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

(Received 24 September 1987; revised 3 February 1988)

Most probable number counts showed that denitrifying species were the numerically predominant NO₃ reducing bacteria in the faeces of five methanogenic individuals [about 10¹⁰ bacteria (g dry wt faeces)⁻¹]. In faecal slurries, however, denitrification was a relatively minor route of NO₃ dissimilation, since only about 3% of the NO₃ was converted to gaseous products, with NO₃⁻ being mainly reduced to NO₂⁻ and NH₄⁺. When KNO₂ was added to the slurries, denitrification became quantitatively more significant with approximately 23% of the NO₃⁻ being lost as gaseous products. The addition of KNO₃ (10 mM) to slurries containing either starch or casein significantly decreased H₂ and CH₄ production. The effect of NO₃⁻ on methanogenesis was twofold: firstly, H₂ accumulation decreased due to diversion of electrons towards NO₃⁻/NO₂⁻ reduction, and as a result of H₂ being used as an electron donor for NO₃⁻ reduction, resulting in the removal of the methanogenic substrate; secondly, there was direct inhibition of methane-producing bacteria by NO₃⁻ and NO₂⁻. In starch-containing slurries, acetate:butyrate molar ratios were increased when NO₃⁻ was added but this effect was not observed when casein replaced starch. These results show that the ability of NO₃⁻/NO₂⁻ 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 H₂, CO₂ and CH₄ (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 NO₃⁻ 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 NO₃ may reach the large gut (Radcliffe et al., 1985). Although this has been challenged by some workers (Bartholomew & Hill, 1984), ¹³N studies in rats and human NO₃⁻ balance studies indicate that a further source of NO₃⁻ results from endogenous formation in the tissues and that NO₃⁻ 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 NO₃⁻ and NO₂⁻. 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 NO₃⁻ 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 H$_2$/CO$_2$/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$_2$O. 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 M.

*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$_2$O to N$_2$. The formation of N$_2$O 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$_2$O 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 CDPI computing integrator. Results were quantified by comparison with authentic gas standards.

*Chemical analysis.* Particulate matter and bacterial cells were removed from samples by centrifugation (27000 g, 10 min). NO$_3^-$ was determined as described by Barnes & Folkard (1951). NO$_2^-$ 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$_2^-$ concentrations were then determined by difference from the NO$_3^-$ analysis described above. NH$_3$ 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).
Intestinal fermentation

1399

Table 1. Most probable number (MPN) counts of NO\textsubscript{3} reducing bacteria and total anaerobes in faeces

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MPN count (g dry wt faeces)\textsuperscript{-1}</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria converting NO\textsubscript{3} to NO\textsubscript{2}</td>
<td></td>
<td>2 x 10\textsuperscript{6}-1 x 10\textsuperscript{8}</td>
<td>2 x 10\textsuperscript{8}</td>
</tr>
<tr>
<td>Bacteria converting NO\textsubscript{3} to gaseous products</td>
<td></td>
<td>2 x 10\textsuperscript{9}-2 x 10\textsuperscript{10}</td>
<td>6 x 10\textsuperscript{9}</td>
</tr>
<tr>
<td>Total bacterial count</td>
<td></td>
<td>6 x 10\textsuperscript{10}-5 x 10\textsuperscript{11}</td>
<td>2 x 10\textsuperscript{11}</td>
</tr>
</tbody>
</table>

RESULTS

Enumeration of bacteria

NO\textsubscript{3} reducing species constituted only a small percentage of bacteria in each of the five faecal samples studied (Table 1). Denitrifying bacteria (N\textsubscript{2}O producers) were the numerically predominant NO\textsubscript{3} reducing species [mean MPN count 6 x 10\textsuperscript{9} (g dry wt faeces)\textsuperscript{-1}], with bacteria that reduced NO\textsubscript{3} to NO\textsubscript{2} occurring in lower numbers [mean MPN count 2 x 10\textsuperscript{8} (g dry wt faeces)\textsuperscript{-1}].

Effect of NO\textsubscript{3} on gas production in faecal slurries

In the absence of added carbohydrate or protein, low levels of CO\textsubscript{2} and CH\textsubscript{4} accumulated in faecal slurries (Fig. 1a). The addition of starch stimulated gas production 3-fold, however (Fig. 1b). Both NO\textsubscript{3} and NO\textsubscript{2} markedly decreased the formation of H\textsubscript{2} and CH\textsubscript{4} in slurries containing starch, although CO\textsubscript{2} production appeared to be relatively unaffected (Fig. 1c, d). Both NO\textsubscript{3} and NO\textsubscript{2} were rapidly reduced in starch slurries resulting in elevated levels of NH\textsubscript{4}\textsuperscript{+} (Fig. 1b, c, d). Substantial quantities of CO\textsubscript{2}, H\textsubscript{2} and CH\textsubscript{4} were also produced by faecal slurries to which casein had been added. As was the case with starch, the addition of NO\textsubscript{3} resulted in a decrease in H\textsubscript{2} production and methanogenesis (Fig. 1e, f). Whilst the quantities of H\textsubscript{2}, CO\textsubscript{2} and CH\textsubscript{4} produced by slurries from different individuals varied markedly, the results when corrected for controls (slurries with no additions), demonstrated that the effects of NO\textsubscript{3} and NO\textsubscript{2} on H\textsubscript{2} and CH\textsubscript{4} formation were similar in every experiment.

