Formate as an Intermediate in the Bovine Rumen Fermentation

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An average of 11 (range, 2 to 47) μmoles of formate per g per hr was produced and used in whole bovine rumen contents incubated in vitro, as calculated from the product of the specific turnover rate constant, k, times the concentration of intercellular formate. The latter varied between 5 and 26 (average, 12) nmoles/g. The concentration of formate in the total rumen contents was as much as 1,000 times greater, presumably owing to formate within the microbial cells. The concentration of formate in rumen contents minus most of the plant solids was varied, and from the rates of methanogenesis the Michaelis constant, Kₘ, for formate conversion to CH₄ was estimated at 30 nmoles/g. Also, the dissolved H₂ was measured in relation to methane production, and a Kₘ of 1 n mole/g was obtained. A pure culture of Methanobacterium ruminantium showed a Kₘ of 1 n mole of H₂/g, but the Kₘ for formate was much higher than the 30 nmoles for the rumen contents. It is concluded that nonmethanogenic microbes metabolize intercellular formate in the rumen. CO₂ and H₂ are the principal substrates for rumen methanogenesis. Eighteen per cent of the rumen methane is derived from formate, as calculated from the intercellular concentration of hydrogen and formate in the rumen, the Michaelis constants for conversion of these substrates by rumen liquid, and the relative capacities of whole rumen contents to ferment these substrates.

Succinate, hydrogen, lactate, ethyl alcohol, and formate, important fermentation products of pure cultures of rumen bacteria and protozoa, occur in the intercellular fluid of the rumen at concentrations so low that the amounts normally absorbed by the ruminant are nutritionally unimportant. These products could be important as intermediates, even at their low concentrations, if the microbes use them as fast as they are formed. This has been demonstrated for succinate (3) by multiplying the concentration (pool size) in the rumen fluid by the specific rate constant, k, for pool turnover, calculated from the rate of disappearance of a pulse of added isotope. Seventy-five per cent of the succinate is converted to propionate. With somewhat different methods, hydrogen has been shown to be an important precursor of rumen methane (7).

Neither lactate nor ethyl alcohol is normally actively metabolized in the rumen. A diminished adenosine triphosphate production per sugar molecule, associated with lactate or ethyl alcohol production, may in part account for their unimportance in the highly competitive rumen environment (6).

It has been difficult to assess the importance of formate as a rumen intermediate. Formate added to the rumen or to incubated rumen contents is rapidly metabolized (2, 4), and methanogenesis increases stoichiometrically according to the equation

\[ 4\text{HCOOH} \rightarrow 3\text{CO}_2 + \text{CH}_4 + 2\text{H}_2\text{O} \]

Formate added to washed rumen bacteria (5) is converted to hydrogen and carbon dioxide. If hydrogenlyase or similar enzymes catalyze this conversion, a reversible equilibrium between hydrogen and formate similar to that found for Escherichia coli (11) would be expected in the rumen. A high concentration of dissolved hydrogen does cause a significant increase in the concentration of formate in rumen liquid incubated in vitro (7).

Although hydrogen and CO₂ appear to be the chief substrates for methanogenesis, formate
could be important as a source of hydrogen. The present report examines this question. In relatively few cases has it been possible to identify with certainty the kind and amount of substrate used by particular bacteria in their natural habitat; this is attempted for *Methanobacterium ruminantium* growing in the rumen.

**MATERIALS AND METHODS**

**Rumen contents.** Rumen contents were obtained from one of several fistulated Jersey heifers fed alfalfa hay twice daily at a maintenance level, or in a few cases a 50:50 grain mixture at a level slightly above maintenance. Rumen liquid containing little plant material or protozoa was used to test the effect of various formate concentrations on the rate of methanogenesis. It was obtained by inserting through the fistula a 3/4-inch (1.6-cm) copper tube, stoppered at the lower end and with many 1/4-inch (0.3-cm) holes drilled through the wall of the lower 8 inches (20 cm) of tube. A 3/4-inch (0.6-cm) plastic tube provided with a suction bulb was used to withdraw ca. 800 ml of liquid from the interior of the copper tube. This was allowed to stand in the laboratory for about 30 min. Fermentation gases carried the solids to the top, and the protozoa settled chiefly on the bottom. The fluid between was used in the experiments.

For rate of methanogenesis and in vitro turnover rate constant measurements with 14C-formate, a sample containing representative proportions of microbes, solids, and liquid was used. This will be called rumen contents.

