Stimulation of Methanogenesis by Slurries of Saltmarsh Sediment after the Addition of Molybdate to Inhibit Sulphate-reducing Bacteria

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The addition of 20 mM-molybdate to sediment slurry in order to inhibit sulphate-reducing bacteria increased the amount of methane formed. Only a small proportion (7·8%) of the total methane came from the $H_2 + CO_2$ pathway, while methanogenesis from acetate was negligible. Conversion of ^{14}C -labelled formaldehyde, methanol and methionine to $^{14}CH_4$ by sediment slurry in the presence of molybdate showed that these were potential precursors of the additional methane, although lack of adequate analytical techniques precluded establishment of the quantitative significance of this turnover; $[^{14}C]$ formate was not converted to $^{14}CH_4$. It is suggested that inhibition of sulphate-reducing bacteria by molybdate immediately increased methanogenesis from formaldehyde, methanol and methionine, and to some extent from $H_2 + CO_2$. There was also evidence for longer term development of an increased methanogenic bacterial population when there was no competition from the sulphate-reducing bacteria for available nutrients.

INTRODUCTION

Molybdate is a potent inhibitor of sulphate-reducing bacteria (Peck, 1959) and has been used as an inhibitor of sulphate reduction in natural microbial communities, including anaerobic sediments (Oremland & Taylor, 1978). A number of workers have used 20 mM-molybdate specifically to inhibit sulphate-reducing bacteria in order to investigate their role in carbon flow in anaerobic sedimentary communities, particularly with regard to competition between sulphate-reducing bacteria and methanogenic bacteria (Oremland & Taylor, 1978; Banat *et al.*, 1981; Sørensen *et al.*, 1981). It has previously been suggested (Winfrey & Zeikus, 1977; Abram & Nedwell, 1978*b*; King & Wiebe, 1980) that the sulphate-reducing bacteria preferentially use both H_2 and acetate in competition with the methanogens. Several workers (Oremland & Taylor, 1978; Banat *et al.*, 1981; Sørensen *et al.*, 1981; Nedwell & Banat, 1981) have reported that inhibition of sulphate-reducing bacteria by molybdate in slurries of anaerobic sediments stimulated the rate of methane formation, and also led to the accumulation of H_2 and acetate (Sørensen *et al.*, 1981; Nedwell & Banat, 1981), thus apparently supporting the hypothesis of competition for these substrates.

The present work was undertaken to investigate whether the additional methane formed upon addition of molybdate to sediment slurry could be accounted for by the stimulation of the $H_2 + CO_2$ or the acetate pathways to methane.

METHODS

Preparation of sediment slurry. Slurry of 50% (v/v) sediment in deoxygenated aged seawater was prepared with the precautions to ensure anaerobiosis described by Banat *et al.* (1981). The sediment used was taken from the top 0–5 cm layer at a site in the bottom of a saltmarsh creek in the Colne Point saltmarsh, Essex, U.K. (Nedwell & Abram, 1978). Samples of slurry (65 ml) were dispensed into 150 ml Erlenmeyer flasks sealed with subaseals (Fisons) and gassed with either N_2/CO_2 (80:20%, v/v) or H_2/CO_2 (80:20%, v/v) (British Oxygen Company,

U.K.). The flasks were incubated at 22 °C on an orbital platform shaker at a speed just sufficient to maintain sediment particles in suspension. Each experiment was carried out with a freshly prepared batch of slurry.

Uptake of H_2 and CH_4 formation. Duplicate flasks of slurry were set up for each of the following treatments under an N_2/CO_2 atmosphere: (a) no additions; (b) with 1 ml $Na_2MoO_4 \cdot 2H_2O$ solution to give a final concentration of 20 mM-molybdate; (c) with 20 mM-molybdate + 0.05% (v/v) chloroform to eliminate methanogenesis (Bauchop, 1967); (d) with chloroform (0.05%, v/v). A second series of duplicate flasks, with identical treatments to the above, were set up under H_2/CO_2 .

