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VIOLOGEN DYE INHIBITION OF METHANE FORMATION BY *METHANOBACILLUS OMELIANSKII*

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ABSTRACT

WOLIN, E. A. (University of Illinois, Urbana), R. S. WOLFE, AND M. J. WOLIN. Viologen dye inhibition of methane formation by *Methanobacillus omelianskii*. J. Bacteriol. **87**:993-998. 1964.—Low concentrations of methyl or benzyl viologen inhibit the formation of CH_4 from ethanol and CO_2 by washed cells of *Methanobacillus omelianskii*. Hydrogen, which is normally formed from ethanol, accumulates in greater quantities when CH_4 formation is inhibited by viologens. The viologens do not stimulate H_2 formation from ethanol in the absence of CO_2. Inhibition of CH_4 formation by the viologens is not reversed by H_2. A variety of other dyes and possible electron acceptors were tested for inhibition, and none was inhibitory in the same low-concentration range at which the viologens were effective.

The production of methane from ethanol and carbon dioxide by *Methanobacillus omelianskii* (*Methanobacterium omelianskii* in *Bergey's manual of determinative bacteriology;* Breed, Murray, and Smith, 1957) was demonstrated by Barker (1943*a*). Methane is formed as a result of the following overall reaction: $2C_2H_5OH + CO_2 \rightarrow 2CH_3CO_2H + CH_4$. Johns and Barker (1960) also showed that, in the absence of CO_2, resting cells of *M. omelianskii* convert ethanol to acetate and hydrogen according to the following equation: $C_2H_5OH + H_2O \rightarrow CH_3CO_2H + 2H_2$.

The present investigation is concerned primarily with the effects of the viologen dyes, benzyl and methyl viologen, on CH_4 formation by resting cells of *M. omelianskii*. We have found that extremely low concentrations of the viologen dyes inhibit the formation of CH_4 from ethanol and CO_2. Concomitant with inhibition of methane formation, low concentrations of viologen dyes cause the accumulation of hydrogen formed from ethanol in the presence of CO_2. A preliminary account of these findings has appeared (Wolin, Wolin, and Wolfe, 1963).

MATERIALS AND METHODS

Preparation of resting-cell suspensions. A culture of *M. omelianskii* was kindly provided by H. A. Barker. The medium used to culture the organism was a modification of Barker's (1940) medium. The modified medium contained the following constituents per 100 ml: 0.1 g of $(NH_4)_2SO_4$, 1 ml of 95% ethanol, 10 ml of phosphate solution, 6 ml of Na_2CO_3 solution, 2 ml of $Na_2S \cdot 9H_2O$ solution, and 1 ml each of a stock mineral and vitamin solution. (All solution compositions are described below.) The pH of the medium was 6.8 to 7.0. The stock mineral solution contained (in g per liter): nitrilotriacetate, 0.5; $MgSO_4 \cdot 7H_2O$, 6.2; $MnSO_4 \cdot 4H_2O$, 0.55; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.17; $CaCl_2 \cdot 2H_2O$, 0.13; $ZnSO_4 \cdot 7H_2O$, 0.18; $CuSO_4$, 0.05; $AlK(SO_4)_2 \cdot 12H_2O$, 0.018; H_3BO_4, 0.01; and $NaMoO_4 \cdot 2H_2O$, 0.011. The nitrilotriacetate was dissolved in 10 ml of 0.5 N NaOH and brought to 500 ml, the salts were added to the solution, and the volume was brought to 1 liter. The stock vitamin solution contained (in mg per liter): biotin, 2; folic acid, 2; pyridoxine·HCl, 10; thiamine·HCl, 5; riboflavine, 5; nicotinic acid, 5; calcium panthothenate, 5; B_{12}, 0.01; *p*-aminobenzoic acid, 5; and thioctic acid, 1. The phosphate solution contained 6 g of K_2HPO_4 and 9 g of KH_2PO_4 per 100 ml. The Na_2CO_3 and the $Na_2S \cdot 9H_2O$ solutions contained 5 g of Na_2CO_3 and 1 g of $Na_2S \cdot 9H_2O$ per 100 ml, respectively, and were sterilized separately and added aseptically.

Stock cultures of *M. omelianskii* were maintained on the above medium in 10-ml amounts in test tubes with agar added to 0.2% and approximately 100 mg of $CaCO_3$ included in each tube. The cultures were transferred weekly and incubated at 37 C under an alkaline pyrogallol plug and rubber stopper. Subcultures which gassed vigorously in 24 to 48 hr were removed from the incubator and stored at 25 C.