**Pure culture of a rumen methanogenic bacterium.** A strain was isolated, just prior to its use, from a high dilution of the rumen contents of the animal supplying the rumen fluid used in the experiments determining the rumen *Km* for formate and for hydrogen. It was identified as *M. ruminantium* from the appearance of the colony.

**Enzymatic measurement of formate in intercellular rumen fluid.** A dialysis sac (1-inch (2.5-cm) diameter) was partially filled with 20 ml of salt solution containing 0.1% NaCl, 0.05% KH2PO4, 0.05% K2HPO4, 0.02% (NH4)2SO4, 0.01% MgSO4, 0.01% CaCl2, and 0.5% NaHCO3, saturated with carbon dioxide gas at atmospheric pressure. The sac was suspended in the rumen, with a 2-oz (57-g) lead weight tied to one end and an 80-lb (36-kg) test nylon cord to the other (and also to the weight) for retrieval. Analysis of the amounts of acetate, propionate, and butyrate in the sac after various periods in the rumen indicated that 1 hr was sufficient for equilibration under these conditions.

After 1 hr, the sac was removed quickly from the rumen, the adherent rumen contents were rapidly rinsed off the exterior with a jet of water from a wash bottle, and the contents were immediately drawn into a 20-ml syringe fitted with an 18-gauge needle. The sample was ejected into a test tube immersed in ice and returned to the laboratory for analysis. The material within the sac will be called the dialysate.

**The formyltetrahydrofolate synthetase method (10)** was employed to measure directly the formate concentration in the dialysates, with spectrophotometric measurement of the absorbancy of 5,10-methylenetetrahydrofolate at 350 nm.

**Purification of 14C-formate.** The commercially obtained sample of H14COONa was converted to the acid and purified by vacuum distillation and by liquid-liquid chromatography as described in the section on chromatographic methods.

**Turnover rate constant, k, for formate conversion.** A representative sample of rumen contents was placed in a warmed Dewar flask and returned to the laboratory within 10 min. At the laboratory, 10-g samples were either weighed out into centrifuge tubes (23 × 120 mm) or were dispensed into them with a ladle holding 10 ± 0.2 g of rumen contents. The gas above the sample in the tube was displaced with O2-free carbon dioxide. The tube was closed anaerobically with a rubber stopper and incubated at 39 °C.

The 14C-sodium formate (2.3 × 104 counts/min, specific activity 1 mc/m mole) for each tube was contained in 0.15 ml of solution in a 1-ml hypodermic syringe equipped with a 21-gauge needle (3.2 cm long) which was inserted through the stopper at a recess in the rubber which left only a 5-mm thickness to penetrate.

Just before and during the addition of tracer, the sample of rumen contents was mixed vigorously with a bent wire rotated at 3,200 rev/min. The shaft of the wire traversed a small hole drilled through the center of the rubber stopper. The wire was of stiff stainless steel, 1 mm in diameter, bent at the tip to give a 9-mm terminal transverse segment, offset to put its center in line with the shaft of the wire. Stirring was continuous during the short exposures to isotopes, but with longer exposures the sample was stirred only briefly when the isotope was added and again when the reaction was stopped.

The reaction was stopped after 4, 8, 16, or 90 sec by injecting 10 ml of a 1 N NaOH solution from a 10-ml syringe with an 18-gauge needle inserted through the rubber at the base of a second depression in the stopper. For very short exposures to the isotopes, the needles of both syringes were pushed through the stopper prior to initiation of stirring. For the zero-time measurements, the alkali was added before the isotope.

The formate was separated chromatographically and its radioactivity was measured.

Initially, anaerobiosis was not maintained during exposure to the isotope, but later air was excluded with a stream of O2-free carbon dioxide delivered inside the reaction tube.

**Chromatographic separation of formate.** A column was prepared by mixing 3.33 g of acid-washed BW-200 Solka-Floc cellulose (Brown Co., Berlin, N.H.) with 0.5 ml of water and 0.5 ml of 0.1 N H3PO4. This was suspended in 50 ml of BA-50 [50% (v/v) acetone in mixed hexanes], and a portion was added as a slurry to the chromatography tube. Air bubbles were removed, the cellulose was lightly tamped with a brass rod which just fitted the lumen of the tube, and further portions of slurry were added and tamped until all the cellulose was used. The BA-50 was washed from the column with BA-5 (5% acetone in mixed hexanes) prior to addition of the cap with sample.
The alkaline sample was centrifuged to separate the solids. A measured volume of supernatant fluid, with added carrier formate, was evaporated to dryness, taken up in 0.4 ml of water, acidified with 0.1 ml of 5 N H₂SO₄, and mixed thoroughly and rapidly with the cap material consisting of 0.74 g of acid-washed and dried cellulose and 0.74 g of anhydrous sodium sulfate. The cap material with sample was transferred to the top of the chromatographic column on which about 1 cm of BA-5 had been left after the column was prepared. The cap material containing the sample was packed carefully on top of the column, and the slight excess of solvent was drained into the column to the level of the top surface. Two 0.5-ml samples of BA-5 were used to wash the sample into the column.