The flasks were incubated and subsamples (50 μ l) of the gas head spaces were periodically removed via the subaseals. Subsamples were analysed for methane concentration by injection into a Perkin-Elmer F33 gas chromatograph with flame-ionization detector (2 m $\times$ 3 mm o.d. stainless steel column packed with Poropak N; isothermally at 70 °C; N_2 carrier gas at 30 ml min⁻¹). In addition, further subsamples were analysed for H_2 concentration in order to detect any H_2 uptake by the slurry under H_2/CO_2 . Analyses for H_2 were by injection of 50 μ l subsamples of the head space into a Perkin-Elmer F33 gas chromatograph with a thermistor detector (2 m $\times$ 3 mm o.d. stainless steel column packed with 80–100 mesh silica gel; isothermally at 100 °C; N_2 carrier gas at 20 ml min⁻¹).

Experiments with $H^{14}CO_3^-$ and $[U-^{14}C]$ acetate. Four flasks were set up with a N_2/CO_2 atmosphere and molybdate (20 mM) was added to two of the flasks. The flasks were incubated for 24 h and CH_4 concentrations in the head spaces were then analysed to ensure that the added molybdate had stimulated the rate of formation of CH_4 . Following this confirmation, 200 μ l of $H^{14}CO_3^-$ solution [10 μ Ci ml⁻¹; 0.1 mCi (3.7 MBq) mmol⁻¹] were then injected into each flask and the incubation continued for a further 3 d. The gas head space of each flask was periodically sampled and 50 μ l subsamples injected into a Pye-Unicam GCD gas chromatograph with flame-ionization detector (2 m $\times$ 4 mm o.d. glass column packed with silica gel, 80–100 mesh) at 100 °C; Ar/CO_2 (95:5%, v/v) carrier gas (British Oxygen Company, U.K.) at 50 ml min⁻¹. At the end of the gas chromatography column a 1:1 stream splitter directed half of the effluent gas stream to the flame-ionization detector and the other half to a gas proportional counter (Model 504, E.S.I. Nuclear, U.K.). The gas stream to the counter was first passed through an oven packed with CuO at 700 °C to oxidize any carbon compounds to CO_2 , and then radioactivity in the CH_4 and CO_2 peaks was measured as $^{14}CO_2$ in a flow-through counting chamber (Balba & Nedwell, 1982). The silica gel gas chromatography column adequately separated CH_4 and CO_2 . Even though the flame-ionization detector did not respond to CO_2 , radioactivity in the CO_2 peak was detected and counted by the gas proportional counter.

A further subsample of the sediment slurry was used to measure titrimetrically the total carbonate content of the slurry (Gran, 1952).

Another set of flasks was used to repeat this experiment, with the same batch of slurry, with 200 μ l of $[U-^{14}C]$ acetate solution [10 μ Ci ml⁻¹; 58 mCi (2.15 GBq) mmol⁻¹] injected into each flask. The acetate concentration in the slurry was measured by ether extraction of an acidified sample of centrifuged slurry and injection of subsamples of the ether extract into a gas chromatograph (Balba & Nedwell, 1982). The concentration of acetate was determined by comparison with extracted aqueous standards.

Methane formation in the presence of fluoroacetate. Two pairs of flasks were set up under N_2/CO_2 . To one pair 20 mM-molybdate was added, while to the second pair 20 mM-molybdate + fluoroacetate (5 mM final concentration) was added. The flasks were incubated as described previously and the gas head spaces periodically sampled and analysed for CH_4 .

