

Hydrogenase Measurement with Photochemically Reduced Methyl Viologen¹

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Methyl viologen was reduced photochemically in the presence of proflavine and ethylenediaminetetraacetic acid. The reduced methyl viologen was oxidized by hydrogenase from *Vibrio succinogenes*. H₂ and oxidized methyl viologen were the products. Hydrogenase activity was determined by spectrophotometric measurement of the disappearance of reduced methyl viologen at 600 nm. The extinction coefficient of reduced methyl viologen was determined and is 8.25 mm⁻¹ × cm⁻¹ at 600 nm. Optimal conditions for assaying *V. succinogenes* hydrogenase were developed. Extracts of *Escherichia coli* and *Desulfovibrio desulfuricans* catalyzed reduced methyl viologen oxidation in the assay system, whereas *Azotobacter vinelandii* extracts were inactive.

Peck and Gest (6) developed an assay for hydrogenase based on the measurement of H₂ evolution from reduced methyl viologen. Methyl viologen was reduced with excess Na₂S₂O₄, and H₂ evolution was measured manometrically. Sweetser (9) developed a spectrophotometric method for measuring O₂ based on the oxidation of photochemically reduced methyl viologen. Methyl viologen was photochemically reduced in the presence of ethylenediaminetetraacetic acid (EDTA) and proflavine. In this report, we show that the hydrogenase of *Vibrio succinogenes* oxidizes photochemically reduced methyl viologen to hydrogen; the development of a spectrophotometric assay for hydrogenase, based on a modification of Sweetser's oxygen-measuring system, is also described.

MATERIALS AND METHODS

Bacteria and extracts. *V. succinogenes* was grown and harvested as previously described (1, 10). *Escherichia coli* B was grown as described for *E. coli* Crookes (2, 5). Cells from 1 liter of medium were washed with deoxygenated water and were suspended in 20 ml of 10 mM potassium phosphate, pH 7.2. *Azotobacter vinelandii* ATCC 12518 was grown on a modified Burk's N-free medium (8) for 16 to 20 hr at 25 C with vigorous aeration. Cells from 1 liter were washed with cold 25 mM potassium phosphate, pH 7.0, and were resuspended in the same buffer.

Crude hydrogenase of *V. succinogenes* was prepared, as described previously (1), by extraction, at pH 11.0, of cell precipitates obtained after cell lysis

with EDTA. Extracts of *E. coli* and *A. vinelandii* were prepared by disruption of cells in a Branson sonic distributor followed by centrifugation to remove debris. The crude extracts of *E. coli* and *A. vinelandii* were used as sources of hydrogenase. A partially purified hydrogenase from *Desulfovibrio desulfuricans* ATCC 8303 was kindly supplied by Richard Haschke, Department of Microbiology, Univ. of Illinois, Urbana.

Hydrogenase assay. The reduction of methylene blue by hydrogen was measured manometrically at 37 C. Enzyme was added to a Warburg vessel side arm to a final volume of 0.5 ml with 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 7.0, and 10 mM glutathione. The main compartment contained 300 μmoles of Tris (pH 8.5), 10 mg of bovine serum albumin, and 15 μmoles of methylene blue in a total volume of 2.35 ml. Fluted filter paper with 0.3 ml of 40% pyrogalllic acid in 5 N NaOH was in the center well. After flushing for 15 min with H₂ and a subsequent 20 min of incubation, the reaction was started by tipping enzyme into the main compartment.

