METHANE FORMATION; FERMENTATION OF ETHANOL IN THE ABSENCE OF CARBON DIOXIDE BY *METHANOBACILLUS OMELIANSKII*[1]

A. T. JOHNS[2] AND H. A. BARKER

Department of Biochemistry, University of California, Berkeley, California

Received for publication May 23, 1960

Methanobacillus omelianskii has been shown to oxidize ethanol anaerobically in the presence of carbon dioxide according to the equation (Barker, 1941)

$$2CH_3 \cdot CH_2OH + CO_2 \rightarrow 2CH_3 \cdot COOH + CH_4. \quad (1)$$

Stadtman and Barker (1949) using C^{14} labeled CO_2 demonstrated that the methane produced in this fermentation is formed entirely by reduction of CO_2. Experiments with growing cultures led to the conclusion (Barker, 1939, 1956) that the oxidation of alcohol is dependent upon the supply of carbon dioxide, and that when the carbon dioxide is consumed, no further oxidation of alcohol occurs. This paper presents evidence that washed suspensions of *M. omelianskii* are able to oxidize ethanol in the absence of carbon dioxide.

EXPERIMENTAL METHODS

Methanobacillus omelianskii strain Mb2 was grown in 20-liter bottles on a medium consisting of: ethanol, 1 per cent; K_2HPO_4, 0.6 per cent; KH_2PO_4, 0.9 per cent; NH_4Cl, 0.5 per cent; $MgCl_2$, 0.1 per cent; $FeSO_4 \cdot 7H_2O$, 0.001 per cent; and $CaCl_2$, 0.001 per cent; tap water. The pH was adjusted to 7.4 by adding solid sodium carbonate. The medium was not sterilized. Methylene blue was added, CO_2 bubbled through vigorously to remove oxygen, and then just enough solid sodium hydrosulfite added to decolorize the dye. By using an actively fermenting 10 per cent inoculum, vigorous gas production occurred within 24 to 36 hr at 37 C. Cells were harvested with a Sharples centrifuge after 3 to 4 days of growth while gas production was still vigorous. The cells were washed with a solution of 0.5 per cent KCl containing 0.02 per cent $Na_2S \cdot 9H_2O$ and were resuspended in this solution. The cells retained their activity for at least 7 days when stored in Thunberg tubes under vacuum at 3 C.

Ethanol was determined by the diffusion method of Widmark (1922). Acetaldehyde was estimated by the total hydrazone method of Friedemann and Haugen (1942). Acetate was determined by titration following steam distillation in the Markham (1942) still after demonstrating by means of gas-liquid chromatography (James and Martin, 1952) that acetate was the only volatile fatty acid present in the fermentation medium. Bacterial protein determinations were made by the method of Lowry et al. (1951). Hydrogen was identified and estimated in a mixture with helium by means of a Consolidated-Nier mass spectrometer. For the quantitative estimation of the ratio of hydrogen (mass 2) to helium (mass 4) the instrument was calibrated with a known mixture of hydrogen and helium.

RESULTS

Fermentation of ethanol. The oxidation of ethanol by cell suspensions of *M. omelianskii* in a bicarbonate buffer (equation 1) can be followed manometrically by observing the pressure increase resulting from the formation of methane. In the absence of carbon dioxide no pressure change would be expected. However, while testing the activity of cell suspensions toward ethanol under various conditions, a pressure increase was observed in the absence as well as in the presence of carbon dioxide.

Typical data on gas production in the absence of carbon dioxide are shown in figure 1. Ethanol, acetate and acetaldehyde were determined in the three blank vessels (with bacteria and 2 ml 2 N H_2SO_4 tipped at the start of the experiment) and in the three flasks in which gas production was observed. The following average values were

[1] This investigation was supported in part by a research grant from the U. S. Public Health Service (E-563) and by a contract with the U. S. Atomic Energy Commission.

[2] This work was done while the senior author held a Commonwealth Fund Fellowship (1954–1955). Present address: Plant Chemistry Division D. S. I. R., Palmerston North, New Zealand.

