight and reduced stool pH are also implicated in reduced risks of colon cancer (6–9). However, the effects of diet on genetic changes which are known in sporadic colorectal cancer have not been established.

G→A transitions at the second G of a GG pair at codon 12 or 13 of *K-ras* are common in colorectal cancer and are characteristic effects of alkylating agents such as *N*-nitroso compounds (NOC*) (10). Human faecal specimens have been shown to contain NOC (11). Alkylated DNA adducts of *O*⁶-methylguanine have been detected in human colonic tissue (12) and *N*-methyl-*N*-nitrosourea induces G→A transitions in codons 12 and 13 of *K-ras* in 30% of rat colon carcinomas (13).

In *p53* mutational C→T transitions at site-specific codons such as 175, 248 and 273 in colorectal cancer (14) are consistent with endogenous deamination mechanisms, for example from nitric oxide generated by nitric oxide synthase (15). Inducible nitric oxide synthase produces continuous amounts of NO[•] from the amino acid arginine (16), so that high protein diets might be expected to increase nitrosation and urinary nitrate, effects which have been shown in animals (17,18). Supplements of nitrate are reduced to nitrite in the colon during dissimilatory nitrate metabolism by the colonic flora and have been shown to elevate faecal NOC levels (11,19). The human colonic lumen is also rich in amines and amides, which are substrates for nitrosation to NOC by NO[•]. The amount of nitrogen as protein and peptides entering the colon and available for amine, ammonia and amide formation is increased by increasing protein intake (20).

We have therefore studied the effect of a 10-fold increase in protein consumption as meat on faecal NOC excretion in humans. The effect of starch and phytate-free bran on NOC excretion and on stool weight, transit time and colonic pH were also assessed. The meat was fed mainly as steaks, fried to maximize heterocyclic amine (HAA) formation (21). Phase I enzymes activate HAA and a greater proportion of patients with large bowel cancer have been shown to be faster oxidizers and acetylators than healthy matched controls (22). We therefore also used the standard caffeine test to phenotype individuals as fast or slow oxidizers on the different diets consumed. The effects of equally high levels of white meat and fish on faecal NOC, nitrite, nitrate and iron levels were also assessed.

Materials and methods

Dietary protocol

Six male volunteers aged 24–32 years were maintained on constant isoenergetic diets for 9 weeks whilst living in the metabolic suite at the Dunn Clinical

Nutrition Centre, where all food was provided and specimens could be collected. Subjects were randomly assigned to either a low (LM) or high red meat (HRM) diet in the first 3 weeks, followed by cross-over for the next 3 weeks and a high red meat, high bran (HRMHB) diet for the last 3 weeks. The diets were isoenergetic and constant in fat throughout. In a subsequent analysis two further male volunteers were fed a low (LM) white meat diet for 5 days, then transferred to a high white meat (HWM) diet for 14 days, followed by a HRM diet for 4 days. The study was approved by the Dunn Nutrition Unit Ethical Committee in 1989.

Diet and energy balance

The LM diet contained 60 g meat/day (Table 1) and the HRM diet 600 g cooked meat, of which 400 g was given as fried beef, lamb or pork steak at the evening meal. The meat was substituted for cream and a glucose syrup drink (Hycal), but the diets were otherwise identical. During the last 21 days the volunteers were fed the HRMHB diet, which was the high red meat diet with a supplement of 20 g bran (Trifyba) which contained 14 g NSP and was free of starch, nitrate and phytate. The HWM diet was similar to the red meat diet except that turkey or white fish (given as sweet and sour, lasagne and fish pie recipes) were used for evening meals and chicken, prawns and tuna were used for lunch.

The diets were designed to be constant and low in nitrate by excluding nitrate-rich foods (23,24) and by using double distilled water for cooking and drinking throughout. Only one piece of fresh fruit from the same supply throughout was allowed each day. Meat was bought in advance, defatted, weighed out into individual portions and deep frozen until required. Before serving, the evening meal steaks in the HRM diet were fried in 20 g butter for 3 min each side so that the outer temperature at the end of cooking was 180°C, to maximize mutagen content (25). All other food was dried, canned or frozen and bought in advance from the same batches. Table 1 shows sample LM and HRM menus calculated to contain 10 MJ. The LM diet contained 27% energy from fat, 44% sugars, 20% starch and 9% protein and the HRM diet 28% energy from fat, 22% sugars, 20% starch and 30% protein. Both diets contained 13 g NSP/day (26).

In order to maintain body weight individual daily diets were adjusted to match estimated energy expenditure. Volunteers were weighed at the beginning of the study and basal metabolic rate calculated (27). Total energy expenditure was estimated by multiplying basal metabolic rate by appropriate factors to allow for the usual activities of each volunteer (27). To match calculated energy expenditure supplements were then added to the basal diet. Each daily 1 MJ supplement consisted of 50 g white bread, 15 g low fat spread and 20 g marmalade and contained 1.1 g NSP, 7% energy as protein, 27% as fat, 24% as sugars and 42% as starch. Subjects required

between a half and six of these supplements in addition to the basal diet of 10 MJ. Subjects weighed themselves daily and small adjustments to the intake of supplements were made in the first week to maintain body weight. Otherwise the intake of supplements and body weight remained constant for each individual throughout the entire study. Individual intakes of energy therefore ranged from 10.5 to 16 MJ/day, of NSP from 13 to 19 g and of starch from 135 to 280 g/day throughout the study. Duplicate samples of 10 MJ HRM and LM diets were prepared, homogenized in deionized water and freeze dried prior to analysis for HAA content.