Experiments with ^{14}C -labelled formate, formaldehyde, methanol and methionine. Similar experiments to those described above were carried out in which flasks were injected with 200 μ l volumes of solutions of: (a) sodium $[^{14}C]$ formate [20 μ Ci ml⁻¹; 10 mCi (0.4 GBq) mmol⁻¹]; (b) $[^{14}C]$ formaldehyde [20 μ Ci ml⁻¹; 10 mCi (0.4 GBq) mmol⁻¹]; (c) $[^{14}C]$ methanol [10 μ Ci ml⁻¹; 58 mCi (2.15 GBq) mmol⁻¹]; or (d) $[methyl-^{14}C]$ methionine [10 μ Ci ml⁻¹; 57 mCi (2.1 GBq) mmol⁻¹]. All radiotracers were obtained from Amersham. The gas head spaces of flasks were periodically sampled to measure any radioactivity appearing in CH_4 or CO_2 . After 3 d, the slurry was acidified with 5 ml 6 M-HCl in order to release all HCO_3^- as CO_2 , and the recovery of ^{14}C as either $^{14}CH_4$ or $^{14}CO_2$ was determined. Each experiment was carried out with a separate batch of freshly-prepared slurry. In all the experiments methane formation was measured and similar results were obtained as for the H_2 + CO_2 /acetate experiment.

These experiments differed from those with $H^{14}CO_3^-$ and $[U-^{14}C]$ acetate in that adequate analytical methods were not available to measure the pool size in the sediment slurry of each of these substrates, and only conversion data could therefore be obtained.

CH_4 oxidation. Radioactive CH_4 was prepared with a culture of unidentified methanogenic bacteria under H_2/CO_2 to which $NaH^{14}CO_3$ was added. When sufficient $^{14}CH_4$ had accumulated in the gas head space of the culture flask, the culture was made strongly alkaline with 5 M-NaOH in order to absorb any residual $^{14}CO_2$. The gas head space of the culture flask was then checked by injecting a 500 μ l subsample into the gas proportional counter. Only $^{14}CH_4$ and no $^{14}CO_2$ was detected in the gas head space. The $^{14}CH_4$ had an activity of 0.03 μ Ci ml⁻¹.

[specific activity $2.2 \mu\text{Ci}$ (81.4 KBq) mmol^{-1}]. Duplicate flasks of slurry were set up under N_2/CO_2 ; while to a second pair of flasks under N_2/CO_2 molybdate solution (20 mM final concentration) was added. Each flask then had 3 ml $^{14}\text{CH}_4$ injected into the head space. The flasks were incubated as before, and the gas head spaces periodically sampled (500 μl subsamples) to determine whether any conversion of $^{14}\text{CH}_4$ to $^{14}\text{CO}_2$ could be detected.

RESULTS

H₂ uptake and CH₄ formation. The formation of CH_4 by slurry in the presence or absence of added molybdate is shown in Fig. 1. The addition of molybdate initially increased the rate of CH_4 formation by sediment slurry under either a N_2/CO_2 or a H_2/CO_2 atmosphere. This initial increase of methanogenesis by molybdate occurred over the first 4 d, following which there was a lag in CH_4 generation. After 8 d, CH_4 was rapidly generated in both the control and molybdate-treated flasks under H_2/CO_2 . In all treatments where chloroform was present there was no formation of CH_4 .

When H_2 uptake from the gas head spaces was followed in those flasks under H_2/CO_2 (Fig. 2) it was evident that in the molybdate-treated slurries there was little stimulation of H_2 uptake during the first 4 d when rapid methanogenesis occurred. The H_2 uptake only increased significantly after 8 d in both the control and molybdate-treated flasks, coincident with the increase of methanogenesis after this time. In those treatments where chloroform was present this increase in H_2 uptake after 8 d did not occur. The presence of chloroform alone had no effect upon the initial rate of H_2 uptake, although it was totally inhibited by molybdate. This agrees with previously reported data (Nedwell & Banat, 1981) that the sulphate-reducing bacteria, not the methanogenic bacteria, are the major H_2 -utilizers in the saltmarsh sediment.

Experiments with $\text{H}^{14}\text{CO}_3^-$ and $[\text{U-}^{14}\text{C}]\text{acetate}$. Methanogenesis was increased by molybdate addition during the first 4 d of incubation (Fig. 1). Since in each experiment an initial 1 d incubation was used to confirm increased methanogenesis in the presence of molybdate, a further 3 d period of incubation was used after radiotracer addition. Three days after addition of either $\text{H}^{14}\text{CO}_3^-$ or $[\text{U-}^{14}\text{C}]\text{acetate}$ only small amounts of radioactivity could be detected in the copious CH_4 which had accumulated in those flasks containing molybdate.