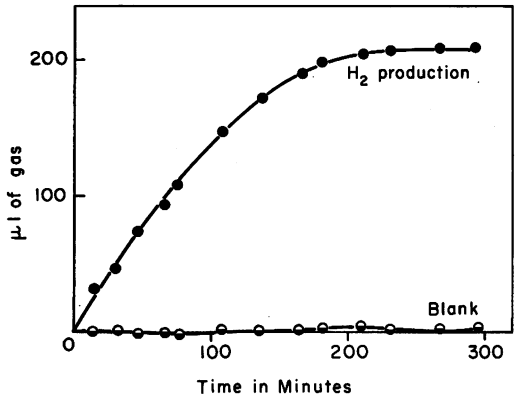

Figure 1. Gas production from ethanol. Each vessel contained 1 ml bacterial suspension (136 mg protein/ml), ethanol 10 μmoles, 0.2 ml 20% KOH (center well), and 400 μmoles potassium phosphate buffer, pH 6.85, in a total volume of 3 ml. The water and buffer were boiled to remove O_2 and CO_2. In the blank the bacteria were inactivated at zero time by adding 2 ml of 2N H_2SO_4 instead of water. Gas phase N_2; temperature 37 C. The gas was calculated as H_2; 22.4 μl = 1 μmole.

observed: ethanol used, 5.47 μmoles; acetate formed, 5.50 μmoles. No acetaldehyde could be detected. On the assumption that the pressure increase was caused entirely by hydrogen it was estimated that 9.05 μmoles were produced.

Washed bacterial suspensions, prepared as described above, were found to take up a considerable amount of H_2 when incubated in a hydrogen atmosphere in the absence of ethanol. To reduce this endogenous H_2 uptake, later experiments were done with suspensions that had been stored under H_2 in a Thunberg tube for at least 24 hr at 3 C. After such treatment the endogenous hydrogen uptake was not more than 0.9 μmole per vessel during a period of 3 hr at 37 C.

Identification of the gas produced. To characterize the gas produced from ethanol, a hydrogen absorbing mixture of palladium and methylene blue was added to the center well and one side arm of a double side arm Warburg vessel in one series of experiments. The experimental conditions and the results are given in table 1.

Vessel 1 showed a formation 15.6 μmoles of gas, calculated as H_2, from ethanol. Vessel 2 showed that there was very little endogenous gas production. Vessel 3 showed that virtually all of the gas produced from ethanol was absorbed by the mixture of palladinized asbestos and methylene blue. This vessel showed a pressure increase corresponding to the production of about 2.7 μmoles of H_2 from 2 to 5 hr, which declined to 0.03 μmole at the end of 12 hr. A comparison of vessels 1 and 3 demonstrated that virtually all of the gas produced was absorbed. This indicates that the gas was hydrogen. Vessel 4, which had a hydrogen atmosphere and no bacteria, showed that the hydrogen absorbant was active and capable of taking up the amount of hydrogen produced by the bacteria from ethanol.

The gas produced from ethanol by *M. omelianskii* was also examined by means of a mass

TABLE 1

Contents of Warburg vessels used to identify gas produced from ethanol by Methanobacillus omelianskii

The Pd asbestos and methylene blue solution were mixed and distributed about equally between the center well and one side arm. The ethanol was added to the cell suspension immediately before gassing the vessels. The vessels were incubated for 12 hr at 37 C before taking the final pressure readings.

	Vessel			
	1	2	3	4
Phosphate buffer; 1 M pH 6.6	0.4 ml	0.4 ml	0.4 ml	0.4 ml
Bacterial suspensions	1.0	1.0	1.0	
5% Pd asbestos			100 mg	100 mg
Methylene blue 10%			0.2	0.2
Ethanol 0.05 M	0.2		0.2	0.2
20% KOH (side arm)	0.2	0.2	0.2	0.2
Water	0.2	0.4		1.0
Gas phase	N_2	N_2	N_2	H_2
Gas change in μmoles, calculated as H_2	15.6	0.85	0.03	−20.1

TABLE 2

Fermentation of ethanol and acetaldehyde by washed suspensions of Methanobacillus omelianskii

After gassing with N_2 and adding the indicated substrates, the vessels were shaken at 37 C for approximately 10 hr, until gas formation ceased. The amount of acetic acid in the reaction mixtures was then determined. In estimating the amount of acetic acid formed, a correction was made for the blank (flask 5).