Acetylation and oxidation

After an overnight fast, at 7 a.m. on day 8 of each LM, HRM and HRMHB dietary period, subjects took three 50 mg 'Proplus' caffeine tablets and collected urine for the next 4 h and then one further specimen was collected 1 h later. Food and drink was permitted 3 h after the caffeine tablets, but no tea or coffee was allowed during days 6–8 inclusive. Aliquots of 15 ml of each specimen were taken and the pH adjusted to 3.5 with HCl before storage at –20°C prior to analysis for caffeine metabolites by HPLC (28). One subject on the LM diet was unable to produce a specimen for either the first 4 h or second 5 h collection and one subject on the HRM diet did not produce a specimen at 5 h. Means have been calculated from the remaining values.

HAA analysis

Twenty four hour urine was collected on day 9 of the LM, HRM and HRMHB dietary periods without preservative and stored at –20°C. The samples were then defrosted and twice shaken for 30 min with 100 mg blue cotton (Funakoshi Pharmaceutical Co. Ltd, Tokyo, Japan). HAAs were then extracted into methanol/ammonia (50:1), evaporated to dryness under nitrogen and the containers were sealed and wrapped in aluminium foil to exclude light. HAAs were analysed in diets, urine and microcapsules (see below) by HPLC (29).

Faecal NOC, nitrite, nitrate, iron and ammonia

In the first protocol fresh faecal specimens were obtained on day 9 of each LM, HRM and HRMHB dietary period and weighed amounts of 50–150 g were stomached for 60 min within 20 min of collection with an approximately equal weight of weighed 0.3 M NaOH made up with double distilled water. Samples were stored at –20°C until analysis for total apparent NOC by denitrosation with HBr and chemiluminescence detection of the released NO using a thermal energy analyser (30,31).

In the second study of white meat and fish, fresh samples were diluted 1 in 4 with nitrate- and nitrite-free deionized water and stomached for 20 min within 20 min of collection. The supernatant after centrifugation at 4500 r.p.m. was stored at –20°C and analysed for NOC as described above. Nitrite was determined by subjecting the supernatant to the conditions for

Table 1. Menus used for HRM diet (10 MJ)^a

Breakfast

Unsweetened orange juice 100 g
Weetabix 20 g
White bread 50 g
Low fat spread 10 g
Marmalade 20 g
Dried skim milk powder 30 g

Lunch

Day 1	Day 2	Day 3
White bread 100 g	White bread 100 g	White bread 100 g
Low fat spread 30 g	Low fat spread 30 g	Low fat spread 30 g
Chicken 100 g ^a	Chicken 100 g ^a	Turkey 100 g ^a
Pork 100 g ^a	Beef 100 g ^a	Pork 100 g ^a
Branson pickle 25 g	Ketchup 20 g	Mayonnaise 12 g
Digestive biscuit 17 g	Crunch biscuits 20 g	Hobnob biscuits 15 g
Granny Smith apple 150 g	Golden delicious apple 150 g	Orange 150 g

Supper

Day 1	Day 2	Day 3
Canned grapefruit juice 100 g	Canned apple juice 100 g	Canned tomato juice 100 g
Beef steaks 2×200 g ^{a,b}	Pork chops 2×200 g ^{a,b}	Lamb steaks 2×200 g ^{a,b}
Bottled tomato relish 40 g	Bottled corn relish 40 g	Bottled cucumber relish 40 g
Frozen mushrooms 30 g	Frozen peas 30 g	Frozen corn 30 g
Frozen green beans 50 g	Frozen carrots 50 g	Frozen onions 50 g
Frozen microwave chips 100 g	Frozen duchess potatoes 100 g	White rice 40 g
Canned peaches fruit 150 g juice 50 g	Canned apricots fruit 150 g juice 50 g	Canned cocktail fruit 150 g juice 50 g

^aItems altered to obtain the LM diet were: 1.5 bottles Hycal and 60 g double cream included; lunch time meats decreased to 25 and 15 g each (40 g total); supper steaks substituted with 20 g roast meat and 10 g butter. ^bCooked as in methods.

NOC determination, but without prior treatment with sulphamic acid, and deducting the nitrite equivalent of NOC. Samples were collected from each volunteer at day 0 (free diet), days 4 and 5 of the LM diet, days 5, 7, 12 and 14 of the HRM diet and day 4 of the HRM diet. In these samples iron was measured by absorbance spectroscopy and nitrate by reduction to NO by Ti^{2+} and measurement of the NO by treatment with ozone and measurement of the resulting chemiluminescence by thermal energy analysis.

To determine faecal ammonia in the LM, HRM and HRMHB periods of the first study weighed amounts of ~50 g fresh faecal specimen were stomached with an approximately three times weighed amount of 0.1 M HCl for 60 min. Two 20 ml aliquots were centrifuged for 30 min at 3000 r.p.m. and the clear supernatant was removed and stored at -20°C prior to analysis. Ammonia concentration was determined colorimetrically (32).