Denitrification by faecal slurries

The use of N\textsubscript{2} 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 N\textsubscript{2} (Sørensen, 1978). A more dependable way of quantifying the process involves the inclusion of acetylene in the assay system, which blocks formation of N\textsubscript{2} from N\textsubscript{2}O, with the result that N\textsubscript{2}O accumulates as the final denitrification product instead of N\textsubscript{2} (Yoshinari & Knowles, 1976). The acetylene blocking method was used in this study. The results showed that denitrification was a minor route of NO\textsubscript{3} dissimilation in faecal slurries with only about 3% of the NO\textsubscript{3} added being reduced to N\textsubscript{2}O (Table 2). In slurries that contained NO\textsubscript{3}, however, denitrification occurred to a greater extent, with approximately 23% of the NO\textsubscript{3} being converted to N\textsubscript{2}O after 48 h.

Effect of NO\textsubscript{3} on VFA and other fermentation products

The addition of NO\textsubscript{3} 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 NO\textsubscript{3}, succinate and lactate did not accumulate in the presence of NO\textsubscript{3}. In contrast, the addition of NO\textsubscript{3} 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$_2^-$. 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$_3^-$; ■, NO$_2^-$; ○, CO$_2$; □, H$_2$; △, CH$_4$.

Table 2. Production of N$_2$O 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 ± SEM of experiments with bacteria from three individuals.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>N$_2$O [μmol (ml slurry)$^{-1}$]</th>
<th>N$_2$ [μmol (ml slurry)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slurry + acetylene</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Slurry + NO$_2^-$ + acetylene</td>
<td>1.16 ± 0.29</td>
<td>ND</td>
</tr>
<tr>
<td>Slurry + NO$_3^-$ + acetylene</td>
<td>0.15 ± 0.02</td>
<td>ND</td>
</tr>
</tbody>
</table>

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

**Fig. 2.** Effect of addition of NO\textsubscript{3} on H\textsubscript{2} and CH\textsubscript{4} formation in faecal slurries. (a) Slurry + starch (0.2\%) + 20 mM-BES; (b) as (a) + 10 mM-NO\textsubscript{3}; (c) slurry + starch (0.2\%) + H\textsubscript{2} (1 atm); (d) as (c) + 10 mM-NO\textsubscript{3}. Data presented are mean results from two methanogenic individuals. ●, CO\textsubscript{2}; □, H\textsubscript{2}; ▲, CH\textsubscript{4}.

**Table 3. Effect of NO\textsubscript{3} on VFA and carboxylic acid production in slurry 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\textsubscript{3} (10 mM). Results are means ± SEM of either three or five separate experiments, and are corrected for endogenous control values.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Total VFA (mM)</th>
<th>VFA molar ratio</th>
<th>Other carboxylic acids (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>Slurry + starch*</td>
<td>19.9 ± 1.9</td>
<td>59 ± 5.8</td>
<td>16 ± 2.0</td>
</tr>
<tr>
<td>Slurry + starch + NO\textsubscript{3}*</td>
<td>20.1 ± 1.1</td>
<td>76 ± 5.4</td>
<td>12 ± 3.1</td>
</tr>
<tr>
<td>Slurry + casein†</td>
<td>58.7 ± 4.0</td>
<td>61 ± 4.6</td>
<td>15 ± 2.9</td>
</tr>
<tr>
<td>Slurry + casein + NO\textsubscript{3}†</td>
<td>51.3 ± 3.4</td>
<td>71 ± 7.5</td>
<td>12 ± 4.6</td>
</tr>
</tbody>
</table>

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\textsubscript{3} on H\textsubscript{2} and CH\textsubscript{4} formation**

Methanogenesis was completely inhibited in faecal slurries containing the inhibitor BES. As a result, substantial quantities of H\textsubscript{2} accumulated in the headspace gas (Fig. 2a). When NO\textsubscript{3} (10 mM) and BES (20 mM) were added to the slurries, H\textsubscript{2} did not accumulate showing that H\textsubscript{2} formation was directly influenced by NO\textsubscript{3} (Fig. 2b). Methanogenesis was stimulated in slurries incubated with excess H\textsubscript{2} (Fig. 2c); however, the addition of 10 mM-NO\textsubscript{3} to these slurries resulted in complete abolition of CH\textsubscript{4} production (Fig. 2d). Production of H\textsubscript{2} and CH\textsubscript{4} was also inhibited in slurries that contained lower concentrations of NO\textsubscript{3} (2 mM) (Fig. 3). The NO\textsubscript{3} was rapidly metabolized, however, and after 12 h incubation no NO\textsubscript{3} or NO\textsubscript{2} was detectable. Once NO\textsubscript{3} and NO\textsubscript{2} were exhausted, H\textsubscript{2} formation and methanogenesis resumed, indicating that the inhibition was reversible.