Large amounts of resting cells were obtained by transferring a stock culture (less than 1 week old) to 500 ml of medium in a 500-ml Florence flask. After 48 to 72 hr, each 500-ml culture was transferred to 3 liters of medium in a 3-liter Florence flask. After 48 to 72 hr, each 3-liter culture was transferred to 20 liters of medium in a 20-liter carboy. These liquid cultures were flushed with 95% N_2 plus 5% CO_2 after inoculation and incubated in that atmosphere at 40 C. The 20-liter cultures were grown in medium which had not been sterilized; before inoculation, sufficient methylene blue to color the medium a light blue was added; then enough $Na_2S_2O_4$ to completely reduce the methylene blue in the medium was added.

The 20-liter cultures were harvested in a Sharples centrifuge after 48 to 72 hr. A 1-g amount of wet cell paste was suspended in 5 ml of a solution containing 0.2 M potassium phosphate buffer at pH 6.5, 0.02% $Na_2S \cdot 9H_2O$, and 0.05% $MgSO_4 \cdot 7H_2O$. The suspension was flushed with H_2 and kept at 0 C until needed. Immediately before use, the suspension was diluted 1:10 in a similar solution except for the substitution of 0.02 M phosphate for 0.2 M phosphate. The diluted suspension was centrifuged at 3,000 $\times$ *g* for 10 min at 4 C and resuspended in the 0.02 M potassium phosphate diluent containing sulfide and Mg^{++}. The volume of the resuspended, washed cells was approximately equal to the volume of the original suspension of the cell paste. The washed cells were immediately flushed with H_2 and kept at 0 C until added to reaction mixtures.

Measurement of gas production. Ordinary single side arm, 15-ml Warburg cups were used as reaction vessels. All solutions were added to the main compartment of each Warburg cup. A serum bottle cap was then used to seal the main compartment of the cup. Gas was introduced into the cup by means of a hypodermic needle injected through the serum cap, and was flushed through the cup for 1 min with the side-arm stem vented to the atmosphere. All gases were passed through a hot, reduced, copper column before introduction into the reaction vessel. The stem was then removed, and the cell suspension was added to the side arm while gas flushing continued. The stem was reinserted, and flushing was continued for 15 sec. Each side-arm stem was then turned to close the system with the simultaneous removal of the hypodermic needle from the serum cap. Each cup was placed in an ice bath until the reaction was started.

Unless otherwise noted, each reaction mixture contained 66.5 mM potassium phosphate buffer (pH 7.2), 405 mM ethanol, 66 mM $NaHCO_3$, and 0.5 ml of washed-cell suspension containing 4.5 to 6.5 mg of protein, in a total volume of 1.53 ml. Reactions were started by tipping the cells into the main compartment after warming the cups to 37 C in a water bath. The cups were incubated at 37 C. Samples of gas were removed at appropriate time intervals with a hypodermic syringe and analyzed for gas composition with an Aerograph A-100 (Wilkens Instruments & Research, Inc., Walnut Creek, Calif.) gas chromatography unit. A silica gel column (5 ft by 0.25 in.) was used with a thermal conductivity cell-detection system. Helium was used as the carrier gas for most methane determinations. When H_2 was measured, N_2 was used as the carrier gas. Methane can also be measured by use of N_2 as the carrier gas. The carrier gas flow-through rate was 60 ml/min.

Chemicals and other methods. Methyl and benzyl viologen were obtained from Mann Research Laboratories, Inc., New York, N.Y. The commercial preparation of benzyl viologen was further purified by recrystallization (Michaelis and Hill, 1933).

The protein concentration of washed-cell suspensions was determined by adding 2.0 ml of 5% trichloroacetic acid to 0.5 ml of suspension. The precipitate was recovered by centrifugation, suspended in 4 ml of 1 M NaOH, steamed for 10 min, and centrifuged. The resulting supernatant solution was analyzed for protein according to the method of Lowry et al. (1951).

RESULTS

The requirement for ethanol and CO_2 for methane production by resting-cell suspensions is shown in Table 1. No methane was produced from ethanol in a N_2 atmosphere in the absence of HCO_3^-. Other analyses showed that H_2 is produced in the presence of N_2 and in the absence of CO_2. This confirms the results of Johns and Barker (1960). No methane was produced from H_2 and CO_2 in the absence of ethanol, although the rate of methane formation from ethanol was greater in an atmosphere of H_2 plus CO_2. The increased rate in the H_2 plus CO_2 atmosphere represents a true stimulation by H_2 rather than a possible inhibition by N_2, since

the rate of methane production is the same in a He plus CO_2 atmosphere as it is in a N_2 plus CO_2 atmosphere.