Faecal and colonic pH

Faecal and colonic pH were determined by radiotelemetry on days 10–13 and days 16–19 of each LM, HRM and HRMHB dietary period (33). The telemetry pills were given after overnight fasting with deionized water at 7 a.m. on days 10 and 16. Subjects took their usual breakfast after the pill left the stomach and monitoring continued until the pill was passed. Caecal pH was taken as the mean of readings at 1, 2 and 3 h after leaving the duodenum. Faecal pH was recorded whilst the pill remained in the faecal specimen until its removal. All telemetry pills were checked for their accuracy before use and after their removal in pH 7 buffer. Only those observations from pills recording pH 6.5–7.5 were used.

Cross-linking and label loss in microcapsules

Cross-linking and label loss was assessed by the semipermeable microcapsule technique (34). Each individual was given 3 ml of an ethanol solution of covalently $^{14}CH_3$ -labelled (12 kBq) polyethyleneimine (PEI) microcapsules in gelatin-coated enteric capsules by mouth on day 10 of the LM, HRM and HRMHB dietary periods as described previously (35). Fifty radio-opaque markers (ROM), also in gelatin-coated enteric capsules were given at the same time to check that the microcapsules had been released from the enteric coating. On day 16 of the dietary periods 3 ml of microcapsules containing copper phthalocyanine (CPTS) for HAA trapping (36) were given with the evening meal, between the main and dessert courses. PEI-containing microcapsules were removed magnetically from faecal collections over days 10–15 of each dietary period and those containing CPTS from specimens collected over days 16–21. The recovered microcapsules were stored in ethanol before analysis for cross-linking as previously described (37).

Urine nitrate excretion

Twenty four hour urine nitrate excretion was measured by anion exchange chromatography (AS4 guard and column; Dionex) in 24 h urine samples collected on days 10–16 of the LM, HRM and HRMHB dietary periods (24). The 2 l urine collection bottles were double washed with deionized water and contained 10 g NaOH pellets as preservative on days 13–16. Collections on days 10–12 contained 4 g/2 l bottle boric acid and were verified for completeness using *p*-aminobenzoic acid by the PABAcheck method (38).

Plasma urea

A fasting blood sample was obtained on days 10 and 16 of the red meat and bran study protocols for plasma urea estimation by hydrolysis with urease to ammonia (Sigma Kit no. 640).

Mean transit time and faecal weight

Faecal weight and mean transit time (MTT) were measured continuously throughout (39). Except where otherwise stated, the subjects took 10 ROM with each meal three times daily. A diary was kept throughout of the times the markers were taken and of faecal collections. All specimens were collected in plastic bags using a frame fitted to the toilet and stored at -20°C prior to X-ray determination of marker content and microcapsule removal. The marker content of each stool and the time the markers were taken was used to calculate MTT and to marker correct faecal weight (39).

Statistics

Results were analysed using Systat 5.2 for the Apple Macintosh. Means, standard errors and Pearson correlation coefficients are shown unless otherwise stated. To test for differences between the LM and HRM diets and other results in the first protocol results were assessed by analysis of variance using a general linear model. Where there were significant dietary effects paired *t*-tests were used to differentiate differences between means on different diets. Results were assessed by unpaired *t*-tests (unless otherwise stated) in the second study.

Results

In the eight volunteers from both protocols who received the LM and HRM diets faecal NOC was 40 ± 7 µg/day with the LM diet. Analysis of variance showed significant dietary effects ($P = 0.031$) and there was a significant ($P < 0.024$) increase by paired *t*-test to 113 ± 25 µg on the HRM diet (Table II). Figure 1a shows the individual values, with those from the two subjects (7 and 8) included from the second protocol indicated. Levels increased in seven of the eight subjects on changing from the LM to HRM diet. In six volunteers in the first investigation who received the HRMHB diet the mean NOC level on the HRMHB diet was 138 ± 41 µg/day, which was also significantly greater than on the LM diet ($P = 0.004$), but not significantly greater than the HRM diet. Figure 1b shows the individual values. Levels increased in three and decreased in three on changing from the HRM to the HRMHB diet.

In six volunteers analysis of variance showed significant dietary effects on plasma urea levels ($P = 0.001$) and 24 h

Table II. Significance of analysis of variance (ANOVA) for dietary effects, mean, standard error and significance of difference in mean results for dietary periods by paired *t*-test (differences at $P > 0.05$ not shown for paired *t*-test)

	<i>P</i> (ANOVA for dietary effects)	LM		<i>P</i> (LM versus HRM)	HRM		<i>P</i> (HRM versus HRMHB)	HRMHB		<i>P</i> (LM versus HRMHB)
		Mean	SE		Mean	SE		Mean	SE	
Faeces										
NOC (µg/day)	0.031	40 ^a	7	0.024	113 ^a	25		138	40	0.004
Faecal NH ₃ (mmol/l)	0.014	2.72	0.50	0.007	6.50	1.08		7.38	1.7	0.021
Faecal pH	0.894	6.77	0.27		7.01	0.27		6.82	0.28	
Caecal pH	0.830	6.07	0.28		6.17	0.43		6.44	0.25	
MTT (h)	0.527	41.5	3.2		40.5	4.8		35.8	2.6	
Faecal weight (g/day)	0.046	172	21	0.02	151	13	0.003	221	20	
Microcapsule cross-linking index	0.961	0.625	0.067		0.585	0.083		0.630	0.069	
Plasma										
Plasma urea (mmol/l)	0.001	4.13	0.35	0.001	8.07	0.67		8.21	0.66	0.001
Plasma creatinine (mmol/l)	0.999	88.4	5.0		90.0	4.6		88.5	4.8	
Urine										
Acetylation index	0.232	0.23	0.03		0.34	0.04		0.30	0.05	
Oxidation index	0.996	6.94	0.74		5.64	0.37		6.87	0.6	0.6
24 h urine NO ₃ (mmol)	0.607	1.10	0.10		1.20	0.06		1.16	0.10	
24 h urine creatinine (mmol)	0.045	17.8	0.68	0.005	20.9	0.74		20.4	0.67	0.029