**Utilization of H\textsubscript{2} as an electron donor for NO\textsubscript{3} reduction**

Only trace amounts of NO\textsubscript{3} were metabolized by washed faecal bacteria in the absence of exogenous electron donor when N\textsubscript{2} was the headspace gas in the serum bottles (Table 4). However, nitrate was stoichiometrically reduced to NO\textsubscript{2} when H\textsubscript{2} was used as an electron donor.
Fig. 3. Inhibition of \( \text{H}_2 \) and \( \text{CH}_4 \) production in faecal slurries by the addition of 2 mM-\( \text{NO}_3^- \). Similar results were obtained with slurries from five different methanogenic subjects. Results presented are from one individual. ■, \( \text{NO}_3^- \); ▲, \( \text{NO}_2^- \); ○, \( \text{CO}_2 \); □, \( \text{H}_2 \); △, \( \text{CH}_4 \).

Table 4. Reduction of \( \text{NO}_3^- \) by washed faecal bacteria with \( \text{H}_2 \) as electron donor

<table>
<thead>
<tr>
<th>Time of sampling (h)</th>
<th>Conditions..</th>
<th>( \text{N}_2 )</th>
<th>( \text{H}_2 )</th>
<th>( \text{H}_2 + \text{starch} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{NO}_3^- )</td>
<td>( \text{NO}_2^- )</td>
<td>( \text{NH}_2^+ )</td>
<td>( \text{NO}_3^- )</td>
</tr>
<tr>
<td>0</td>
<td>8.4</td>
<td>ND</td>
<td>0.87</td>
<td>8.1</td>
</tr>
<tr>
<td>20</td>
<td>8.3</td>
<td>0.15</td>
<td>1.61</td>
<td>4.6</td>
</tr>
</tbody>
</table>

ND, Not detected.

donor. The production of \( \text{NO}_3^- \) instead of \( \text{NH}_2^+ \) by faecal bacteria when \( \text{H}_2 \) was the sole electron donor showed that these cultures were effectively electron-donor-limited. When starch was added to bottles with \( \text{H}_2 \) headspace, more \( \text{NO}_3^- \) was reduced, less \( \text{NO}_2^- \) accumulated and more \( \text{NH}_2^+ \) was formed, showing that under these conditions the bacteria were electron-acceptor-limited.

DISCUSSION

The anaerobic metabolism of \( \text{NO}_3^- \) is carried out by many species of facultative and strictly anaerobic bacteria. Two major pathways of dissimilatory \( \text{NO}_3^- \) metabolism exist: reduction of \( \text{NO}_3^- \) to \( \text{NO}_2^- \) or \( \text{NH}_2^+ \), which is termed \( \text{NO}_3^- \) dissimilation, and reduction of \( \text{NO}_3^- \) to gaseous products (\( \text{N}_2 \), \( \text{N}_2 \)), a process known as denitrification (Herbert, 1982). \( \text{NO}_3^- \) 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 \( \text{NO}_3^- \) as the main product of \( \text{NO}_3^- \) dissimilation when grown under carbon-limited conditions and that \( \text{NH}_2^+ \) is produced during nitrogen-limited growth (Dunn et al., 1979; Keith et al., 1982; Macfarlane & Herbert, 1982).

In this study, \( \text{NO}_3^- \) and \( \text{NH}_2^+ \) were found to be the major products of \( \text{NO}_3^- \) reduction by human intestinal bacteria, irrespective of whether starch or casein was added to faecal slurries. \( \text{NH}_2^+ \), the end-product of \( \text{NO}_3^- \) dissimilation, is toxic to mammalian cells (Visek, 1978); however, the production of \( \text{NH}_2^+ \) from \( \text{NO}_3^- \) is likely to be unimportant quantitatively in the colon, compared
to the \( \text{NH}_4^+ \) that is formed as a result of amino acid fermentation (Macfarlane et al., 1986). \( \text{NO}_2^- \) 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 \( \text{N}-\text{nitrosation} \) reactions, as a result of dissimilatory \( \text{NO}_3^- \) reduction.

