Formation of Methane by Bacterial Extracts

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The oxidation of ethanol to acetate by cultures of Methanobacillus omelianskii was found by Barker to be dependent upon carbon dioxide (1); using C14O2, Stadtman and Barker demonstrated conclusively that carbon dioxide was the precursor of methane (2). Recently Johns and Barker have reported hydrogen formation from ethanol by resting cells of M. omelianskii in the absence of carbon dioxide (3). The mechanism of carbon dioxide reduction to methane by M. omelianskii as well as the mechanism of methane formation by other organisms is unknown. Current knowledge of the nutrition and fermentative patterns of the methane bacteria has been reviewed by Barker (4). The present communication is concerned with the formation of methane by cell-free extracts of M. omelianskii; a brief report of some of these findings has appeared (5).

EXPERIMENTAL PROCEDURE

The original strain of M. omelianskii, isolated in 1937, was kindly supplied by Dr. H. A. Barker and was mass cultured as described by Johns and Barker (3), except that distilled water supplemented with 10 ml per liter of mineral solution1 and 10 ml per liter of vitamin solution2 was used instead of tap water. Although the nutrition of this organism is simple, growth was more reproducible in our hands when the vitamin mixture was added. For many experiments the level of iron in the medium was increased by adding 19 mg of ferrous sulfate per 20-liter carboy. Harvested cells from an actively gassing culture (16 g, wet weight) were washed under a hydrogen atmosphere in 200 ml of water containing 100 mg of MgSO4 and 50 mg of Na2S. After centrifugation the cells were crushed at -20° in a Hughes press. Crushed cells were suspended in 16 ml of 0.5 potassium phosphate buffer at pH 7.0 containing about 100 μg of DNase. After evacuation and flushing with hydrogen for 10 minutes the suspension was centrifuged at -20° in a Hughes press. Crushed cells were suspended in 16 ml of 0.5 potassium phosphate buffer at pH 7.0 containing about 100 μg of DNase. After evacuation and flushing with hydrogen for 10 minutes the suspension was centrifuged at 20,000 x g to remove cell debris. The cell-free extract prepared in this manner contained about 40 mg of protein per ml as measured by the biuret procedure (6) and was used immediately.

The reaction vessel was a Warburg flask fitted tightly with a rubber serum cap as shown in Fig. 1. The desired gas atmosphere was provided from high purity gas cylinders at a rate of 500 cc per minute to a manifold connected to six flasks; additions to the reaction mixture in each flask were made by removing the side arm as gassing continued. Gas mixtures were prepared using a gas proportioner (Matheson Company, Joliet, Illinois) and were passed through a heated copper train before contact with the reaction mixture. Extract was placed in the main compartment, other components being tipped from the side arm to start the reaction after isolation of the flask. To follow methane formation, gas samples of 0.4 cc were removed through the serum cap by means of a hypodermic needle attached to a syringe. Methane was determined with a Beckman GC-2 gas chromatograph which contained a silicone gel column connected to a hydrogen flame detector. Data presented represent total CH4 formed.

RESULTS

General Properties of Crude Extracts—When crushed cells were suspended in water no detectable methane was produced by the resulting extract. The most active extracts were prepared only when crushed cells were suspended in neutral buffer; for a reaction time of 40 minutes, 0.5 m potassium phosphate buffer at pH 7.0 proved to be satisfactory. The extracts were extremely sensitive to oxygen and were kept near 0° under hydrogen before placement in the reaction vessel. To obtain maximal activity, the enzyme preparation was diluted only by the necessary additions from the side arm of the reaction vessel. The specific activity, micromoles of CH4 per mg of protein per hour, varied as well as the condition of the cells at the time of extract preparation. Since growth incurred a variable lag period and required 2 to 3 days for a maximal cell yield, it was not always possible to harvest a carboy at the precise time when methane formation was most active.