**n* = 8. For all other analyses *n* = 6.

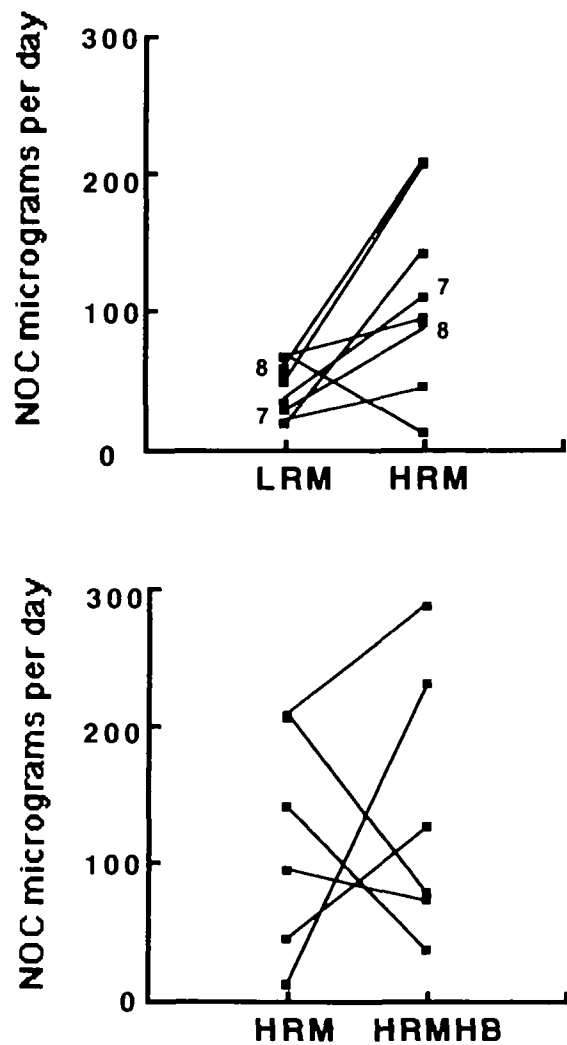


Fig. 1. Total faecal NOC output (µg/day) (a) on low red meat (LRM) and HRM diets in eight volunteers with values for two subjects studied in the second protocol indicated and (b) on HRM and HRMHB diets in six volunteers.

urine creatinine ($P = 0.045$). Table II shows that in response to the HRM diet plasma urea increased significantly ($P = 0.001$), with no change on increasing bran intake. Plasma creatinine did not increase with the HRM diet, although there was a significant ($P = 0.005$) increase in urinary creatinine output. There were no significant dietary effects on acetylation index, oxidation or 24 h urine nitrate excretion by analysis of variance (Table II).

Analysis of variance showed significant ($P = 0.014$) dietary effects on faecal ammonia, which increased from 2.72 to 6.50 mmol/l supernatant with the HRM diet. There was a further (non-significant) increase with the HRMHB diet (Table II). There were no dietary effects on MTT or faecal or caecal pH with any of the dietary protocols by analysis of variance, although the HRMHB diet significantly increased faecal weight compared with the HRM diet ($P = 0.003$). Table III shows that MTT was inversely related to faecal weight ($r = -0.644$) and faecal pH was related to caecal pH ($r = 0.619$). Faecal ammonia was related to plasma urea ($r = 0.688$).

There was no change in cross-linking index in microcapsules with any of the dietary protocols (Table II). However, cross linking was inversely related to faecal weight (Table III). The

Table III. Significant ($P < 0.05$) Pearson correlation coefficients between continuous variables

Faecal weight versus	MTT	-0.644
	Cross-linking index	-0.536
Cross-linking versus	Starch intake	0.624
	Starch intake	-0.773
Faecal pH versus	Starch intake	-0.642
	Caecal pH	0.619
Faecal ammonia versus	Plasma urea	0.688