Four-carbon and higher acids were eluted with 30 ml of BA-5. Propionic and acetic acids, and most of the formic acid, were eluted by 200 ml of BA-10. Complete elution of formic acid and elution of succinic and lactic acids required an additional 250 ml of BA-30. The method gave clean separation of all acids except the butyric and higher acids in the first band. By eluting slowly, it was possible to separate a few micromoles of formate from 250 μmoles of acetate. In some cases in which no carrier was added, a formate band could be barely detected and titrated.

The eluate from the column dripped into the titration vessel of an electrometric titrimeter (Titrigraph, Type SBR2c, Radiometer, Copenhagen, Denmark) equipped with a glass electrode which dripped through the upper solvent layer into a lower aqueous layer. The titrator was ordinarily operated to titrate to pH 9.5. The liquids were stirred rapidly with a small electric stirrer, and a gentle stream of carbon dioxide-free air was bubbled through the aqueous and hexane-acetone layers. When the chart recording the titrations showed that it was time for the formate band to come off the column, the water in the titration vessel was changed, and after the band had come off all of the water and the eluate accumulated during elution of the formate band were pooled and evaporated to dryness. The sodium formate was taken up in 0.5 ml of water, added to a scintillation vial containing 10 ml of scintillation fluid, and counted with a Packard Tri-Carb scintillation counter, series 3000. Quenching corrections were estimated by the channel count ratio method. Recovery of formate was 100 ± 5%.

Rate of exchange. In experiments to measure the back reaction rate of conversion of ¹⁴CO₂ into H⁺¹⁴COO⁻, ¹⁴C-sodium bicarbonate (7.5 × 10⁷ counts/min, 5 mc/m mole) contained in 0.1 ml of solution was injected into the 10-g sample of rumen contents under anaerobic conditions. The reaction was stopped with H₂SO₄. In these experiments, the CO₂ was first removed from the acidified rumen fluid and the formate was then oxidized with mercuric chloride.

Initially, the carbon dioxide was removed by gentle boiling of the rumen fluid in a tube provided with a reflux condenser to prevent escape of the formic acid. Under these conditions very little H⁺¹⁴COO⁻ was recovered. Recovery was 90% or better if the ¹⁴C-formate was heated in an inorganic solution. Apparently, some component in the boiling rumen fluid destroyed the formate.

Measurement of formate radioactivity in the exchange experiments. The method of Yang (personal communication) was used. A 5-ml amount of supernatant fluid obtained by centrifuging acidified rumen contents at 20,000 × g for 30 min was placed in a 50-ml Erlenmeyer flask in crushed ice, and 1 ml of 3 M phosphate buffer, pH 2.5, was added. Nitrogen gas was bubbled through for 30 min to drive off CO₂. Then 0.5 g of HgCl₂ was added to oxidize the formic acid to CO₃, and the flask was closed with a serum cap from which a plastic center well was suspended. Some of the gas in the flask was removed with a syringe to create a slight vacuum, and the flask was heated to 80°C for 50 min. It was cooled, and 0.3 ml of ethanollamine was injected through the cap into the center well to absorb the CO₂ from oxidation of the formic acid. The flask was left overnight at room temperature, and the radioactivity in the ethanollamine measured with a Beckmann scintillation counter, LSC 100, with 1 part of methylcellulose and 2 parts 5%, 2,5-diphenyloxazole in toluene.

Rate of methanogenesis. A 10-ml sample of rumen contents in a 50-ml centrifuge tube was passed with CO₂, the tube was stoppered, and excess gas was allowed to escape into a water-lubricated syringe through a needle, which was then withdrawn. The tube was incubated at 39°C along with the samples used to measure turnover rate constants. At periodic intervals, the sample was thoroughly shaken to equilibrate the gas phase with the fermentation gas bubbles caught in the solids. The gas produced was measured with the syringe, the gas was replaced, and a 1-ml gas sample for analysis was withdrawn.

Methane and hydrogen were measured on a thermal conductivity gas chromatograph (Perkin-Elmer Model 154B) with a silica gel column; nitrogen was used as carrier gas.

