Effect of Nitrate on Methane Production and Fermentation by Slurries of Human Faecal Bacteria

By C. ALLISON AND G. T. MACFARLANE*

MRC Dunn Clinical Nutrition Centre, 100 Tennis Court Road, Cambridge CB2 1QL, UK

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Most probable number counts showed that denitrifying species were the numerically predominant NO_3^- reducing bacteria in the faeces of five methanogenic individuals [about 10^{10} bacteria (g dry wt faeces) $^{-1}$]. In faecal slurries, however, denitrification was a relatively minor route of NO_3^- dissimilation, since only about 3% of the NO_3^- was converted to gaseous products, with NO_3^- being mainly reduced to NO_2^- and NH_4^+ . When KNO_2 was added to the slurries, denitrification became quantitatively more significant with approximately 23% of the NO_2^- being lost as gaseous products. The addition of KNO_3 (10 mM) to slurries containing either starch or casein significantly decreased H_2 and CH_4 production. The effect of NO_3^- on methanogenesis was twofold: firstly, H_2 accumulation decreased due to diversion of electrons towards $\text{NO}_3^-/\text{NO}_2^-$ reduction, and as a result of H_2 being used as an electron donor for NO_3^- reduction, resulting in the removal of the methanogenic substrate; secondly, there was direct inhibition of methane-producing bacteria by NO_3^- and NO_2^- . In starch-containing slurries, acetate:butyrate molar ratios were increased when NO_3^- was added but this effect was not observed when casein replaced starch. These results show that the ability of $\text{NO}_3^-/\text{NO}_2^-$ to act as an electron sink can significantly influence the major products of the human colonic fermentation.

INTRODUCTION

The human large intestine is a site of intense microbial activity. The major end products of the colonic fermentation are the volatile fatty acids (VFA) acetate, propionate and butyrate, and the gases H_2 , CO_2 and CH_4 (Cummings, 1981), although only about 30–50% of individuals in Western populations carry large populations of methanogens (Wolin & Miller, 1983). VFA are known to be important in that they take part in a wide range of reactions within the body (Cummings *et al.*, 1987); however, the significance of gas production is less well understood.

Considerable quantities of NO_3^- are consumed by man (about 120 mg d $^{-1}$) (Walker, 1975; Knight *et al.*, 1987) and studies with small intestinal contents have suggested that a substantial proportion of this NO_3^- may reach the large gut (Radcliffe *et al.*, 1985). Although this has been challenged by some workers (Bartholomew & Hill, 1984), ^{13}N studies in rats and human NO_3^- balance studies indicate that a further source of NO_3^- results from endogenous formation in the tissues and that NO_3^- is secreted into the gastrointestinal tract at several locations (Witter *et al.*, 1979; Lee *et al.*, 1986). Furthermore, recent work by Iyengar *et al.* (1987) has shown that stimulated macrophages synthesize and secrete NO_2^- and NO_3^- . Since the large intestinal epithelium harbours large populations of macrophages which are constantly exposed to immunological stimuli (Beeken *et al.*, 1987) it is likely that these cells contribute significantly towards NO_3^- production in the colon. Nitrate is rapidly metabolized by faecal bacteria (Archer *et al.*, 1981) and it seems possible therefore that the colon may function as a sink for this metabolite.

Abbreviations: BES, 2-bromoethanesulphonic acid; MPN, most probable number; VFA, volatile fatty acid(s).

There is increasing interest in the formation of *N*-nitroso compounds in the large gut (Suzuki & Mitsuoka, 1984) but the effect of NO_3^- on the colonic fermentation has so far received little attention. The major objectives of this study therefore were to determine the effect of NO_3^- on fermentation by intestinal bacteria and to ascertain the major route of NO_3^- dissimilation in the large gut.

METHODS

Most probable number (MPN) counts. Cell population densities of denitrifying bacteria and bacteria that dissimilated NO_3^- to NO_2^- were determined in faeces from five methanogenic individuals using a modification of the five-replicate, 10-fold dilution technique described by Alexander (1965). Fresh faeces (1 g) was serially diluted in anaerobic half-strength Wilkins–Chalgren broth in an anaerobic cabinet, containing an atmosphere of $\text{H}_2/\text{CO}_2/\text{N}_2$ (10:10:80, by vol.). Samples (1 ml) from each dilution were used to inoculate two sets of replicate universal bottles, each containing 9 ml anaerobic Wilkins–Chalgren broth. One set of bottles contained KNO_3 (1%, w/v) whilst the other set had no KNO_3 added. The bottles were sealed prior to incubation (5 d, 37 °C) and uninoculated bottles were used as controls. After incubation, bottles showing turbidity were recorded as positive for growth and provided the total anaerobe count. Samples of headspace gas were removed using a gas-tight syringe and were analysed for N_2O . Liquid samples were then taken for measurement of NO_3^- and NO_2^- .