The standard assay system for measuring the oxidation of photochemically reduced methyl viologen contained 4 mM methyl viologen, 0.04 mM proflavine, and 40 mM EDTA (pH 5.0) in 3 ml in a 1-cm light path cuvette with a Thunberg side arm. Enzyme (0.2 ml) was in the side arm. The cuvette was evacuated and flushed with O₂-free N₂ about 10 times. N₂ was freed from O₂ by passage through a hot, reduced Cu column and two 500-ml gas washing bottles containing photochemically reduced methyl viologen. The gas washing solutions were identical to the assay solution and were reduced by exposure to a fluorescent lamp. The evacuated and flushed cuvettes were exposed to a Gates white light lamp at a distance of 8 cm for approximately 3 min to obtain a concentration of approximately 0.23 mM reduced methyl viologen; the amount of reduction was monitored by the absorbancy

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at 600 nm. The side arm was wrapped in aluminum foil to eliminate any possible effect of light on the enzyme. The reaction was initiated by tipping enzyme into the cuvette. All spectrophotometric assays were done at room temperature and activity was determined by measuring the absorbancy decrease at 600 nm.

A unit of hydrogenase is defined as the amount of hydrogenase catalyzing the uptake of 1 μ mole of H_2 per min in the manometric assay and the oxidation of 2 μ moles of reduced methyl viologen per min in the spectrophotometric assay (equivalent to the production of 1 μ mole of H_2 per min from reduced methyl viologen).

Hydrogen gas determination. Gas chromatography was used as described previously (7).

Protein determination. The method of Lowry et al. (3) was used with crystalline bovine albumin as a standard.

Chemicals. EDTA was obtained from the Sigma Chemical Co., benzyl and methyl viologen were from Mann Research Laboratories, and proflavine was from the National Aniline Division of the Allied Chemical Corp.

RESULTS

Spectrum of the reduced assay system. Figure 1 shows the spectrum of the assay system after

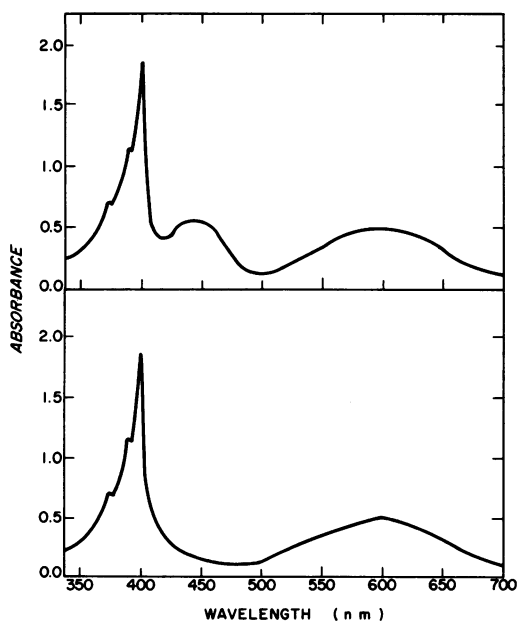


FIG. 1. Absorption spectrum of photoreduced methyl viologen. A 3.2-ml reaction mixture contained 0.21 μ mole of methyl viologen, 0.12 μ mole of proflavine, and 120 μ moles of EDTA, pH 5.0. After complete photoreduction, the spectrum was recorded with a Cary model 14 spectrophotometer. The top part shows the absorbancy measured against air; the bottom part shows the absorbancy measured against a reaction mixture which was not photoreduced.

methyl viologen was photochemically reduced. Comparison of the spectrum obtained by reading against an oxidized control shows that the 450-nm peak of proflavine does not interfere with measurement of the 400- and 600-nm peaks of reduced methyl viologen. The 600-nm peak was chosen for subsequent studies. The extinction coefficient of reduced methyl viologen at 600 nm was determined after complete reduction of methyl viologen by three different reducing systems. Methyl viologen was reduced with 0.2 ml of 0.1 M $Na_2S_2O_4$, added from the side arm of a modified Thunberg cuvette, in a N_2 atmosphere. Reduction was also carried out by heating a methyl viologen solution in 0.1 M Na_2CO_3 with 3 mg of glucose, in a N_2 atmosphere, at 50 C until the absorbancy reached a maximum at 600 nm. Both methods gave identical extinction coefficients of $8.25 \text{ mM}^{-1} \times \text{cm}^{-1}$. Complete reduction by light in the photochemical system gave a slightly lower extinction coefficient of $7.60 \text{ mM}^{-1} \times \text{cm}^{-1}$. We will show that completely reduced methyl viologen is unstable in the photochemical system and the higher extinction coefficient was chosen for all subsequent calculations.