Table 2 shows the inhibition by methyl and benzyl viologen of methane formation from ethanol and CO_2 in the presence of an 80% N_2 plus 20% CO_2 atmosphere. It can be seen that very low concentrations of the viologen dyes completely inhibited methane formation. Methyl viologen was more inhibitory than benzyl viologen. Variations were noted in the exact amount of viologen dyes necessary to inhibit different preparations of cell suspensions. More active preparations were somewhat less sensitive to the viologens, but the concentration necessary to completely inhibit methane formation was never more than tenfold greater than the concentrations shown in Table 2.

A variety of other dyes were tested for their ability to inhibit methane formation from ethanol and CO_2. Dyes which were inhibitory between 0.1 and 1.0 mM concentrations but not lower than 0.1 mM were methylene blue, 2,6-dichlorophenol indophenol, malachite green, and crystal violet. Compounds which were slightly inhibitory or noninhibitory at 1.0 mM were resazurin, indigo carmine, methyl orange, phenazine methosulfate, tetrazolium blue, tetrazolium violet, and potassium ferricyanide. Thus, none of the compounds tested was an effective inhibitor in the low range of concentrations at which the viologens were active.

The viologen dyes are reduced at low oxidation-reduction potentials. The E_0' of these dyes are -0.440 and -0.359 v for methyl and benzyl viologen, respectively (Clark, 1960). Viologen dyes are known to react in systems which produce or activate molecular H_2, such as the formic hydrogenlyase system (Peck and Gest, 1957*a*) and hydrogenase (Peck and Gest, 1957*b*). It seemed possible that the inhibition of methane formation from ethanol and CO_2 could be due to interference by the viologens with the normal flow of electrons to CO_2, with an accompanying shift of electrons towards the production of molecular H_2. Analysis of the viologen-inhibited system for H_2 showed that H_2 did accumulate. With increasing concentrations of benzyl viologen, methane production decreased and H_2 production increased (Fig. 1). Experiments with

TABLE 1. *Requirements for CH_4 formation**

Expt	Addition	Gas phase	CH_4 formed per 135 min
			μmoles
1	EtOH, HCO_3^-	$H_2 + CO_2$	78
	HCO_3^-	$H_2 + CO_2$	0
	EtOH, HCO_3^-	$N_2 + CO_2$	52
	EtOH	N_2	0
2	EtOH, HCO_3^-	$N_2 + CO_2$	33
	EtOH, HCO_3^-	$He + CO_2$	37

* See Materials and Methods for experimental details. Concentration of CO_2, where used, was 20%.

TABLE 2. *Inhibition of CH_4 formation by viologens**

Expt	Viologen	Concn	CH_4 formed per 135 min	Inhibition
		mM	*μmoles*	%
1	None	—	52.0	0
	Benzyl	1.3×10^{-3}	49.4	5
	Benzyl	2.6×10^{-3}	35.4	32
	Benzyl	5.2×10^{-3}	0.0	100
2	None	—	58.0	0
	Methyl	3.3×10^{-4}	58.0	0
	Methyl	1.6×10^{-3}	0.0	100

* CH_4 measured in an 80% N_2 plus 20% CO_2 atmosphere. See Materials and Methods for experimental details.

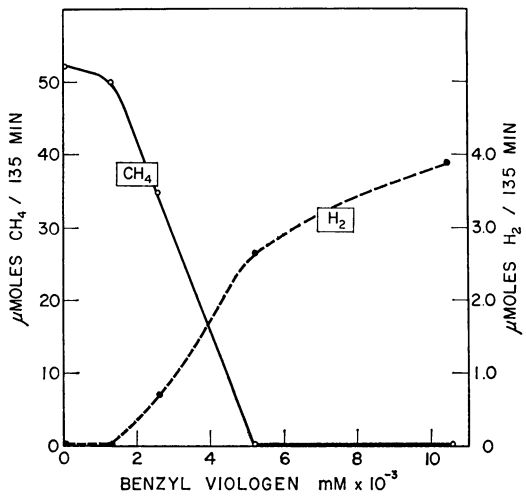

FIG. 1. *Effect of varying concentrations of benzyl viologen on CH_4 and H_2 production in the presence of ethanol and CO_2. Gas phase = 80% N_2 + 20% CO_2. See Materials and Methods for experimental details.*