The influence of hydrogen and formate concentrations on the rate of methanogenesis was measured in vitro with 400 ml of rumen liquid in a system similar to that previously described (7) except that hydrogen was not metered into the system. It was added only at the end of a run when formate was in excess, to check that additional hydrogen increased the rate of methanogenesis. Sodium formate (2 m) was fed at various rates with a Harvard pump.

The initial feed rate was the lowest tested; later rates were increased by steps until formate accumulated in excess. Just before the feed rate was increased, the dialysate was removed into a syringe. A 3-ml sample for formate analysis was refrigerated immediately, and the remainder was analyzed for dissolved hydrogen, usually within 1 hr. At frequent intervals, the gas circulating through the system was analyzed for methane and hydrogen, and the pressure increase (or decrease when hydrogen was added) in the system was noted on the manometer containing Brodie solution. The amount of methane or hydrogen corresponding to the pressure changes was determined by noting the pressure change when 10 ml of hydrogen was injected from a syringe into the system. The rate of methane production was approximately constant.
during each period in which the formate feed rate was constant.

The duration of an experiment was usually less than 8 hr with rumen liquid, but the pure culture could be studied in the apparatus over a period of several days. Changes due to growth of the culture could be detected by differences with time in the rate of methanogenesis when excess hydrogen gas was provided. By starting with a culture nearing the stationary phase, these differences were not large during an experiment.

For quantitative estimation of dissolved H₂, the dissolved gas in a sample of dialysate was converted into a gas phase by using the apparatus shown in Fig. 1. Water (20 ml) was injected into the 100-ml flask through the rubber stopper. The syringe was detached from the needle, leaving the latter in place as a channel for escape of air and water vapor. The water was heated with a small flame and kept boiling for about 2 min until all air was driven from the flask. While the water was still gently boiling, the syringe containing the dialysate was connected to the needle, and the syringe contents were injected into the flask. The needle was then quickly withdrawn, leaving in the flask only water plus the sample.

A 5-ml syringe fitted with a water jacket (Fig. 1) was filled with 3 ml of 1.5 N NaOH, the needle was inserted through the stopper, and the alkaline solution was injected. The needle was left in the stopper with the syringe still attached. The flask was thoroughly shaken to absorb CO₂, and then was heated to boiling with a small flame. The rate of boiling was regulated so that the plunger of the syringe was forced up 2 or 3 cm by the escaping water vapor and gas. The water condensed in the syringe. The excess could be forced back into the flask, with a little retained as a seal against escape of the collected gas.

After about 1 min of boiling, all dissolved gas had collected. Heating was stopped and the tip of the needle was pulled up into the rubber of the stopper, sealing off the syringe contents. The contained gas quickly cooled and the needle was completely withdrawn. Excess water was expelled, air was drawn in to a total volume of 1 ml of gas in the syringe, and this was injected into the chromatograph.

The reliability of the method was tested by drawing 50 ml of water into a 50-ml syringe, together with a few ml of H₂. The needle tip was sealed by sticking it into a rubber stopper. The H₂ and water were shaken to equilibrate them and then left overnight. The next morning, the gas was expelled from the syringe, and 10-ml samples of the water were withdrawn for analysis of dissolved H₂. The peak heights for triplicate analyses were 87.5, 88.5, and 89. The amount of hydrogen recovered was about that expected from the solubility coefficient of H₂ in water at room temperature, taking into account that some nitrogen and other gases dissolved in the water had entered the gas phase, so that the gas was no longer 100% hydrogen.

**RESULTS**

The rates of methanogenesis during the first hour of in vitro incubation of rumen contents are shown in Table 1, together with formate concentration and the rate constant, k, for formate turnover. It was not feasible to measure rates of methanogenesis, turnover rate constant, and formate concentration in each experiment. In the experiment of 13 March, the formate concentration measured in rumen contents incubated in vitro resembled that found in the rumen itself.

In Table 2 are summarized the results of all valid experiments we have made concerning formate utilization.

In the initial measurement of turnover rate

![Diagram of apparatus for quantitative recovery of dissolved gas for gas chromatographic estimation.]