Faecal slurries. Fresh faeces were obtained from five methanogenic subjects. Individual faecal slurries (5%, w/v) were prepared by homogenizing the faeces in 0.1 M-sodium phosphate buffer, pH 7.0, saturated with argon. Samples (240 ml) were dispensed into glass bottles (280 ml capacity) and immediately sparged with high-purity argon for 15 min via a three-way valve system. The bottles were incubated at 37 °C in a rotary shaker. Samples of headspace gas (17 ml) and liquid samples (3 ml) were taken at 0, 1.5, 3, 6, 12, 24 and 48 h, using a 20 ml capacity plastic syringe fitted with a three-way valve. The samples were replaced by the addition of 20 ml high-purity argon. Initial studies showed that H_2 could be stored in syringes for up to 6 h without significant loss of gas.

Addition of NO_3^- and NO_2^- to starch and casein containing slurries. Faecal slurries were amended where appropriate with either Lintner's starch (0.2%) or casein (1%, w/v). Either KNO_3 or KNO_2 was added, to give final concentrations of 10 mM or 2 mM.

Effect of NO_3^- on gas formation. To determine the effect of NO_3^- on hydrogen production, the methanogenesis inhibitor 2-bromoethanesulphonic acid (BES) was added to faecal slurries, (final concentration 20 mM). In experiments to determine the effect of NO_3^- on CH_4 production, bottles were pressurized with H_2 (1 atm) to ensure that excess H_2 was available. CO_2 was always produced in excess by faecal slurries. Denitrification was measured using the acetylene blocking technique (Yoshinari & Knowles, 1976). In these experiments the bottles were incubated with acetylene (5%, v/v) which inhibits the reduction of N_2O to N_2 . The formation of N_2O was used to measure denitrification.

H_2 as an electron donor for NO_3^- reduction. Washed faecal bacterial cell suspensions were prepared from faeces obtained from two individuals in anaerobic 0.1 M-sodium phosphate buffer, pH 7.0, as described by Macfarlane & Englyst (1986). Samples (20 ml) were added in duplicate to 58 ml capacity serum bottles containing KNO_3 (10 mM) which were sealed with butyl rubber stoppers (Miller & Wolin, 1974). Starch (0.2%) was added to some of the bottles. The serum bottles were then gassed with either high-purity N_2 or H_2 and pressurized to 1 atm before being incubated at 37 °C on a rotary shaker. Samples for chemical analysis were taken periodically using a gas-tight syringe.

Gas analysis. CH_4 , H_2 , CO_2 , N_2 and N_2O were measured by GC using a Pye series 104 gas chromatograph, equipped with a 1 ml injection loop. The gases were separated on a 3 ft (about 0.9 m) glass column (4 mm i.d.) containing Porapack Q (Waters). Column and detector temperatures were 40 °C and 110 °C respectively. Argon was the carrier gas and detection was by a thermal conductivity detector connected to a Pye Unicam CDP1 computing integrator. Results were quantified by comparison with authentic gas standards.

Chemical analysis. Particulate matter and bacterial cells were removed from samples by centrifugation (27 000 g, 10 min). NO_2^- was determined as described by Barnes & Folkard (1951). NO_3^- was enzymically reduced to NO_2^- by a crude membrane fraction prepared from *Escherichia coli*, using procedures described by Dunn *et al.* (1979). Nitrate reductase assays were done as described by Lowe & Evans (1964) using reduced dithionite as the electron donor. NO_3^- concentrations were then determined by difference from the NO_2^- analysis described above. NH_4^+ was measured using the phenol–hypochlorite method of Solorzano (1969). VFA and other carboxylic acids were detected by GC by the procedures of Holdeman *et al.* (1977).

Statistical analysis of data. Molar ratios of acetate and butyrate in NO_3^- and non- NO_3^- slurries were statistically compared using the paired *t*-test (Bradford-Hill, 1971).