Denitrification accounted for approximately 3\% of the \( \text{NO}_3^- \) metabolized in faecal slurries, but when \( \text{NO}_2^- \) was added to the slurries in place of \( \text{NO}_3^- \), 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 \( \text{NO}_3^- \) dissimilating bacteria in faeces (Table 1). Nevertheless, the results unequivocally show that bacteria dissimilating \( \text{NO}_3^- \) to \( \text{NO}_2^- \) or \( \text{NH}_4^+ \) were able to outcompete the denitrifying species for \( \text{NO}_3^- \) in faecal slurries. Denitrifying bacteria have been shown to outcompete \( \text{NO}_3^- \) dissimilating species in saltmarsh sediment slurries when high concentrations of \( \text{NO}_3^- \) were present, whereas bacteria producing \( \text{NH}_4^+ \) competed most successfully when small amounts of \( \text{NO}_3^- \) were added (King & Nedwell, 1985). This was attributed by the authors to the fact that \( \text{NO}_3^- \) accumulated in the high \( \text{NO}_3^- \) slurries and this \( \text{NO}_3^- \) was preferentially denitrified.

\( \text{H}_2 \) and \( \text{CH}_4 \) production were stimulated in faecal slurries by addition of either starch or casein. The addition of \( \text{NO}_3^- \) or \( \text{NO}_2^- \) had profound effects on microbial gas metabolism however. \( \text{CO}_2 \) production was relatively unaffected but neither \( \text{H}_2 \) nor \( \text{CH}_4 \) accumulated in slurries to which \( \text{NO}_3^- \) or \( \text{NO}_2^- \) 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 \( \text{NO}_3^- \) or \( \text{NO}_2^- \). Firstly, since \( \text{H}_2 \) 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 \( \text{H}_2 \) did not accumulate in slurries containing \( \text{NO}_3^-/\text{NO}_2^- \) was that \( \text{H}_2 \) produced during fermentation by non-\( \text{NO}_3^- \)-reducing bacteria was used to reduce \( \text{NO}_3^- \) by \( \text{NO}_3^- \) dissimilating species (Table 4). The data suggest that even when starch was present in the faecal slurries, \( \text{NO}_3^- \) metabolizing species were electron-donor-limited and this probably explains why \( \text{NO}_2^- \) accumulated in the early stages of the fermentations. In \( \text{NO}_3^- \) reducing bacteria, \( \text{H}_2 \) production could also be inhibited by electrons being used to reduce \( \text{NO}_3^- \) and \( \text{NO}_2^- \) instead of being disposed of via \( \text{H}_2 \) generating reactions (Schlegel & Schneider, 1978). There is some evidence for this occurring in faecal slurries, since small quantities of \( \text{H}_2 \) were always produced initially in the \( \text{NO}_3^- \) slurries, before \( \text{NO}_2^- \) accumulated, but \( \text{H}_2 \) was never detected in slurries to which \( \text{NO}_2^- \) was added, unless the \( \text{NO}_2^- \) had been completely metabolized (Fig. 3). Since three times as many electrons are needed to reduce \( \text{NO}_2^- \) to \( \text{NH}_4^+ \) than are required to reduce \( \text{NO}_3^- \) to \( \text{NO}_2^- \), the disposal of reducing equivalents in this way would be of considerable benefit to the bacteria.

The addition of \( \text{NO}_2^- \) to washed suspensions of rumen bacteria has been reported to inhibit \( \text{CH}_4 \) production (Jones, 1972), although the mechanisms of action of \( \text{NO}_3^- \) were not elucidated. The work of Lewis (1951), and later of Jones (1972), showed that \( \text{H}_2 \) and formate were preferred electron donors for the reduction of \( \text{NO}_3^- \) by rumen bacteria.

The addition of \( \text{NO}_3^- \) 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 \( \text{NO}_3^- \) resulted in less butyrate and more acetate being formed. The production of more oxidized fermentation products in the presence of \( \text{NO}_3^- \) or \( \text{NO}_2^- \) is consistent with findings from a number of studies with pure cultures of fermentative bacteria such as \( \text{E. coli} \) (Cole & Brown, 1980), \( \text{Clostridium perfringens} \) (Ishimoto et al., 1974), \( \text{Clostridium butyricum} \) (Keith et al., 1982) and \( \text{Propionibacterium acnes} \) (Allison & Macfarlane, 1987). Dissimilatory reduction of \( \text{NO}_3^- \) or \( \text{NO}_2^- \) 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\textsubscript{3}\textsuperscript{-} to influence fermentation product ratios was not due to 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\textsubscript{3}\textsuperscript{-} 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 & Laboisse, 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\textsubscript{3}\textsuperscript{-} 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\textsubscript{3}\textsuperscript{-} 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\textsubscript{3}\textsuperscript{-} in the large gut fermentation be established.

We are grateful to Drs M. J. Wolin and T. L. Miller for helpful discussions.

REFERENCES


Intestinal fermentation