Stoichiometry of reduced methyl viologen oxidation. *V. succinogenes* hydrogenase was used in the photochemical assay system. After all of the photochemically reduced methyl viologen was oxidized, 0.5 ml of the gas phase was removed with a syringe attached to the gassing port of the modified cuvette. The hydrogen content was measured by gas chromatography, and the total volume of the cuvette was determined. The results of the two experiments shown in Table 1 show a stoichiometry which fits the following equation: 2 reduced methyl viologen \rightarrow 2 oxidized methyl viologen + H_2 . Oxidation of the viologen dye cannot be observed in an atmosphere of H_2 .

Proportionality between enzyme concentration

TABLE 1. Stoichiometry of reduced methyl viologen oxidation and hydrogen formation^a

Expt	(A) Reduced methyl viologen oxidized	(B) H_2 produced	(A)/(B)
	μ moles	μ moles	
1	0.639	0.320	2.00
2	0.310	0.160	1.94

^a Complete oxidation of photoreduced methyl viologen by 0.32 unit (spectrophotometric assay) of *V. succinogenes* hydrogenase was carried out under the standard assay conditions. After the reaction was completed, 0.5 ml of the gas phase of the modified cuvette was analyzed by gas chromatography.

and rate. The rate of oxidation of photochemically reduced methyl viologen is proportional to *V. succinogenes* enzyme concentration (Fig. 2). A linear relationship is obtained below 0.03 units of enzyme, and assays were usually performed with 0.01 to 0.03 enzyme units.

We compared the rate of gas evolution from photochemically reduced methyl viologen with the spectrophotometric oxidation rate. Gas evolution was measured manometrically with the assumption that the produced gas was H₂. The conditions for manometry were similar to those for spectrophotometry, except that all reagents were increased approximately 10-fold to permit the generation of sufficient reduced methyl viologen for measurable gas production. Enzyme was also increased 10-fold because of the decreased sensitivity of the manometric assay. An additional stopcock was placed between the cup and the manometer fluid of a standard manometer to allow evacuation and flushing of the system with N₂, without disturbance of the manometer fluid, before photochemical reduction. *V. succinogenes* enzyme was added from the side arm to the photochemically reduced system in the main compartment after equilibration. The rate of gas evolution was compared with the rate of reduced methyl viologen oxidation calculated from an assay performed with the same prepara-

tion on the same day. The rate of gas evolution from reduced methyl viologen was almost the same as the rate expected from the spectrophotometric oxidation rate (Table 2).

pH optimum. The effect of pH on *V. succinogenes* hydrogenase was measured. An apparent optimum was found at pH 5.0 (Fig. 3). The increasing activity as the pH is decreased to 5.0 is probably related to the increasing difference between the E_0' of the methyl viologen system and the hydrogen half cell as the pH is decreased. The E_0' of the methyl viologen half cell is independent of pH and is -460 mv (3). The E_0' of the hydrogen half cell decreases with increasing pH by the factor -0.06 (pH). Rates could not be determined below pH 3.5, because methyl viologen could not be photochemically reduced below pH 3.5.