**Table 1. Detailed results of some experiments with bovine rumen contents**

<table>
<thead>
<tr>
<th>Date of experiment</th>
<th>Rate of CH₄ production (μmoles per g per hr)</th>
<th>Formate concn (μmoles/ml)</th>
<th>k, rate constant (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 May 1968</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 April 1968</td>
<td>6:00-7:25*</td>
<td>5.9</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>7:30-9:00</td>
<td>10.3</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>9:15-10:45</td>
<td>9.7</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>12:15-12:45</td>
<td>6.6</td>
<td>7.2</td>
</tr>
<tr>
<td>15 April 1968</td>
<td>12:45-15:45</td>
<td>5.0</td>
<td>5.6</td>
</tr>
<tr>
<td>13 March 1968</td>
<td>8.4, 7.3</td>
<td>5.4*</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* The animal was feeding when this sample was taken.

* Dialysis sac in the rumen.

* Dialysis sac in rumen contents shaken in vitro.
first-order curves similar to those of the dis-appearances of labeled formate during administration of free carbon dioxide was used to show the influence of formate (Fig. 3) and hydrogen (Fig. 4) concentrations on the rate of methanogenesis by samples of rumen fluid incubated anaerobically in vitro. Dialysis sacs of sterile salt solution were immersed in the incubating fluid, and the dialysate was withdrawn at intervals to measure the concentrations of formate and hydrogen. The differences in slopes of the curves in Fig. 3 and 4 are assumed to be due to differences in the number of active methanogenic bacteria in the rumen fluid used in the various experiments.

If disappearance of label from H\textsuperscript{14}COO\textsuperscript{-} was due in part to exchange with carbon dioxide, the rate constants found for formate turnover are too large. To test for exchange with carbon dioxide, \textsuperscript{14}CO\textsubscript{2} was added to 10 ml of rumen contents, and the appearance of label in formate was examined by the chemical method for formate analysis. If exchange with \textsuperscript{14}CO\textsubscript{2} was the chief pathway by which label appeared in formate, the increment of label would be greatest initially, diminishing logarithmically until equilibrium was reached. The results in Table 3 indicate that the increment was least during the first 4 sec and increased rapidly up to 16 sec. This indicates that the carbon dioxide participated in a large number of reactions leading to formate. Under these circumstances, the technique is not adequate to measure the rate of CO\textsubscript{2}-HCOOH exchange. To the extent that formate carbon exchanges with carbon dioxide, the rate constants for formate turnover are too high.

At an average turnover rate constant for formate of 16/min or 960/hr (Table 2), and an average formate concentration of 12 \textmu moles/g, the average turnover (flux) of formate would be 11.52 \textmu moles per g per hr, equivalent to 2.88 \textmu moles of methane per g per hr. This is 37\% of the observed average rate of methane production. In the one experiment in which CH\textsubscript{4} production, formate concentration, and \( k \) were all measured, the calculated formate produced would account for 7\% of the methane.

Lineweaver-Burk plots were constructed to show the influence of formate (Fig. 3) and hydrogen (Fig. 4) concentrations on the rate of methanogenesis by samples of rumen liquid incubated anaerobically in vitro. Dialysis sacs of sterile salt solution were immersed in the incubating fluid, and the dialysate was withdrawn at intervals to measure the concentrations of formate and hydrogen. The differences in slopes of the curves in Fig. 3 and 4 are assumed to be due to differences in the number of active methanogenic bacteria in the rumen fluid used in the various experiments.

**TABLE 2. Comparison of formate turnover with methane production**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Rate of CH\textsubscript{4} production (\textmu moles per g per hr)</th>
<th>Formate concn in intercellular rumen fluid (\textmu moles/g)</th>
<th>( k ), Specific rate constant for formate turnover (hr\textsuperscript{-1})</th>
<th>Formate turnover, ( k \times ) formate concn (\textmu moles per g per hr)</th>
<th>CH\textsubscript{4} equivalent of formate turnover (\textmu moles per hr)</th>
<th>CH\textsubscript{4} accounted for by formate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average value</td>
<td>7.7 (10)*</td>
<td>12 (13)</td>
<td>960 (15)</td>
<td>11.52</td>
<td>2.88</td>
<td>37</td>
</tr>
<tr>
<td>Range</td>
<td>3.5-10.3</td>
<td>5-26</td>
<td>360-1,800</td>
<td>1.8-46.8</td>
<td>0.45-11.7</td>
<td>13-104</td>
</tr>
</tbody>
</table>

* The figure in parentheses is the number of measurements made.

In the one experiment in which CH\textsubscript{4} production, formate concentration, and \( k \) were all measured, the calculated formate produced would account for 7\% of the methane.