The slurry had a total carbonate content of $9610 \text{ nmol carbonate (ml slurry)}^{-1}$, and after 3 d, the turnover of $\text{H}^{14}\text{CO}_3^-$ to $^{14}\text{CH}_4$ in the presence of molybdate was 0.09% of the added radioactivity. Therefore it could be calculated that the $\text{H}_2 + \text{CO}_2$ pathway to CH_4 accounted for $9610 \times 0.0009 = 9.61 \text{ nmol CH}_4 \text{ (ml slurry)}^{-1}$. However, total CH_4 formation during the same 3 d period was $111 \text{ nmol CH}_4 \text{ (ml slurry)}^{-1}$ and the $\text{H}_2 + \text{CO}_2$ pathway therefore accounted for only $9.61/111 \times 100 = 7.8\%$ of the total CH_4 formed.

Similarly, after 3 d there was a proportionate turnover to $^{14}\text{CH}_4$ of only 0.07% of the added $[\text{U-}^{14}\text{C}]\text{acetate}$ in the presence of 20 mM-molybdate. Acetate concentration in the slurry was $65 \text{ nmol (ml slurry)}^{-1}$, giving a rate of acetate methanogenesis over 3 d of $65 \times 0.0007 = 0.04 \text{ nmol CH}_4 \text{ (ml slurry)}^{-1}$. This was only $0.04/111 \times 100 = 0.04\%$ of the total CH_4 formed during the 3 d period.

Methane formation in the presence of fluoroacetate. The accumulation of CH_4 in the molybdate-treated flasks of slurry was identical whether 5 mM-fluoroacetate was present or not. The result therefore confirmed that acetate was not a significant precursor of the CH_4 formed by the molybdate-treated slurry.

Experiments with ^{14}C -labelled formate, formaldehyde, methanol or methionine. Table 1 shows the proportional conversion to $^{14}\text{CH}_4$ or $^{14}\text{CO}_2$ of each of these added substrates after 3 d, in the presence or absence of 20 mM-molybdate.

CH₄ oxidation. No decrease of radioactivity from the $^{14}\text{CH}_4$ in the head spaces of the flasks could be detected in any of the treatments for up to 12 d of incubation. This showed that $^{14}\text{CH}_4$ was not being metabolized by sedimentary bacteria.

Fig. 1

Fig. 2

Fig. 1. Evolution of CH_4 by sediment slurry. Solid symbols indicate H_2/CO_2 atmosphere; open symbols indicate N_2/CO_2 atmosphere. $\bullet$, $\circ$, Controls, with no additions; $\blacktriangle$, $\triangle$, with 20 mM-molybdate; $\times$, with 20 mM-molybdate + 0.05% (v/v) chloroform, and also with 0.05% (v/v) chloroform only. CH_4 was not formed in either of the flasks containing chloroform. Bars indicate spread of duplicates.

Fig. 2. H_2 uptake from the gas head spaces of flasks by slurry under H_2/CO_2 atmosphere. $\bullet$, Control with no additions; $\blacktriangle$, with 20 mM-molybdate; $\blacksquare$, with 0.05% (v/v) chloroform; $\blacktriangledown$, with 20 mM-molybdate + 0.05% (v/v) chloroform. Bars indicate spread of duplicates.

Table 1. *Conversion of potential precursors to CH_4 by sediment slurry*

The conversion of ^{14}C -labelled precursors to $^{14}\text{CH}_4$ or $^{14}\text{CO}_2$ was measured in the presence and absence of 20 mM-molybdate. Measurements were made after 3 d, as it was over this period that methanogenesis was enhanced by 20 mM-molybdate.