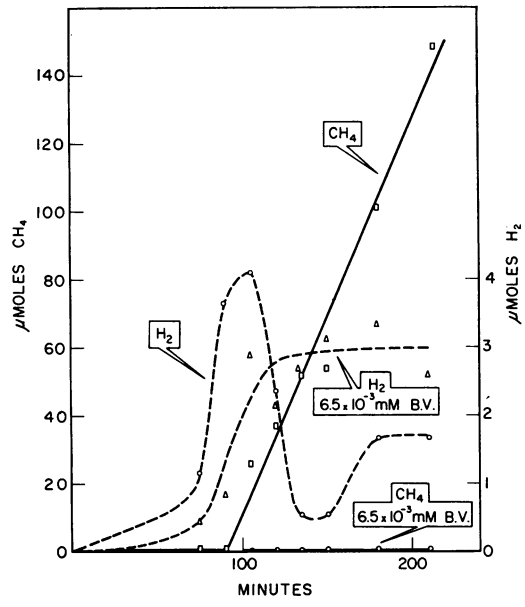

FIG. 2. *Production of CH_4 and H_2 from ethanol in the presence and absence of 6.5 × 10^{-3} mM benzyl viologen. Gas phase = 80% N_2 + 20% CO_2. See Materials and Methods for experimental details.*

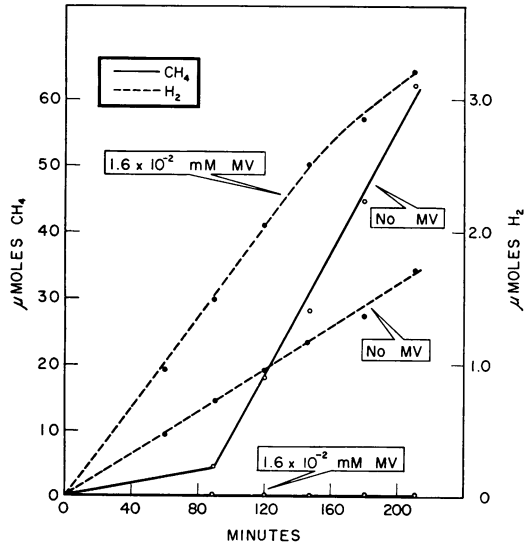

FIG. 3. *Production of CH_4 and H_2 from ethanol in the presence and absence of 1.6 × 10^{-2} mM methyl viologen. Gas phase = 80% N_2 + 20% CO_2. See Materials and Methods for experimental details.*

methyl viologen as the inhibitor showed a similar pattern. Thus, low concentrations of viologen dyes, in addition to inhibiting methane production, caused an apparent increase in the production of H_2 from ethanol.

Further experiments demonstrated, however, that the accumulation of H_2 in the presence of viologens is not due to a diversion of electrons (from ethanol) to H_2 production at the expense of methane production. H_2 was produced from ethanol whether or not the viologen dye was present (Fig. 2). In the absence of benzyl viologen, H_2 was produced from ethanol and appeared before methane was formed, but as soon as CH_4 production began H_2 was utilized. In the presence of benzyl viologen, there was a delay in H_2 production, but no utilization took place (presumably because CO_2 reduction was inhibited). Thus, the net effect was a greater accumulation of H_2 in the viologen-inhibited system. It should be pointed out that the amount of H_2 evolved was of a lower order of magnitude than the amount of CH_4 produced.

In another experiment, H_2 and CH_4 formation were followed with time in the presence and absence of methyl viologen. The patterns of H_2 and CH_4 evolution shown in Fig. 3 were somewhat different than in the experiment described above. Methyl viologen stimulated H_2 production and inhibited CH_4 formation. A decrease in H_2 production concomitant with the initiation of CH_4 formation was not observed in the uninhibited reaction mixture.

The inhibition of CH_4 formation by the viologen dyes was not reversed by replacing the N_2 plus CO_2 atmosphere with a H_2 plus CO_2 atmosphere. Table 3 shows the inhibitory activity of benzyl viologen in N_2 plus CO_2 and H_2 plus CO_2 atmospheres. Similar results were obtained with methyl viologen as inhibitor.