Formation of Methane from H2-CO2—Formation of methane was found to be dependent upon the addition of hydrogen, carbon dioxide, ATP, and CoA as components of the reaction mixture. The effect of carbon dioxide on the formation of methane is shown in Fig. 2; maximal production occurred in a hydrogen atmosphere containing 20% carbon dioxide. The pH of the reaction mixture at the end of the reaction period was 6.8 for concentrations of carbon dioxide at 20% and below. No detectable methane was formed by a similar reaction mixture containing nitrogen in place of hydrogen; both hydrogen and carbon dioxide were required, the most effective ratio of H2 to CO2 being 8:2. Methane formation from this gas mixture was dependent upon ATP and was stimulated by CoA as shown in...
Fig. 3. A concentration of 7.5 μmoles of ATP per ml of reaction mixture effectively saturated the system (Fig. 4).

Effect of Pyruvate—In a hydrogen atmosphere, pyruvate was found to elicit methane formation. As shown in Fig. 5, 0.04 M sodium pyruvate was the most effective initial concentration. Pyruvate at a concentration of 60 μmoles per ml of reaction mixture was found to produce a linear formation of methane for

![Graph showing the effect of ATP concentration on methane formation](image)

**Fig. 4.** Effect of ATP concentration on the formation of methane from H₂ and CO₂. The reaction mixture contained crude extract, 40 mg of protein; CoA, 0.05 μmole; ATP as indicated; potassium phosphate buffer at pH 7.0, 650 μmoles. Total volume, 1.3 ml. Gas atmosphere, 80% H₂ and 20% CO₂. Reaction time, 20 minutes at 37°.

![Graph showing the effect of CO₂ addition on methane formation](image)

**Fig. 2.** Effect of CO₂ addition on methane formation in an H₂ atmosphere. Each reaction vessel contained crude extract, 45 mg of protein; CoA, 0.05 μmole; ATP, 10 μmoles; potassium phosphate buffer at pH 7.0, 500 μmoles; H₂ and CO₂ as indicated. Reaction time was 40 minutes at 37°. The volume of the reaction mixture was 1.2 ml.

![Graph showing the effect of methyl-CoA on methane formation](image)

**Fig. 1.** The reaction vessel for following methane formation. The vessel is a standard Warburg flask of 20-ml capacity fitted with a rubber serum cap.
0 0.02 0.04 0.06 0.08
MOLARITY PYRUVATE

Vol. 238, No. 8
Methane Formation
2884

0.0 0.1 0.2 0.3 0.4 0.5
µmoles METHANE FORMED

0 0.02 0.04 0.06 0.08
MOLARITY PYRUVATE

Fra. 5. Effect of pyruvate concentration on the formation of methane. Each reaction vessel contained crude extract, 40 mg of protein; KPO4 buffer at pH 7.0, 500 µmoles; ferredoxin, 0.5 mg; sodium pyruvate as indicated; and an H2 atmosphere. Total volume of liquid, 1.2 ml. Reaction time was 20 minutes at 37°.

% N2 OR He
0 20 40 60 80 100

FIG. 5. Effect of pyruvate concentration on the formation of methane. Each reaction vessel contained crude extract, 40 mg of protein; KPO4 buffer at pH 7.0, 500 µmoles; ferredoxin, 0.5 mg; sodium pyruvate as indicated; and an H2 atmosphere. Total volume of liquid, 1.2 ml. Reaction time was 20 minutes at 37°.

% H2
0 10 20 30 40 50

µmoles CH4 FORMED

0 0.5 1.0 1.5 2.0

FIG. 6. Effect of decreasing H2 concentration on the formation of methane. Each vessel contained sodium pyruvate, 60 µmoles; CoA, 0.05 µmole; ATP, 3 µmoles; extract, 42 mg of protein; potassium phosphate buffer, 500 µmoles; gas atmosphere as indicated. Reaction time, 40 minutes at 37°. Total liquid volume, 1.3 ml.