Radiotracer	Recovery of added radioactivity (%)			
	Without molybdate		With 20 mM-molybdate	
	$^{14}\text{CH}_4$	$^{14}\text{CO}_2$	$^{14}\text{CH}_4$	$^{14}\text{CO}_2$
[^{14}C]Formate	0	45.0	0	79.0
[^{14}C]Formaldehyde	0	4.8	5.2	34.1
[^{14}C]Methanol	0.91	96.1	26.0	47.0
[methyl- ^{14}C]Methionine	0	53.0	12.6	23.1

DISCUSSION

The results of the first experiment showed that molybdate addition initially increased methanogenesis from sediment slurry to an equal extent under both N_2/CO_2 and H_2/CO_2 . This suggested that the source of the extra CH_4 was not from the $\text{H}_2 + \text{CO}_2$ pathway. Also, the lack of any stimulation of H_2 uptake by molybdate (Fig. 2) during this initial 4 d period supported this contention. It was only after 8 d that H_2 uptake and methanogenesis greatly increased from

slurry under H_2/CO_2 , suggesting that methanogenesis from this pathway was initially limited by low numbers of active methanogenic bacteria. The 8 d lag before methanogenesis increased was presumably the time required for the population of methanogens to respond to the enhanced concentrations of $H_2 + CO_2$ available in the experimental flasks.

The experiment with added $H^{14}CO_3^-$ confirmed quantitatively that the $H_2 + CO_2$ pathway was not the major source of the additional CH_4 formed during the initial period. Only 7.8% of the total CH_4 formed in the presence of molybdate during the 3 d after addition of $H^{14}CO_3^-$ was from this pathway. Molybdate addition does, however, stimulate the $H_2 + CO_2$ pathway to some extent as the 9.6 nmol CH_4 (ml slurry) $^{-1}$ formed under N_2/CO_2 has to be compared with a total methanogenesis of only 2.8 nmol CH_4 (ml slurry) $^{-1}$ in the control without molybdate.

It has previously been suggested that sulphate-reducing bacteria preferentially oxidize available H_2 in competition with methanogenic bacteria (Winfrey & Zeikus, 1977; Abram & Nedwell, 1978a; Oremland & Taylor, 1978) resulting in inhibition of methanogenesis in sulphate-rich environments such as anaerobic marine sediments. The increase in methanogenesis on addition of molybdate has, therefore, been attributed to the removal of this competition (Oremland & Taylor, 1978; Sørensen *et al.*, 1981; Nedwell & Banat, 1981). None of these workers used radiotracers, however, and their data and conclusions are therefore less direct than those reported here.

Again, our experiment with $[U-^{14}C]$ acetate showed quantitatively that only a negligible proportion (0.04%) of the CH_4 formed in the presence of molybdate was derived from acetate. The experiment with fluoroacetate, an inhibitor of acetate turnover (Banat *et al.*, 1981), confirmed that acetate was also not a significant precursor of the additional CH_4 .

Although molybdate addition initially increased CH_4 formation, a lag subsequently developed after 4 d under both N_2/CO_2 and H_2/CO_2 . This suggested that molybdate permitted the rapid metabolism to CH_4 of precursors in the sediment which became depleted after 4 d. Formate, formaldehyde and methanol have been proposed as intermediates in the reduction of CO_2 to CH_4 and have also been shown to be converted to CH_4 by various methanogenic bacteria (Wolfe, 1971; Wolfe & Higgins, 1979). Amino acids such as methionine may also be used as CH_4 precursors (Zinder & Brock, 1978). These, therefore, represented possible alternative precursors for CH_4 in the sediment. Our experiments with ^{14}C -labelled isotopes of these potential precursors of CH_4 showed that in the absence of molybdate they were converted to $^{14}CO_2$, not $^{14}CH_4$ (Table 1). In the presence of molybdate, $[^{14}C]$ formate did not give rise to any $^{14}CH_4$, but 5.2% of the $[^{14}C]$ formaldehyde, 26% of the $[^{14}C]$ methanol, and 12.6% of the terminal methyl group of $[methyl-^{14}C]$ methionine, were converted to $^{14}CH_4$ without any lag period for adaptation. We do not at the moment have adequate analytical techniques for sedimentary concentrations of these CH_4 precursors and so, unlike HCO_3^- and acetate, we cannot at present determine the quantitative contribution of each of these precursors to the additional CH_4 formed in the presence of molybdate. The results clearly show, however, that formaldehyde, methanol and methionine were converted to CH_4 in the presence of molybdate and these must therefore be strong candidates as the source of the extra CH_4 . In agreement with these findings, it is interesting to note that Oremland *et al.* (1982a) have recently shown that methanogenesis from sediment in Big Soda Lake, Nevada, was stimulated by addition of methanol, trimethylamine, and methionine, but not by addition of H_2 , acetate or formate. Oremland *et al.* (1982b) have also demonstrated that the majority of CH_4 from a North American saltmarsh sediment was from methanol and methylated amines, not from acetate or HCO_3^- . These workers suggested that sulphate-reducing and methanogenic bacteria did not compete for methanol which was used only for methane formation. In contrast, our results suggest competition even for methanol in the Colne Point sediment, and sulphate-reducing bacteria are known to utilize it as an electron donor (Postgate, 1979).