Chemicals. All chemicals were obtained from Sigma with the exception of Lintner's starch (BDH) and the formulated bacteriological media (Oxoid).

Table 1. *Most probable number (MPN) counts of NO₃⁻ reducing bacteria and total anaerobes in faeces*

MPN counts were done as described in Methods, using faeces from five methanogenic individuals.

Bacteria	MPN count (g dry wt faeces) ⁻¹	
	Range	Mean
Bacteria converting NO ₃ ⁻ to NO ₂ ⁻	2 × 10 ⁶ –1 × 10 ⁹	2 × 10 ⁸
Bacteria converting NO ₃ ⁻ to gaseous products	2 × 10 ⁹ –2 × 10 ¹⁰	6 × 10 ⁹
Total bacterial count	6 × 10 ¹⁰ –5 × 10 ¹¹	2 × 10 ¹¹

RESULTS

Enumeration of bacteria

NO₃⁻ reducing species constituted only a small percentage of bacteria in each of the five faecal samples studied (Table 1). Denitrifying bacteria (N₂O producers) were the numerically predominant NO₃⁻ reducing species [mean MPN count 6 × 10⁹ (g dry wt faeces)⁻¹], with bacteria that reduced NO₃⁻ to NO₂⁻ occurring in lower numbers [mean MPN count 2 × 10⁸ (g dry wt faeces)⁻¹].

Effect of NO₃⁻ on gas production in faecal slurries

In the absence of added carbohydrate or protein, low levels of CO₂ and CH₄ accumulated in faecal slurries (Fig. 1*a*). The addition of starch stimulated gas production 3-fold, however (Fig. 1*b*). Both NO₃⁻ and NO₂⁻ markedly decreased the formation of H₂ and CH₄ in slurries containing starch, although CO₂ production appeared to be relatively unaffected (Fig. 1*c, d*). Both NO₃⁻ and NO₂⁻ were rapidly reduced in starch slurries resulting in elevated levels of NH₄⁺ (Fig. 1*b, c, d*). Substantial quantities of CO₂, H₂ and CH₄ were also produced by faecal slurries to which casein had been added. As was the case with starch, the addition of NO₃⁻ resulted in a decrease in H₂ production and methanogenesis (Fig. 1*e, f*). Whilst the quantities of H₂, CO₂ and CH₄ produced by slurries from different individuals varied markedly, the results when corrected for controls (slurries with no additions), demonstrated that the effects of NO₃⁻ and NO₂⁻ on H₂ and CH₄ formation were similar in every experiment.

Denitrification by faecal slurries

The use of N₂ production as an index of denitrification is unreliable (Balderston *et al.* 1976) since a major source of error is contamination of the samples by atmospheric N₂ (Sørensen, 1978). A more dependable way of quantifying the process involves the inclusion of acetylene in the assay system, which blocks formation of N₂ from N₂O, with the result that N₂O accumulates as the final denitrification product instead of N₂ (Yoshinari & Knowles, 1976). The acetylene blocking method was used in this study. The results showed that denitrification was a minor route of NO₃⁻ dissimilation in faecal slurries with only about 3% of the NO₃⁻ added being reduced to N₂O (Table 2). In slurries that contained NO₂⁻, however, denitrification occurred to a greater extent, with approximately 23% of the NO₂⁻ being converted to N₂O after 48 h.

Effect of NO₃⁻ on VFA and other fermentation products

The addition of NO₃⁻ to faecal slurries containing starch had little effect on total levels of VFA produced (Table 3). However, molar ratios of acetate were significantly increased ($P < 0.005$) whereas those of butyrate were significantly reduced ($P < 0.025$). Whilst propionate formation was not significantly influenced by NO₃⁻, succinate and lactate did not accumulate in the presence of NO₃⁻. In contrast, the addition of NO₃⁻ to slurries containing casein resulted in less total VFA being produced, but had little effect on VFA molar ratios or the production of other carboxylic acids.

Fig. 1. Effect of NO_3^- and NO_2^- on gas production in faecal slurries. (a) Slurry with no additions; (b) slurry + starch (0.2%); (c) as (b) + 10 mM- NO_3^- ; (d) as (b) + 10 mM- NO_2^- ; (e) slurry + casein (1%); (f) as (e) + 10 mM- NO_3^- . Similar results were obtained with slurries from either five (a, b, c and d) or three (e and f) methanogenic subjects. Results presented are from one individual in each group. ●, NH_4^+ ; ▲, NO_2^- ; ■, NO_3^- ; ○, CO_2 ; □, H_2 ; △, CH_4 .