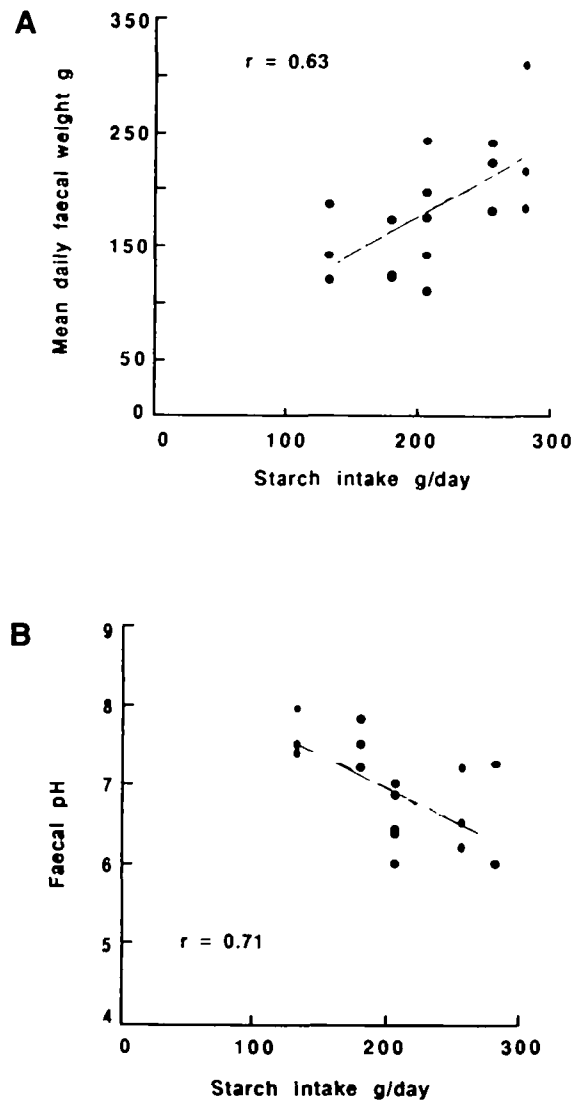


Fig. 2. Daily starch (and NSP) intake (g/day), mainly from bread, in relation to (a) faecal weight (g/day) and (b) faecal pH in six volunteers fed three different diets.

use of bread as a supplement to maintain the energy balance in individuals created a continuous dietary variable for starch (and a small amount of NSP) which was inversely related to cross-linking in the microcapsules ($r = -0.773$) and faecal pH ($r = -0.642$) and positively correlated with faecal weight ($r = 0.624$) (Table III). Figure 2a and b shows the positive association between starch and faecal weight and the inverse association with faecal pH. Figure 3a and b shows the inverse

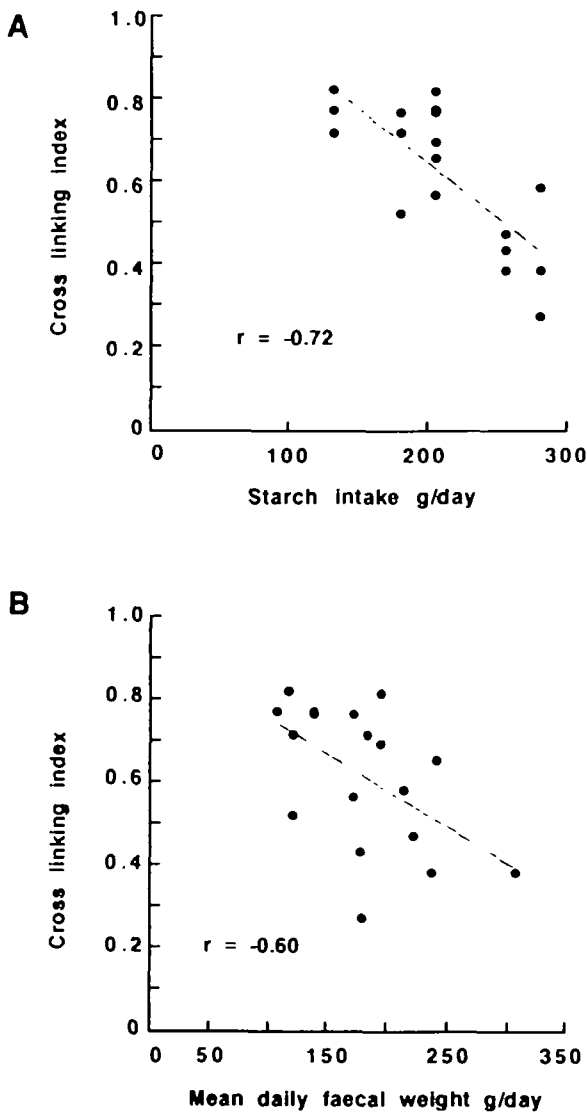


Fig. 3. Cross-linking index in microcapsules in relation to (a) daily starch (and NSP) intake (g/day), mainly from bread, and (b) faecal weight (g/day) in six volunteers fed three different diets.

Table IV. Content of heterocyclic amines in food samples ($\mu\text{g/day}$)

	LM		HRM	
	Mean	SE	Mean	SE
MeIQx	<0.2		0.90	0.60
DiMeQx	<0.2		0.27	0.07
IQ	<0.6		2.80	1.81
PhIP	<0.2		<0.2	
Norharman	10.17	3.02	11.3	4.13
Harman	7.53	1.84	6.63	0.93
Total MeIQ etc.	<1.2		4.17	2.48
Total MEIQ etc. + others	18.9	4.62	22.1	2.25

All differences in means are not significant ($P > 0.05$).
DiMeIQx, 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline; MeIQx, 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline.

relationships between cross-linking index and starch and cross-linking and faecal weight.