Lineweaver-Burk plots were constructed to show the influence of formate (Fig. 3) and hydrogen (Fig. 4) concentrations on the rate of methanogenesis by samples of rumen liquid incubated anaerobically in vitro. Dialysis sacs of sterile salt solution were immersed in the incubating fluid, and the dialysate was withdrawn at intervals to measure the concentrations of formate and hydrogen. The differences in slopes of the curves in Fig. 3 and 4 are assumed to be due to differences in the number of active methanogenic bacteria in the rumen fluid used in the various experiments.
If the methanogenic bacteria all had the same enzyme affinity for formate, the intercept on the X axis should be the same for each curve and should represent \(-1/K_m\), in which \(K_m\) is the Michaelis constant designating the substrate concentration at which the enzyme is half-saturated. Inspection of Fig. 3 shows that the curves tend to converge on the abscissa at approximately \(-33\) nmol of formate/ml. This suggests that the Michaelis constant for utilization of formate in this system is about \(3 \times 10^{-4}\) M.

Similarly, the curves for hydrogen in Fig. 4 indicate a Michaelis constant of about \(10^{-4}\) M, a value significantly lower than the constant for formate.

These experiments on the effects of hydrogen and formate concentrations were repeated with the freshly isolated pure culture of \(M.\ ruminantium\), grown on autoclaved 33% rumen fluid shaken with 80% \(\text{H}_2\)-20% \(\text{CO}_2\). The active culture was transferred aseptically and anaerobically to the experimental vessel (7), and carbon dioxide was recirculated through it. Before the formate feed was started, traces of hydrogen remaining in the culture could be absorbed out on a bypass through a CuO column heated to 350 C. After hydrogen was absorbed and the bypass was closed off, it took about 40 min for the hydrogen in the gas phase to increase to the concentration in equilibrium with the hydrogen generated in solution by the culture from the low concentration of formate first fed. With higher concentrations of formate, obtained by increasing the feed rate, the equilibrium was reached more rapidly and the concentration of hydrogen was greater. In some experiments in which this equilibrium was achieved, the amount of dissolved hydrogen was calculated from the solubility coefficient for hydrogen at 39 C and the concentration of hydrogen in the equilibrated gas phase.

The results for hydrogen with the pure culture are shown as a Lineweaver-Burk plot in Fig. 5.

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**TABLE 3. Appearance of label in formate when \(^{14}\text{CO}_2\) was added to incubated rumen contents**

<table>
<thead>
<tr>
<th>Time of sampling (sec)</th>
<th>Counts per min per 5 ml</th>
<th>Count increment/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>269</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6,987</td>
<td>1,749</td>
</tr>
<tr>
<td>8</td>
<td>22,944</td>
<td>2,810</td>
</tr>
<tr>
<td>16</td>
<td>110,384</td>
<td>10,930</td>
</tr>
<tr>
<td>90</td>
<td>485,936</td>
<td>5,070</td>
</tr>
</tbody>
</table>

* *\(^{14}\text{CO}_2\)-was added at time zero, and successive samples were removed for analysis of formate by the chemical method.*

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**FIG. 3. Curves from three experiments with rumen contents, showing the reciprocal of the rate of methane formation against the reciprocal of the formate concentration.**

**FIG. 4. Curves from three experiments with rumen contents, showing the reciprocal of the rate of methane formation against the reciprocal of the dissolved \(\text{H}_2\) concentration.**

**FIG. 5. Curves from two experiments (○ and □) with a pure culture of Methanobacterium ruminantium, showing the reciprocal of the rate of methane formation against the reciprocal of the dissolved \(\text{H}_2\) concentration. The rate of methanogenesis was quite low in the two unplotted experiments (△ and ●).**
The $K_{m}$ of the pure culture for hydrogen is approximately the same as that of the rumen liquid.

It was not possible to measure methane production from low concentrations of formate, because even in starved pure cultures the concentration never dropped below 260 nmoles/ml. This was in contrast to dissolved hydrogen which readily diminished to values below 1 nmoles/ml. Higher formate concentrations gave methane but the results were too erratic to permit an estimate of the $K_{m}$ for formate. The $K_{m}$ for formate in the pure culture must have been much higher than the 33 nmoles/ml for rumen liquid, since 260 nmoles/ml (the lowest observed concentration of formate in the pure culture) did not support measurable methane production, and the concentration of formate did not decrease.

In some experiments with the pure culture, the formate concentrations in the liquid within the dialysis sac and in a sample of rumen liquid withdrawn directly from the experimental vessel were measured by the enzymatic method. As shown in Table 4, the values agreed fairly well. In contrast, with rumen contents, the intercellular formate pool (from the liquid in the dialysate sac) was 8 nmoles/ml, whereas chromatography of the total rumen contents and titration of the formate band gave a value of 8 µmoles/ml, a 1,000-fold higher concentration.