Table 2. Production of N_2O by faecal slurries

Slurries were incubated at 37 °C for 48 h with starch (0.2%). The incubation bottles contained acetylene (5% v/v). NO_3^- and NO_2^- concentrations were each 10 mM. Results are means $\pm$ SEM of experiments with bacteria from three individuals.

Conditions	Gas produced [$\mu\text{mol (ml slurry)}^{-1}$]	
	N_2O	N_2
Slurry + acetylene	ND	ND
Slurry + NO_2^- + acetylene	1.16 ± 0.29	ND
Slurry + NO_3^- + acetylene	0.15 ± 0.02	ND

ND, Not detected. Limit of detection was 0.5 nmol ml^{-1} .

Fig. 2. Effect of addition of NO_3^- on H_2 and CH_4 formation in faecal slurries. (a) Slurry + starch (0.2%) + 20 mM-BES; (b) as (a) + 10 mM- NO_3^- ; (c) slurry + starch (0.2%) + H_2 (1 atm); (d) as (c) + 10 mM- NO_3^- . Data presented are mean results from two methanogenic individuals. ●, CO_2 ; ■, H_2 ; ▲, CH_4 .

Table 3. Effect of NO_3^- on VFA and carboxylic acid production in slurries containing starch or casein

Faecal slurries were incubated for 48 h at 37 °C with starch (0.2%) or casein (1%, w/v) with and without NO_3^- (10 mM). Results are means $\pm$ SEM of either three or five separate experiments, and are corrected for endogenous control values.

Incubation conditions	Total VFA (mM)	VFA molar ratio			Other carboxylic acids (mM)		
		Acetate	Propionate	Butyrate	Lactate	Fumarate	Succinate
Slurry + starch*	19.9 $\pm$ 1.9	59 $\pm$ 5.8	16 $\pm$ 2.0	25 $\pm$ 4.4	2.2 $\pm$ 0.9	ND	5.9 $\pm$ 1.6
Slurry + starch + NO_3^- *	20.1 $\pm$ 1.1	76 $\pm$ 5.4	12 $\pm$ 3.1	10 $\pm$ 3.7	ND	ND	ND
Slurry + casein†	58.7 $\pm$ 4.0	61 $\pm$ 4.6	15 $\pm$ 2.9	24 $\pm$ 5.8	1.8 $\pm$ 0.7	4.0 $\pm$ 2.0	6.3 $\pm$ 2.4
Slurry + casein + NO_3^- †	51.3 $\pm$ 3.4	71 $\pm$ 7.5	12 $\pm$ 4.6	17 $\pm$ 6.4	ND	4.9 $\pm$ 1.7	6.5 $\pm$ 2.0

ND, Not detected; values were less than 0.1 mM.

* Results from faecal slurries from five individuals.

† Results from faecal slurries from three individuals.

Effect of NO_3^- on H_2 and CH_4 formation

Methanogenesis was completely inhibited in faecal slurries containing the inhibitor BES. As a result, substantial quantities of H_2 accumulated in the headspace gas (Fig. 2a). When NO_3^- (10 mM) and BES (20 mM) were added to the slurries, H_2 did not accumulate showing that H_2 formation was directly influenced by NO_3^- (Fig. 2b). Methanogenesis was stimulated in slurries incubated with excess H_2 (Fig. 2c); however, the addition of 10 mM- NO_3^- to these slurries resulted in complete abolition of CH_4 production (Fig. 2d). Production of H_2 and CH_4 was also inhibited in slurries that contained lower concentrations of NO_3^- (2 mM) (Fig. 3). The NO_3^- was rapidly metabolized, however, and after 12 h incubation no NO_3^- or NO_2^- was detectable. Once NO_3^- and NO_2^- were exhausted, H_2 formation and methanogenesis resumed, indicating that the inhibition was reversible.

Utilization of H_2 as an electron donor for NO_3^- reduction

Only trace amounts of NO_3^- were metabolized by washed faecal bacteria in the absence of exogenous electron donor when N_2 was the headspace gas in the serum bottles (Table 4). However, nitrate was stoichiometrically reduced to NO_2^- when H_2 was used as an electron

Fig. 3. Inhibition of H_2 and CH_4 production in faecal slurries by the addition of 2 mM- NO_3^- . Similar results were obtained with slurries from five different methanogenic subjects. Results presented are from one individual. ■, NO_3^- ; ▲, NO_2^- ; ○, CO_2 ; □, H_2 ; △, CH_4 .