The viologen dyes had little or no effect on H_2 evolution from ethanol when N_2 was used as the gas phase and CO_2 was absent from the system. The dyes were tested at concentrations which inhibited CH_4 formation in the presence of

TABLE 3. *Effect of gas phase on viologen inhibition**

Gas phase	CH_4 produced (μmoles)	
	No viologen	Benzyl viologen (0.01 mM)
H_2 + CO_2.....	13.6	0.0
N_2 + CO_2.....	5.2	0.0

* CH_4 measured at 90 min. See Materials and Methods for experimental details. Gas phase contained 20% CO_2.

CO_2. H_2 production from ethanol was not stimulated by the viologens and was slightly inhibited at high concentrations of the viologens (Table 4).

TABLE 4. *Effect of viologen dyes on H_2 production from ethanol in a N_2 atmosphere**

Expt	Addition	H_2 produced (μmoles)	
		90 min	180 min
1	None	3.70	6.92
	0.065 mM benzyl viologen	2.57	5.10
	0.0065 mM benzyl viologen	3.03	6.20
2	None	2.00	4.32
	0.0033 mM methyl viologen	1.90	4.05
	0.0165 mM methyl viologen	1.71	4.09

* Gas phase, 100% N_2. See Materials and Methods for experimental details.

DISCUSSION

The results presented above demonstrate that low concentrations of methyl and benzyl viologen prevent the use of electrons from ethanol for the reduction of CO_2 to CH_4. Electrons, which are normally used for both H_2 production and CO_2 reduction, are used only for H_2 production when the viologen dyes are present. The inhibition of CO_2 reduction is not caused by a viologen-dependent diversion of electrons to H_2 formation. Further evidence that the viologens do not divert electrons (from ethanol) to the production of H_2 is that the dyes have no stimulatory effect on H_2 production from ethanol in a N_2 atmosphere.

Differences in the patterns of H_2 and CH_4 formation in the presence of ethanol in the uninhibited system (Fig. 2 and 3) are not completely understood. The differences may be due to variations in cell suspensions which lead to different rates of H_2 evolution from ethanol relative to CH_4 formation, in addition to variations in the lag periods observed before CH_4 production can be detected. In the case where H_2 disappears at the same time as CH_4 production begins (Fig. 2), the CO_2 reduction steps may proceed at rates sufficient to use all the available electrons in the system, including those available in the molecular H_2 present. Where H_2 does not disappear (Fig. 3), it is possible that the availability of electrons is not a limiting factor in CH_4 formation, and a certain fraction of the electrons derived from ethanol are continuously used for H_2 formation. The inhibition of CH_4 formation by the viologens would thus lead to increased availability of electrons for H_2 formation, which is reflected in the accumulation of H_2 (Fig. 2) or an increased rate of H_2 formation (Fig. 3).

Other examples of the inhibition of normal electron-transport reactions by the viologen dyes are known. Methyl viologen prevents N_2 fixation in extracts of *Clostridium pasteurianum* (Mortenson and Sizelover, 1963). Electrons from pyruvate are diverted into butyric acid formation at the expense of the normal formation of H_2 and acetyl coenzyme A in the absence of methyl viologen. Benzyl viologen, in addition to other dyes such as phenazine methosulfate and Janus Green B, inhibits the photoevolution of H_2 in *Rhodospirillum rubrum* (Gest, Ormerod, and Ormerod, 1962). The mechanism by which the viologen dyes inhibit these systems and CH_4 formation is not known. Perhaps the viologens compete with a natural electron carrier for an enzyme site. Another possible mechanism could involve a viologen-catalyzed irreversible oxidation of a reduced natural electron carrier.

The inability of the cell suspensions used in these studies to produce CH_4 from H_2 and CO_2 would seem to be at variance with the results of other investigators (Barker, 1943*b*; Pine, 1958). Unwashed cells formed CH_4 from H_2 and CO_2, but the washed cells used for the experiments reported formed no CH_4 or trace amounts of CH_4 from H_2 and CO_2, although H_2 disappeared when ethanol was used as a substrate for methane production (Fig. 2). Barker (1943*b*) used washed cells for his studies on CH_4 formation from H_2 and CO_2 and found that it was often necessary to activate cell suspensions by long periods of incubation under H_2 to obtain CH_4 formation. It is possible that differences in the method of preparation of cell suspensions and length of exposure to H_2 are responsible for the lack of CH_4 formation from H_2 plus CO_2 by the cells used in the present study.

ACKNOWLEDGMENT

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