**DISCUSSION**

The speed (within 8 sec) with which formate conversion diminished when the rumen contents were exposed to air was unexpected. Previous experience had indicated that a fairly large sample of rumen contents containing the normal complement of solids could be left for some time in the laboratory without observed deleterious effects except on the surface. The efficiency of mixing was apparently great enough in the present experiments to aerate the entire mixture very rapidly. The results indicate that the important rumen enzymes for conversion of formate are sensitive to oxygen.

There has seemed to be a discrepancy between the finding that formate utilization is markedly inhibited by air, and the earlier success of Doetsch et al. (5) in demonstrating conversion of formate into hydrogen and carbon dioxide with a suspension of bacteria obtained directly from the rumen. The discrepancy is resolved if formate hydrogenlyase is reversibly inhibited by oxygen, whereas methanogenesis is irreversibly inhibited.

In one experiment during the present study, an actively methanogenic culture of *M. ruminantium* was accidentally exposed to air during transfer from the culture to the experimental vessel. The medium gradually became reduced again, but the rate of methanogenesis was very low. Formate was fed to this system, and although with time the rate of methanogenesis remained low there was a gradual build-up of hydrogen in the circulating gas. Enzyme systems converting formate to hydrogen and carbon dioxide were apparently less inhibited than were the enzymes forming methane. In the experiments of Doetsch et al., sodium sulfide was added to the bacterial suspension after it was prepared. This may have restored the capacity to convert formate, at least partially, but the capacity for methanogenesis was not restored.

The fact (Table 4) that the culture of *M. ruminantium* (including the cells) contained about the same concentration of formate as did the cell-free dialysate suggests that there is no marked active accumulation of free formate by these cells. The high formate concentration in the total rumen contents as compared with the intercellular fluid (assumed to be in equilibrium with the liquid in the dialysis sac) indicates that certain components of the population either actively accumulate formate from the medium or retain intracellularly formed material. The rapid specific rate constant for conversion of added formate suggests that it rapidly enters the cells converting it to CO$_2$ and H$_2$. This entrance is probably the reaction limiting the rate at which formate leaves the intercellular pool. The inter- and intracellular pools are postulated not to be in equilibrium with each other.

The formate concentrations reported by investigators from the usual analyses of rumen volatile fatty acids represent the intracellular pool, rather than the very small intercellular pool.

**Table 4. Comparison of the formate concentrations in the dialysate and the culture of *Methanobacterium ruminantium***

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Dialysate µmoles/ml</th>
<th>Total culture µmoles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>First day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:45</td>
<td>0.66</td>
<td>1.05</td>
</tr>
<tr>
<td>16:42</td>
<td>0.95</td>
<td>1.10</td>
</tr>
<tr>
<td>Second day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>1.19</td>
<td>1.05</td>
</tr>
<tr>
<td>14:40</td>
<td>1.09</td>
<td>1.0</td>
</tr>
<tr>
<td>15:51</td>
<td>0.42</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* A pure mass culture of *M. ruminantium* was fed sodium formate at various rates, and the formate concentration within a dialysis bag was compared with the concentration in the whole culture, including cells. The enzymatic method for formate measurement was used.
Some of the high formate concentrations found in analyses of rumen contents may be due to formate contained in the feed.

The lack of affinity of \textit{M. ruminantium} for formate, as compared with the mixed rumen population, shows that in the rumen intercellular formate is not used by this species. In contrast, the similarity of its affinity for hydrogen with that of the rumen shows that hydrogen in the rumen can be used by this organism. Since hydrogen does occur in the rumen and since formate and hydrogen are the only substrates known to be used for carbon dioxide reduction to methane by this organism, it is concluded that in the rumen the only substrates providing energy to \textit{M. ruminantium} are hydrogen and carbon dioxide. This agrees with the previous finding (7) that the rate of methanogenesis in the rumen can be accounted for on the basis of the dissolved hydrogen. Carbon dioxide is in excess in the rumen.

\textit{M. mobilis} (8), the only other important methanogenic bacterium thus far isolated from the bovine rumen, must have a \(K_\text{m} \) for hydrogen similar to that of \textit{M. ruminantium}. It can use only hydrogen, formate, and carbon dioxide as energy sources in pure culture. If, like \textit{M. ruminantium}, it has also a high \(K_\text{m} \) for formate, some of the non-methanogenic bacteria must be chiefly responsible for formate decomposition in the rumen. Others presumably produce formate. The concentration of formate in the intercellular pool is at any moment the resultant of formate production and utilization.