	Vessel				
	1	2	3	4	5
Phosphate buffer pH 6.6	0.4	0.4	0.4	0.4	0.4
Bacterial suspension (100 mg protein/ml)	1.0	1.0	1.0	1.0	1.0
Ethanol, ~0.05 M	0.2	0.1			
Acetaldehyde, ~0.06 M			0.2	0.2	
Water	0.4	0.5	0.4	0.4	0.6
H_2 formed, μmoles	14.0	7.3	11.0	10.3	0.03
Acetate formed, μmoles	7.6	3.9	11.2	10.85	
Hydrogen/acetate, in moles/mole	1.84	1.87	0.98	0.95	

spectrometer. The experiment was done in the following manner. To a 100-ml round bottom flask was added 10 ml bacterial suspension (120 mg protein per ml), 40 ml 0.1 M potassium phosphate buffer pH 6.8 and 6 ml 0.05 M ethanol. A sample was immediately removed for the determination of the initial acetate concentration and the flask was evacuated for 15 min on a water pump. The evacuated and closed flask was then incubated for 3 hr at 37 C. The gas formed was pumped from the fermentation flask, diluted with a known amount of helium, and passed into a container of known volume. The ratio of the two gases in the mixture was determined by measuring the relative abundance of the mass 2 and mass 4 peaks with a mass spectrometer and making a comparison with a known hydrogen-helium mixture. The mass analysis permitted identification of the fermentation gas as hydrogen. From the ratio of the gases and the volume of the container and the total gas pressure, the amount of hydrogen in the fermentation flask was calculated to be 329 μmoles. The quantity of acetate formed from ethanol was 163 μmoles. Therefore the H_2/acetate molar ratio was 2.02. This indicated that the oxidation of ethanol in the absence of carbon dioxide proceeded according to equation 2.

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \quad (2)$$

Fermentation of acetaldehyde. As acetaldehyde appeared to be a likely intermediate in the oxidation of ethanol, the products of the fermentation of ethanol and acetaldehyde were compared. The bacterial suspension used in this experiment had not been stored under hydrogen, so the manometers were gassed with H_2 and allowed to equilibrate with hydrogen for 2 hr;

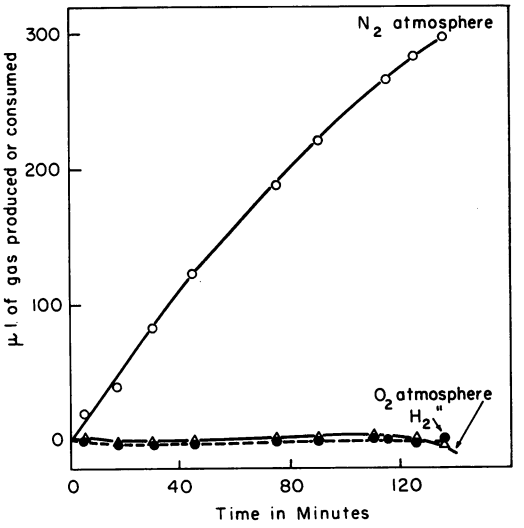

Figure 2. Influence of N_2, H_2, and O_2 on hydrogen formation from ethanol. Each vessel contained 1.0 ml bacterial suspension, 400 μmoles potassium phosphate buffer, pH 6.6, 10 μmoles ethanol (side arm), 0.2 ml 20% KOH (center well) in a total volume of 2.0 ml. Gas phase: H_2, N_2, or O_2, as indicated. A small amount of methylene blue was added to the flask with the oxygen atmosphere. Flasks were allowed to equilibrate for 2 hr before the addition of ethanol.