Table 4. Reduction of NO_3^- by washed faecal bacteria with H_2 as electron donor

Suspensions of washed faecal bacteria were incubated anaerobically at 37 °C with KNO_3 (10 mM). N_2 or H_2 was the headspace gas (1 atm). Starch (0.2%) was added to some bottles. Data are means of results from experiments with bacteria obtained from two different individuals.

Time of sampling (h)	Conditions...	Inorganic nitrogen (mM)							
		N_2			H_2			H_2 + starch	
		NO_3^-	NO_2^-	NH_4^+	NO_3^-	NO_2^-	NH_4^+	NO_3^-	NO_2^-
0		8.4	ND	0.87	8.1	ND	0.94	8.00	ND
20		8.3	0.15	1.61	4.6	4.30	1.19	1.95	1.70

ND, Not detected.

donor. The production of NO_2^- instead of NH_4^+ by faecal bacteria when H_2 was the sole electron donor showed that these cultures were effectively electron-donor-limited. When starch was added to bottles with H_2 headspace, more NO_3^- was reduced, less NO_2^- accumulated and more NH_4^+ was formed, showing that under these conditions the bacteria were electron-acceptor-limited.

DISCUSSION

The anaerobic metabolism of NO_3^- is carried out by many species of facultative and strictly anaerobic bacteria. Two major pathways of dissimilatory NO_3^- metabolism exist: reduction of NO_3^- to NO_2^- or NH_4^+ , which is termed NO_3^- dissimilation, and reduction of NO_3^- to gaseous products (N_2O , N_2), a process known as denitrification (Herbert, 1982). NO_3^- dissimilation occurs in many environments, such as sewage sludge (Kaspar & Tiedje, 1981), soils (Stanford *et al.*, 1975) and sediments (Macfarlane & Herbert, 1984). The process has also been reported to occur in ruminant animals (Lewis, 1951; Wang *et al.*, 1961; Jones, 1972). Physiological studies have shown that fermentative bacteria form NO_2^- as the main product of NO_3^- dissimilation when grown under carbon-limited conditions and that NH_4^+ is produced during nitrogen-limited growth (Dunn *et al.*, 1979; Keith *et al.*, 1982; Macfarlane & Herbert, 1982).

In this study, NO_2^- and NH_4^+ were found to be the major products of NO_3^- reduction by human intestinal bacteria, irrespective of whether starch or casein was added to faecal slurries. NH_4^+ , the end-product of NO_3^- dissimilation, is toxic to mammalian cells (Vissek, 1978); however, the production of NH_4^+ from NO_3^- is likely to be unimportant quantitatively in the colon, compared

to the NH_4^+ that is formed as a result of amino acid fermentation (Macfarlane *et al.*, 1986). NO_2^- accumulated transiently in the faecal slurries showing that they were not electron-acceptor-limited in the initial stages of the fermentation (Keith *et al.*, 1982), and indicating that in the large gut this metabolite could potentially become available for *N*-nitrosation reactions, as a result of dissimilatory NO_3^- reduction.

Denitrification accounted for approximately 3% of the NO_3^- metabolized in faecal slurries, but when NO_2^- was added to the slurries in place of NO_3^- , denitrification was stimulated 7-fold (Table 2). It seems paradoxical that denitrification should be such a minor process, when MPN counts showed that denitrifying species were present in substantially greater numbers than NO_3^- dissimilating bacteria in faeces (Table 1). Nevertheless, the results unequivocally show that bacteria dissimilating NO_3^- to NO_2^- or NH_4^+ were able to outcompete the denitrifying species for NO_3^- in faecal slurries. Denitrifying bacteria have been shown to outcompete NO_3^- dissimilating species in saltmarsh sediment slurries when high concentrations of NO_3^- were present, whereas bacteria producing NH_4^+ competed most successfully when small amounts of NO_3^- were added (King & Nedwell, 1985). This was attributed by the authors to the fact that NO_2^- accumulated in the high NO_3^- slurries and that this NO_2^- was preferentially denitrified.