Since the fermentation rate in the rumen is fairly steady under natural conditions, we have adopted the continuous-fermentation model to describe the system and have assumed that entry and disappearance rates of the formate intercellular pool are equal and that the formate concentration remains constant. Although the analytical results show that this is not true for an animal fed twice a day, by using the average of a number of measurements made at different times in relation to feeding and applying it to the steady-state model, a value approximating the average of actual rates is obtained.

Failure to measure the rate of \(^{14}\text{C} \) exchange between carbon dioxide and formate prevents the use of isotope disappearance rates to calculate accurately the turnover rate constant for formate and the amount of formate produced as an intercellular intermediate in the rumen fermentation. The importance of formate in rumen methane genesis can be estimated by comparing the rates of methane production from the average formate and hydrogen concentrations in the intercellular rumen fluid, since the total of these substrates used in methanogenesis is absorbed from the intercellular pool.

For hydrogen, the average concentration has been estimated to be 1 nmole/g of intercellular liquid phase (7), coincidentally the same value as the \(K_\text{m} \) for hydrogen. On the average, the rate-limiting enzyme for conversion of \(H_2 \) to methane in the rumen is approximately half-saturated, producing methane at half the rate obtainable with saturating \(H_2 \) concentrations. For formate, the average concentration is 12 nmole/g, as compared with a \(K_\text{m} \) of 30 nmole/g.

The maximal rate of \(H_2 \) conversion to methane (4), studied in a Warburg vessel with rapid shaking, was 1.42 \(\mu\text{mole} / \text{min per g of rumen contents} \), as compared with a maximal formate conversion to methane of 0.63 \(\mu\text{mole} / \text{min per g of rumen contents} \), a ratio of 2.25. A lower ratio was found in larger-scale experiments (7) in which \(H_2 \) was bubbled through rumen liquid, but these conditions were not as favorable as the Warburg technique for rapid solution of \(H_2 \).

Since methane production from formate is almost linear with formate concentration between 0 and 30 nmole/g of intercellular liquid, the methane produced from formate is \((0.63 \times 12)/(1.42 \times 30) = 0.18 \) of the amount produced from \(H_2 \). Since \(H_2 \) is an intermediate in the production of methane from formate, 18\% of the rumen methane arises from formate.

Because of the errors and approximations involved in the experiments and calculations leading to this figure, it must be regarded as a rough estimate of the average rather than a precise value applicable in particular instances. Comparison with the average of 37\%, calculated from the intercellular formate concentration and turnover rate constant, \(k \) (uncorrected for exchange), suggests that the rate of disappearance of formate label through conversion to methane is about equal to its rate of disappearance through exchange and absorption into nonmethanogenic cells. Since carbon dioxide so readily enters intracellular pools of formate (Table 3), absorption from the intercellular pool may not be important for those nonmethanogenic bacteria not metabolizing formate to CO\(_2 \) and \(H_2 \).

In the rumen, with substrates available for fermentation, more formate may be assimilated, and a very small amount may be absorbed through the rumen wall. But these in vivo and in vitro differences do not affect the validity of the present estimate of the fraction of methane derived from formate. This estimate is based on the concentrations of formate and hydrogen in the intercellular fluid in the rumen itself, on the relative affinities of the rumen microbiota to ferment formate and hydrogen to methane, and on the relative abundance of the enzyme systems attacking these substrates. The affinities are not likely to change during brief in vitro maintenance.
under conditions simulating the rumen. The relative abundance of the enzymes attacking formate and hydrogen may vary in rumens receiving different feeds but would be expected to be fairly constant in animals on the same feed. The present estimates are based chiefly on alfalfa-fed animals. It is concluded that the estimate of formate participation in bovine rumen methanogenesis is valid for the rumen itself.

A great affinity of methanogenic cells for hydrogen is requisite for rapid methanogenesis at the very low concentrations of dissolved hydrogen in the rumen. Hydrogen does not normally accumulate in the rumen unless specific inhibitors of methanogenesis are provided (1) or the animal is starved for an extended period (9). The 128 nmoles of methane per min per g of rumen contents (Table 2) would require a hydrogen production of 512 nmoles per min per g. This would require that a pool of 1 n mole of H2/g of rumen contents turn over 512 times/min; i.e., the turnover rate constant, k, is 512/min, a much greater value than that for the other rumen intermediates studied.

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LITERATURE CITED