Table IV shows levels of HAA in duplicate samples of the LM and HRM diets. Levels of all HAA measured were higher

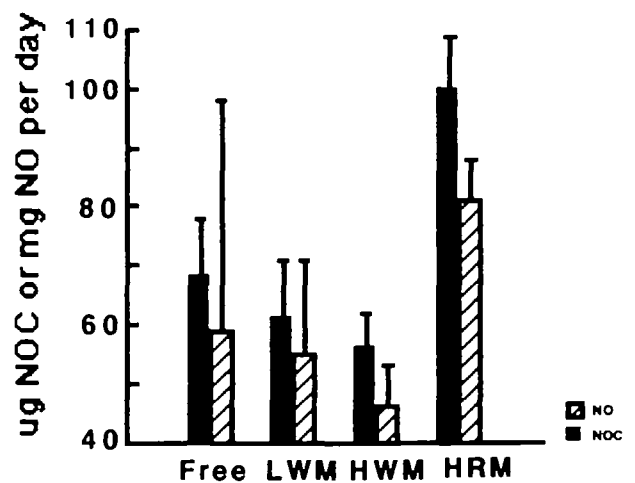


Fig. 4. Mean (\pm SE) total NOC ($\mu\text{g/day}$) and NO (mg/day) faecal output on the free, LWM, HWM and HRM diets in two volunteers.

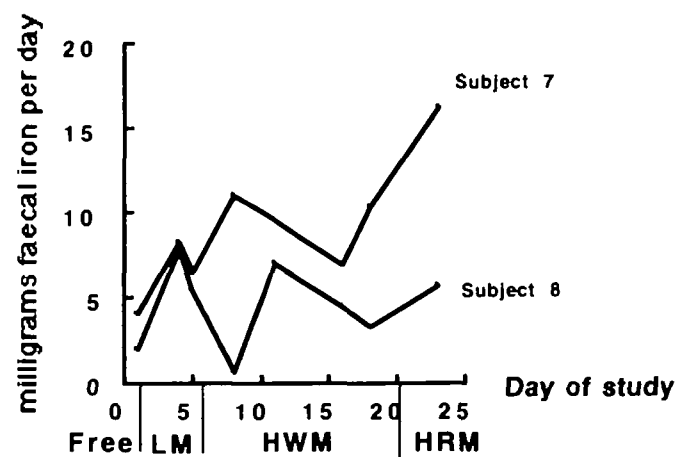


Fig. 5. Faecal output of iron (mg/day) on the free, LM, HWM and HRM diets in two volunteers.

in the high meat diets, although these differences failed to reach significance. The co-mutagens norharman and harman were found in greatest amounts, followed by 2-amino-3-methylimidazo(4,5-f)quinoxaline (IQ). There were no detectable levels of 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) present. No HAA were found to be present in the extracts of urine nor in microcapsules recovered from faeces.

Mean NOC levels in the two subjects fed the LM diet over 5 days were $61 \pm 10 \mu\text{g/day}$, similar to levels found whilst they had been eating a free diet ($68 \pm 10 \mu\text{g/day}$). Over 15 days there was no change with the HWM diet ($56 \pm 6 \mu\text{g/day}$, $P > 0.05$), but a significant ($P < 0.05$) increase to $100 \pm 9 \mu\text{g/day}$ after 4 days on the HRM diet. Faecal NOC and nitrite levels were correlated ($r = 0.76$) and mean levels of nitrite in response to red or white meat showed a similar pattern to those of NOC (Figure 4). Mean levels of nitrite were 59 ± 39 , 54 ± 16 , 46 ± 7 and $80 \pm 7 \text{ mg/day}$ in the free, LM, HWM and HRM dietary periods respectively, $P < 0.05$ HRM versus HWM.

Faecal nitrate levels were low and not significantly different throughout the dietary periods. Mean levels were 0.17 ± 0.33 , 0.26 ± 0.13 , 0.31 ± 0.11 and $0.21 \pm 0.03 \text{ mg/day}$ in the free, LM, HWM and HRM dietary periods respectively. When both subjects were considered together there was no significant

correlation between faecal nitrate and nitrite levels ($r = -0.05$) nor with faecal NOC ($r = -0.15$). Faecal iron on the LM diet was 7.0 ± 0.65 mg/day and 6.7 ± 1.3 mg/day on the HRM diet. The increase to 11.0 ± 5.2 mg/day on the HRM diet was not significant.

Faecal iron levels were significantly higher throughout the study period in volunteer 7 (9.1 ± 1.29 mg/day) compared with volunteer 8 (4.5 ± 0.9 mg/day, $P < 0.001$). Figure 5 shows the trends with time in faecal iron in the separate individuals. Faecal iron levels were correlated with faecal NOC in subject 7 ($r = 0.714$) and with nitrite ($r = 0.769$), but less so in subject 8 (NOC, $r = 0.442$; nitrite, $r = 0.49$). In subject 8 there were inverse associations between faecal nitrate and NOC ($r = -0.53$) and between faecal iron and nitrate ($r = -0.51$), but positive associations ($r = 0.54$ and 0.20) in subject 8.

Discussion

This is the first demonstration in humans that intestinal N-nitrosation is raised when red meat intake is increased. About a 4-fold increase was shown, with seven of the eight individuals showing an increase with red meat (Figure 1a). The average levels of 113–138 $\mu\text{g/day}$ NOC found on the HRM diets were comparable with other sources of NOC, e.g. tobacco smoke aerosol levels (40). Approximately 30 $\mu\text{g/day}$ of tobacco-specific carcinogenic NOC are obtained from smoking 40 cigarettes/day and the lifetime exposure to faecal NOC, at 2.2 mmol/kg body wt/day, is of the same order as the lowest dose of NOC found to be tumourigenic in rodents (41). This effect of red meat has been confirmed in our more recent study in which a 7-fold increase in faecal NOC levels from 281 ± 63 to 1940 ± 1330 ng/g faeces was found in eight volunteers fed the same amounts of red meat (42). The present report also shows that, in contrast to the effect of red meat, no effect on faecal NOC levels was found when white meat and fish intake was increased to the same level (600 g/day).