Autooxidation of reduced methyl viologen. The rate of autooxidation is proportional to the ratio of reduced to oxidized methyl viologen after photochemical reduction and, presumably, is related to the oxidation-reduction (O-R) potential. Figure 4 shows this relationship. There is a low, concentration-independent rate of autooxidation above -380 mv, but below -380 mv the rate is dependent on the ratio of reduced methyl viologen to oxidized methyl viologen. The O-R potentials (E_h) were calculated from the equation: $E_h = E_0 - RT/nF \ln$ (reduced methyl viologen)/(oxidized methyl viologen), with $E_0 = -460$ mv and $n = 1$ (3). The autooxidation rate

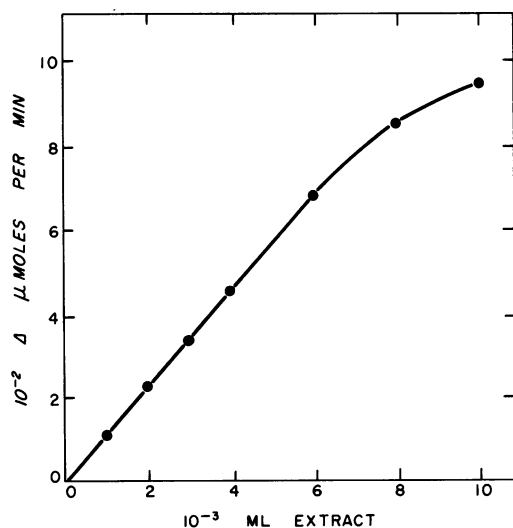


FIG. 2. Effect of enzyme concentration on the rate of oxidation of photoreduced methyl viologen. The standard assay system was employed to measure *V. succinogenes* hydrogenase. A blank rate of 0.021 absorbancy unit per min was subtracted to obtain the indicated rates.

TABLE 2. Comparison of the rate of H₂ evolution with the rate of photoreduced methyl viologen oxidation

Determination	Rate
	μmoles per min
Reduced methyl viologen oxidized	
Measured spectrophotometrically ^a	0.102
Calculated for 10× enzyme used in manometric experiment	1.02
H ₂ produced	
Expected from spectrophotometric assay	0.51
Found manometrically ^b	0.43

^a The enzyme was 0.051 unit (spectrophotometric assay) of *V. succinogenes* hydrogenase in the standard spectrophotometric assay.

^b The Warburg vessel contained 40 mM methyl viologen, 0.1 mM proflavine, and 400 mM EDTA, pH 5.0, in 3.2 ml and 0.51 unit (spectrophotometric assay) of *V. succinogenes* hydrogenase in the side arm. O₂ was removed and methyl viologen was photoreduced as in the spectrophotometric system. Gas evolution was measured at 25 C.

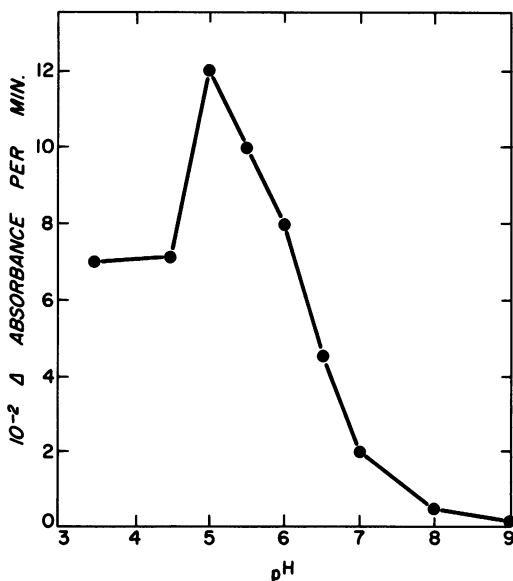


FIG. 3. Effect of pH on the enzymatic oxidation of photoreduced methyl viologen. The standard assay was employed, except that 0.01 M Tris was added in the pH range of 6.5 to 8.5 and 0.01 M glycine was added in the pH range of 8.5 to 11.0. The 0.04 M EDTA was used as the buffer in the pH range of 3.5 to 7.0. *V. succinogenes* hydrogenase was used. Blank (autooxidation) rates at each pH were subtracted to obtain the indicated rates.

increased with decreasing pH when the ratio of reduced to oxidized methyl viologen was that of the standard assay system (E_h approximately -380 mv). At pH 5.0 the absorbance change without enzyme was 0.065 min^{-1} whereas at pH 7.0 the change was 0.010 min^{-1} . The effect of pH on autooxidation is probably related to the decrease of the hydrogen half cell potential with increasing pH.