10.3 μmoles of H_2 were consumed. The substrates were then added and the vessels were immediately gassed with N_2. The quantities of hydrogen and acetate produced were determined.

The results given in table 2 show that acetaldehyde is readily converted to acetate with the formation of approximately 1 mole of hydrogen per mole of acetate, according to equation 3

$$CH_3CHO + H_2O \rightarrow CH_3COO^- + H^+ + H_2 \quad (3)$$

Inhibition of hydrogen production by a hydrogen atmosphere. The effect of various gases on ethanol oxidation and hydrogen production by *M. omelianskii* was tested manometrically. Three vessels were used with atmospheres of hydrogen, nitrogen, and oxygen, respectively. The results, shown in figure 2, demonstrate that both hydrogen and oxygen at a pressure of 1 atm completely inhibit hydrogen production. No indication of an aerobic oxidation of ethanol was obtained. The possibility that the alcohol oxidizing or hydrogen producing system was inactivated by exposure to oxygen for 2 hr is not excluded.

DISCUSSION

The oxidation of ethanol by *M. omelianskii* in the absence of carbon dioxide was unexpected because the growth of the organism with ethanol had previously been found to be dependent upon a supply of carbon dioxide which served as the ultimate oxidant. However, since the organism is known to have an active hydrogenase system (Barker, 1943) it is not surprising that this system can be coupled with the alcohol and aldehyde dehydrogenase systems to cause the evolution of hydrogen gas when carbon dioxide, the normal oxidant, is lacking. The thermodynamics of these systems permits the evolution of hydrogen under the experimental conditions used. The oxidation of acetaldehyde according to equation 3, at 25 C, pH 7, 1 atm H_2 pressure, and 0.01 M acetaldehyde and acetate, involves a free energy change of −8.1 kcal. The free energy change for the oxidation of ethanol according to equation 2, under the same conditions, is less favorable, namely, 1.5 kcal. This unfavorable equilibrium largely explains why an atmosphere of hydrogen inhibits hydrogen production from ethanol. By using a nitrogen atmosphere, the final hydrogen pressure is decreased to approximately 0.01 atm. At this pressure the free energy change for reaction 2 under conditions otherwise identical with those mentioned above, is approximately −3.9 kcal; consequently, the reaction goes almost to completion.

With acetaldehyde as a substrate, a dismutation to ethanol and acetate might first occur, followed by an oxidation of the ethanol according to equation 2. If this sequential transformation occurred, the molar ratio of H_2 to acetate would be expected to change from zero to 2 during the course of the experiment. Unfortunately, acetate was estimated only after hydrogen evolution had virtually ceased, so no evidence for or against a possible dismutation of acetaldehyde was obtained.

M. omelianskii would not be expected to grow at the expense of ethanol oxidation according to equation 2 because of the small negative free energy change involved even under optimal conditions and because the medium would quickly become saturated with hydrogen, causing the reaction to stop. A fermentation of acetaldehyde in the absence of carbon dioxide might be able to support growth of methane bacteria, just as a fermentation of pyruvate is able to support the growth of sulfate reducing bacteria in the absence of sulfate (Postgate, 1952), although this has not yet been demonstrated experimentally. A fermentation of acetaldehyde involving reduction of carbon dioxide, according to equation 4,

$$CH_3CHO + \tfrac{1}{2} H_2O + \tfrac{1}{4} CO_2 \rightarrow CH_3COO^- + H^+ + \tfrac{1}{4} CH_4 \quad (4)$$

would be much more favorable energetically, since the free energy change under physiological conditions is approximately −17 kcal or about twice that of the fermentation of acetaldehyde according to equation 3.

ACKNOWLEDGMENT

We are greatly indebted to Dr. C. C. Delwiche of the Kearney Foundation, University of California, for the measurements made with the mass spectrometer and for their interpretation.

SUMMARY

Washed suspensions of *Methanobacillus omelianskii* convert ethanol or acetaldehyde anaerobically, in the absence of carbon dioxide, to acetic acid and hydrogen. An atmosphere of hydrogen inhibits the oxidation of ethanol under these conditions.

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