Nature and production of NOC

The alkaline preservation technique, used with samples from the first six subjects to minimize artefactual formation of NOC, would have destroyed nitrosamides. Subsequent extraction and *in vitro* treatment of samples homogenized with water instead of alkali have not identified considerable amounts of nitrosamides or nitrosated guanidines, but have shown that both acidic and basic nitrosamines are present. We are presently carrying out further work to characterize the faecal NOC produced, but the presence of carcinogenic or mutagenic NOC cannot as yet be defined. There are conflicting reports on the effects of high meat diets in modulating promotion of colon carcinogenesis in animal models (43,44), but NOC would be important in initiation rather than promotion.

The origin of NOC in faeces is likely to be endogenous. In rats fed diets containing undetectable levels of NOC synthesis was shown to be possible in the large intestine (11) and a number of facultative and anaerobic colonic bacteria are able to catalyse their formation (45). The differential effects of red and white meat in increasing levels could arise from differential effects in the digestion of protein and, hence, the availability of nitrogenous substrates and amines for nitrosation in the colon. However, this was not amenable to study in the present report.

Dissimilatory nitrate metabolism within the colon and, hence, nitrite formation is likely to be important. Nitrate

originating from food and drink that reaches the colon is reduced to nitrite and faecal NOC levels increased from an average of 8 $\mu\text{g}/100$ g faeces on a low nitrate diet to 30 $\mu\text{g}/100$ g faeces with a supplement of 300 mg nitrate (11). The levels of NOC found with the nitrate supplement were similar to those established here with the LM diets and of the same order as those found in a rural African population (636 ± 148 $\mu\text{g}/\text{kg}$ faeces homogenized in NaOH and 573 ± 165 $\mu\text{g}/\text{kg}$ homogenized in water) (31).

Faecal nitrite and O- and S-nitrosating agents were increased with increased red meat, as evidenced by the significant increase from 46 ± 7 to 80 ± 7 mg/day in faecal nitrite levels obtained when replacing 600 g white meat and fish with 600 g red meat (Figure 4). However, there was no effect on faecal nitrite on changing from a low (60 g) to high (600 g) white meat and fish diet. This suggests that the increase in faecal NOC and nitrosating agents is brought about by a specific effect of red meat not seen with white meat. A major difference between red and white meat is in their content of iron, which is poorly absorbed from the small intestine. However, there was individual variation and an effect of red meat consumption on faecal iron content was only evident in subject 7 (Figure 5). In this subject there were highly significant correlations with faecal iron and both nitrite ($r = 0.769$) and NOC ($r = 0.714$).

Could iron have a role in the production of NO within the colon? Iron is a catalyst for NOC formation and iron and molybdenum are integral components of nitrate reductase and are essential for enzyme activity (46). A previous study in F344 rats maintained with human faecal flora in their intestine and fed human diets showed a 3-fold increase in faecal nitrate reductase with 3-fold increased red meat consumption (47). Faecal nitrate reductase may be a key step in determining the levels of production of NOC and nitrosating agents, such as nitrite from nitrate, and hence in total NOC levels, but we were unable to measure faecal nitrate reductase in the present work. When subjects 7 and 8 were considered together we were also unable to show a significant correlation between faecal nitrite and nitrate ($r = -0.05$) and that there were significant differences in faecal nitrate with the different diets. This and the fact that faecal nitrate levels were low throughout might suggest that iron-dependent activity of faecal nitrate reductase was not the rate limiting step in the conversion of nitrate to nitrite in the colon. However, there were individual differences in faecal iron and nitrate excretion and therefore perhaps in faecal nitrate reductase levels.

Intakes of nitrate and nitrite were constant and low throughout the studies reported here and, hence, the increase in faecal nitrite and other nitrosating agent levels could not have arisen through increased intake. However, NO does not arise from dietary sources of nitrate alone. Increased endogenous production of nitrate via NO synthase is possible from increased dietary arginine levels, from the substantial increase in dietary protein with either red or white meat. The NO would be produced in the epithelium and possibly lead to a localized production of NOC in the adjacent lumen. Studies in animals have shown evidence of increased endogenous nitrosation and increased urinary nitrate with high protein diets (17,18). We found no increase in urine nitrate with the HRM diet and, also in humans, Castillo *et al.* (48) found no evidence of increased urine nitrate production on a high versus low arginine diet, but did not measure faecal NOC or nitrite levels. It is possible that increased colonic iron levels may be related to colonic

NO synthase activity and, hence, the increase in nitrite and NOC with red but not white meat, but we have no evidence to demonstrate this at present.

We were unable to show that bran inhibited NOC formation (Table II). There is a possibility that normal bran, which contains substantial amounts of phytate, might have inhibited NOC formation because it inhibits faecal nitrate reductase (49). A role for phytate in protecting against colorectal cancer due to its ability to chelate iron has been proposed (50) and chemopreventive effects of phytate have been shown (51). The original hypothesis related to the formation of reactive oxygen species, but subsequently it has been shown that increased iron did not increase lipid peroxidation products in a rodent colon cancer model (52).