One further hypothesis to explain the additional CH_4 in the presence of molybdate needed to be considered. Recent work (Davis & Yarborough, 1966; Reeburgh, 1980; Devol & Ahmed, 1981; Iversen & Blackburn, 1981) has suggested that sulphate-reducing bacteria may oxidize CH_4 anaerobically. The apparent stimulation by molybdate of CH_4 accumulation in the gas head spaces of flasks could also have been explained, therefore, if molybdate addition inhibited

oxidation of CH_4 by sulphate-reducing bacteria rather than stimulated CH_4 formation. However, the result of the experiment with added $^{14}\text{CH}_4$ discounted this hypothesis as no removal of $^{14}\text{CH}_4$ by bacteria in the slurry could be detected.

Our observations do not necessarily contradict the general hypothesis that competition by the sulphate-reducers for H_2 and acetate inhibits CH_4 formation from these precursors. Under conditions of such competition in the presence of sulphate it might be expected that only small populations of methanogenic bacteria would normally be present in the sediment, metabolizing these substrates at very low rates of activity. Senior *et al.* (1982) have shown, for example, that methanogenesis from H^{14}CO_3 proceeds in the Colne Point saltmarsh sediment, but only at rates three orders of magnitude lower than the rates of sulphate reduction. Again, in marine sediments acetate is converted to CO_2 not CH_4 (Banat *et al.*, 1981; Sørensen *et al.*, 1981), whereas in freshwater sediments acetate is a precursor of CH_4 (Cappenberg & Prins, 1974; Winfrey & Zeikus, 1979). Thus, molybdate addition to sulphate-rich sediments may not result in any great or immediate stimulation of either the $\text{H}_2 + \text{CO}_2$ or the acetate pathways to CH_4 , until the methanogenic bacterial populations have time to develop in response to the greater availability of these substrates. Such a development of the $\text{H}_2 + \text{CO}_2$ pathways to CH_4 was seen after 8 d in our first experiment (Fig. 1).

Only *Methanosarcina barkeri* and *Methanococcus mazei* are known to metabolize methanol (Weimer & Zeikus, 1978; Hippe *et al.*, 1979). The latter organism has been isolated from Colne Point saltmarsh sediment (Abram & Nedwell, 1978a). It has been demonstrated that methanol is converted much more rapidly to methane by *Methanosarcina barkeri* than is $\text{H}_2 + \text{CO}_2$ or acetate (Hutten *et al.*, 1980). Thus the rate at which methanol may be converted to methane may be less restricted by a small methanogenic population size than is the conversion of $\text{H}_2 + \text{CO}_2$.

Thus the addition of 20 mM-molybdate would seem to stimulate methanogenesis by a twofold process. Firstly, precursors of CH_4 which are usually oxidized to CO_2 are made available to methanogenic bacteria by the addition of molybdate. These include formaldehyde, methanol and methionine, but not acetate or formate. The $\text{H}_2 + \text{CO}_2$ pathway to CH_4 is also stimulated to a limited extent. Secondly, the longer term development of a larger methanogenic bacterial population is permitted because of the removal of competitive inhibition by the sulphate-reducing bacteria.

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