H_2 and CH_4 production were stimulated in faecal slurries by addition of either starch or casein. The addition of NO_3^- or NO_2^- had profound effects on microbial gas metabolism however. CO_2 production was relatively unaffected but neither H_2 nor CH_4 accumulated in slurries to which NO_3^- or NO_2^- had been added (Fig. 1).

The studies on gas production suggested that at least two mechanisms existed whereby methanogenesis could be inhibited by the addition of NO_3^- or NO_2^- . Firstly, since H_2 did not accumulate in the slurries, methanogenic substrate was not available; secondly, direct inhibition of the methanogenic bacteria occurred (Figs 1 and 2). These effects were transitory and depended on the presence of $\text{NO}_3^-/\text{NO}_2^-$ (Fig. 3).

One reason why H_2 did not accumulate in slurries containing $\text{NO}_3^-/\text{NO}_2^-$ was that H_2 produced during fermentation by non- NO_3^- -reducing bacteria was used to reduce NO_3^- by NO_3^- dissimilating species (Table 4). The data suggest that even when starch was present in the faecal slurries, NO_3^- metabolizing species were electron-donor-limited and this probably explains why NO_2^- accumulated in the early stages of the fermentations. In NO_3^- reducing bacteria, H_2 production could also be inhibited by electrons being used to reduce NO_3^- and NO_2^- instead of being disposed of via H_2 generating reactions (Schlegel & Schneider, 1978). There is some evidence for this occurring in faecal slurries, since small quantities of H_2 were always produced initially in the NO_3^- slurries, before NO_2^- accumulated, but H_2 was never detected in slurries to which NO_2^- was added, unless the NO_2^- had been completely metabolized (Fig. 3). Since three times as many electrons are needed to reduce NO_2^- to NH_4^+ than are required to reduce NO_3^- to NO_2^- , the disposal of reducing equivalents in this way would be of considerable benefit to the bacteria.

The addition of NO_3^- to washed suspensions of rumen bacteria has been reported to inhibit CH_4 production (Jones, 1972), although the mechanisms of action of NO_3^- were not elucidated. The work of Lewis (1951), and later of Jones (1972), showed that H_2 and formate were preferred electron donors for the reduction of NO_3^- by rumen bacteria.

The addition of NO_3^- to faecal slurries containing starch had marked effects on the fermentation products formed, but did not significantly influence the products of the casein fermentation (Table 3), indicating that saccharolytic bacteria were primarily affected. In the starch slurries, addition of NO_3^- resulted in less butyrate and more acetate being formed. The production of more oxidized fermentation products in the presence of NO_3^- or NO_2^- is consistent with findings from a number of studies with pure cultures of fermentative bacteria such as *E. coli* (Cole & Brown, 1980), *Clostridium perfringens* (Ishimoto *et al.*, 1974), *Clostridium butyricum* (Keith *et al.*, 1982) and *Propionibacterium acnes* (Allison & Macfarlane, 1987). Dissimilatory reduction of NO_3^- or NO_2^- is energetically advantageous since it enables bacteria to regenerate oxidized coenzymes during fermentation, with the result that more oxidized fermentation products are formed and, due to increased substrate level phosphorylation, higher cell yields achieved.

It was found in parallel experiments that the ability of NO_3^- to influence fermentation product ratios was not the result of any inhibitory effect on methanogenesis, since identical effects on VFA production were observed with faecal slurries obtained from non-methanogenic individuals (unpublished results).

The effect of NO_3^- on butyrate production may be important from the viewpoint of gastrointestinal physiology since it is a major fermentation product in the human large gut, and is the major energy yielding substrate for colonic epithelial cells (Roediger, 1980). Moreover, butyrate has been shown to promote differentiation of malignant colonocytes (Augeron & Labois, 1984), and in the rat its deficiency results in the onset of clinical and biochemical lesions characteristic of ulcerative colitis (Roediger & Nance, 1986).

A major problem in estimating the importance of NO_3^- with respect to bacterial metabolism in the large intestine of man resides in the fact that the amount of this substance entering the large gut is largely unknown. Whilst it is true that quantities of NO_3^- are consumed in the diet (Walker, 1975; Knight *et al.*, 1987) and that considerable levels are produced endogenously by the tissues (Lee *et al.*, 1986), an accurate assessment of the role played by this metabolite requires detailed knowledge of how much actually becomes available to bacteria in the colon. Only then can the significance of NO_3^- in the large gut fermentation be established.

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