Assay of other organisms. Crude hydrogenases of two organisms and a partially purified hydrogenase of *D. desulfuricans* were assayed for hydrogenase activity in a manometric assay, with a H_2 -methylene blue couple, and in the spectrophotometric assay. Only the *A. vinelandii* extract was inactive in the spectrophotometric assay (Table 3). Extracts active in the spectrophotometric assay were inactivated by heating in a boiling-water bath (20 min for *V. succinogenes*, 10 min for the other extracts).

DISCUSSION

Comparison of activities between the organisms tested in the methylene blue reduction and reduced methyl viologen oxidation assays should

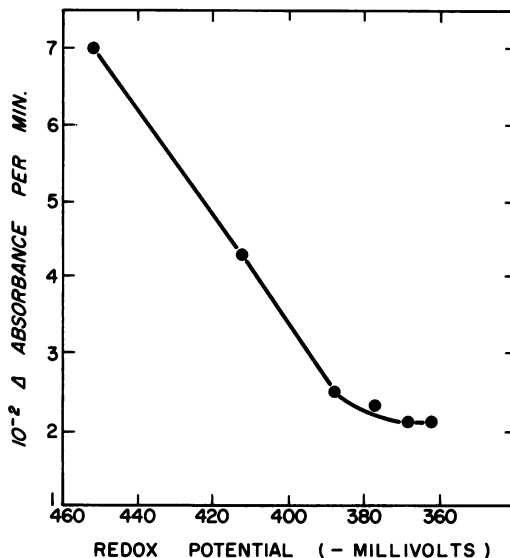


FIG. 4. Autooxidation rates of reduced methyl viologen as a function of the redox potential. The standard assay system was used without enzyme. The total methyl viologen added was varied from 0.23 to 4.74 mM, and the oxidation rate was measured after 0.23 mM reduced methyl viologen was generated by photoreduction.

TABLE 3. Comparison of activity of hydrogenase of different organisms assayed by methylene blue reduction and photoreduced methyl viologen oxidation

Source of hydrogenase	Activity by methylene blue reduction assay (units/mg of protein)	Activity by photoreduced methyl viologen oxidation assay (units/mg of protein)
<i>Escherichia coli</i>	1.8	1.3
<i>Desulfovibrio desulfuricans</i> ..	0	4.5
<i>Azotobacter vinelandii</i>	0.32	0
<i>Vibrio succinogenes</i>	7.1	1.0

be made with caution. Optimal assay conditions were developed only for *V. succinogenes* hydrogenase. The limited results, however, suggest that the assay system can be used with hydrogenases other than the *V. succinogenes* hydrogenase. The lack of activity of *A. vinelandii* in the spectrophotometric assay is in agreement with the results of Peck and Gest (6), who showed that *A. vinelandii* hydrogenase did not catalyze the production of H_2 from methyl viologen reduced with $\text{Na}_2\text{S}_2\text{O}_4$. Methylene blue reduction activity has been reported for *D. desulfuricans* hydro-

genase (6). We do not know why the preparation we used was inactive in the methylene blue reduction system.

Several types of assay systems have been employed for measuring hydrogenase (6). The spectrophotometric assay we developed is essentially a modification of the hydrogen evolution assay of Peck and Gest (6). The use of photochemical reduction, rather than $\text{Na}_2\text{S}_2\text{O}_4$ reduction, of methyl viologen permits the level of reduced methyl viologen to be easily controlled for spectrophotometric measurements of the oxidation of the reduced viologen. The photochemical reduction system may be useful for the study of other enzyme reactions which require an electron donor with a low redox potential.

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