50 minutes; the initial rate of methane formation was not as great as with 40 µmoles of pyruvate per ml, but there was not an initial lag as observed with higher concentrations. The addition of ethanol to similar reaction mixtures with or without addition of carbon dioxide elicited the formation of only negligible amounts of methane. Pyruvate effectively replaced the requirements for ATP and carbon dioxide for the formation of methane; additions of CoA (0.05 µmole) and ATP (3 µmole) produced only a slight stimulation and were inhibitory at higher concentrations. Pyruvate was not detectable in the reaction mixture when methane formation ceased. When nitrogen replaced hydrogen as the gas atmosphere, an 80% inhibition of methane formation resulted. As shown in Fig. 6, this effect was not due to a selective oxidation by nitrogen but rather to a dearth of hydrogen, since the degree of inhibition by a helium atmosphere was approximately the same.

Effect of pH—To test the effect of pH on the formation of methane (pyruvate system) without dilution of the extract in the reaction vessel, a mass of crushed cells was divided into portions; each portion was suspended in an appropriate amount of separate phosphate buffer of desired pH, and each of these cell-free extracts was prepared in the manner described previously. Fig. 7 presents the results of this experiment, pH 7.0 being clearly the pH of choice.

Effect of Ferredoxin—Ferredoxin stimulated methane production as shown in Fig. 8, but because of the lability of these extracts, it was not possible to recover active extracts from a DEAE-cellulose column which had removed ferredoxin as described by Mortenson, Valentine, and Carnahan (7) and Valentine, Jackson, and Wolfe (8). Ferredoxin from M. omelianskii was active in the resolved pyruvate elastic reaction of Clostridium pasteuriunum when tested for us by Dr. R. C. Valentine. Ferredoxin, 60 units per mg (7), from C. pasteuriunum was used in the experiment presented in Fig. 8. A stimulation of methane formation upon the addition of ferredoxin (0.5 mg) to the reaction mixture occurred only when extracts were used which had been prepared from cells grown in the medium without the additional 19 mg of ferrous sulfate per
DISCUSSION

The strictly anaerobic nature of methane formation has been one of the obstacles to an elucidation of its biochemical mechanism. The experiments reported here indicate that it is possible to prepare active extracts by conventional techniques provided that exposure to air occurs for only short periods. Sensitivity to oxygen, however, has thus far prevented fractionation of the component enzymes. The marked drop in extract activity observed upon dilution of the extract in buffer points to the probable presence of dissociable intermediates or active cofactors. In this investigation addition of various cofactors, acids, and alcohols did not reverse the disproportionate loss of activity on dilution of the extract. Although ethanol is the substrate for growth, it was ineffective for methane formation when added to extracts of *M. omelianskii*. The observation of Johns and Barker (3) that hydrogen is produced from ethanol by whole cells in the absence of carbon dioxide may have considerable pertinence to the requirement of hydrogen for methane formation by extracts. Since ethanol was not utilized as a source of electrons for methane formation, the role of hydrogen may have been more clearly demonstrable with only the reductive portion of the methane pathway being active.

The role of ferredoxin in this system is not clear; a stimulation of methane formation was noted only when ferredoxin was added to a reaction mixture containing pyruvate. The most active extracts from cells grown in high iron-containing medium were not stimulated by normal levels of ferredoxin, and at higher concentrations ferredoxin had an inhibitory effect.

The formation of methane in the presence of serine has interesting implications. Experiments are now in progress to discover whether carbon atoms from serine or pyruvate are converted to methane. Serine could yield pyruvate, or an active C₅ compound from carbon 3, or possibly formate. An analogy may be made to the requirement for pyruvate in the nitrogen fixation system of *Clostridium pasteurianum* (9), in which the ratio (in micromoles) of pyruvate utilized to ammonia formed may be as great as 100:1. In the methane-forming system described here the ratio has varied from 20:1 to 100:1.

SUMMARY

Cell-free extracts of *Methanobacillus omelianskii* produced methane in phosphate buffer at pH 7.0 when hydrogen, carbon dioxide, coenzyme A, and adenosine triphosphate were components of the reaction mixture. Pyruvate, serine, or O-phosphoserine substituted for ATP and carbon dioxide. A hydrogen atmosphere was essential for optimal methane formation.

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