Effects of diet on MTT, ammonia, faecal weight and cross-linking

Despite the inability of the bran used here to modulate faecal NOC levels, faecal weight was increased and, hence, the contents of the lumen diluted. MTT is inversely related to faecal weight (Table III). The net result would have been less contact between NOC arising from the HRM diet and the colonic mucosa with the HRMHB diet. The high meat diet elevated blood urea levels and increased faecal ammonia concentration. The effect of high meat diets in increasing faecal ammonia concentration has been shown before (53). Ammonia in drinking water enhances epithelial cell proliferation in the gastric mucosa and promotes *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced adenocarcinomas in rodents (54,55). Visek (56) implicated elevated faecal ammonia levels in large bowel carcinogenesis. Although NSP can reduce faecal ammonia levels due to a high protein diet via increased fermentation by bacterial flora in the large intestine (53), there was no effect with the bran used in this study. The bran was chosen because it had been treated to be free of starch, nitrate and phytate, but these procedures also render this bran largely unfermentable (57).

Bread intake varied from subject to subject, due to the need to maintain energy balance throughout the study. White bread contains ~1% resistant starch and 1.5% NSP, which reaches the large intestine and is fermented. This should lead to any available nitrogen, as ammonia, being incorporated into bacterial cell walls. The inverse relation between starch intake and faecal ammonia did not reach statistical significance ($r = -0.468$; data not shown), but the increase in biomass produced during fermentation increased faecal weight and there were strong correlations between dietary starch and NSP and faecal weight (Figure 2a).

Short chain fatty acids are also produced during fermentation and the effects of the consequent reduction in stool pH has been discussed in relation to carcinogenesis in the colon (8). In this study there was no effect of unfermentable bran on caecal and faecal pH (Table II), but faecal pH was reduced in response to an increase in starch and NSP from bread ($r = -0.642$, Figure 2b). Fermentation is most rapid in the caecum and, as has been demonstrated elsewhere, caecal pH was lower than faecal pH (33). Caecal and faecal pH were individually correlated ($r = 0.619$; Table III), but caecal pH was not significantly related to intake of starch and NSP from bread ($r = -0.205$; data not shown).

Cross-linking in microcapsules was not increased by high protein diets (Table II), but was significantly reduced in relation to starch intake ($r = -0.773$) and faecal weight ($r = -0.644$)

(Figure 3a and b). Cross-linking is indicative of the presence of bi-functional alkylating agents within the colonic lumen (37) and reduced cross-linking with increased starch consumption and, therefore, increased faecal weight is likely to be associated with altered metabolism or dilution of these agents due to increased biomass within the lumen. The effect of reduced cross-linking with increased faecal weight has been shown previously in humans (35).

HAA and phenotyping

The amounts of meat consumed on the high meat diet were within the normal range of day-to-day variation, but contained relatively low levels of HAA. Although little or no PhIP was detected in the duplicate diets analysed, probably because higher cooking temperatures are required (58), levels of HAA were (not significantly) greater on the high protein diets. Microcapsules have previously been shown to trap HAA (36), but no HAA could be detected in extracts from CPTS microcapsules in the present study of humans. No obvious evidence of the typical mutations in *ras* or *p53* have been shown in colon cancers induced in rats by PhIP or IQ (59). When meat is cooked in a conventional manner the finding of elevated faecal NOC may be more consistent with known mutational effects in colon cancer. NOC rather than HAA may therefore be the important factor relating increased meat consumption to colon cancer risk.

We investigated phenotypic changes because HAA and certain *N*-nitrosamines are activated by P450 enzymes present in the liver and small intestinal mucosa. CYP1A2, which *N*-oxidizes aromatic amines, also catalyses the demethylation of caffeine and, using caffeine as a surrogate to phenotype individuals as fast or slow oxidizers, patients with large bowel cancer have been shown to be faster oxidizers and acetylators than healthy matched controls (21). However, as a consequence of increasing meat intake, protein intake was also altered in this study and there is extensive literature showing a general reduction in P450 enzyme system activity when protein intake is reduced in animals, probably because protein synthesis and liver cell proliferation are retarded (60). These findings also apply to humans, since low protein diets decrease antipyrine and theophylline clearance (61). Changes may occur relatively rapidly, within 1–2 weeks of a change in protein intake in rats (62). Phase II enzyme activity may also be reduced, so that the net result may be an increase or decrease in toxicity of xenobiotics in protein-deficient animals (60). Although diet may therefore affect phenotypic studies of cases and controls in large bowel cancer, we were unable to establish significant dietary effects on acetylation or oxidation in the present study using the caffeine test.

Conclusion

Several changes in intraluminal metabolism in the colon that are related to risk of colon cancer were brought about by the changes in diet in this study. Our present finding of evidence of increased faecal production of NOC and nitrite when red meat consumption is increased is in line with the suggestion that meat may enhance endogenous faecal nitrosation via elevated colonic amine levels and faecal nitrite (62). The increase in endogenous NOC production in the colon from red meat is rapid, occurring within days of a change in diet, and has been now confirmed in three of our study protocols, here and elsewhere (42). The lack of effect of white meat and fish is unexpected, but may relate to faecal iron levels and epidemiological findings that red and processed meat are

associated with increased risk of colon cancer, whereas chicken and fish are associated with decreased risk (2,3).

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