THE REDUCTION OF METHYLENE BLUE BY HYDROGENASE[1]

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Hydrogenase activity in biological systems has been measured by a variety of procedures. These include the orth-para conversion of hydrogen and the deuterium exchange reaction (Krasna and Rittenberg, 1954), the fixation of tritium (Smith and Marshall, 1952), and the catalytic hydrogenation of a variety of acceptor substances (Farkas and Fischer, 1947; Gest, 1954). Catalytic hydrogenation of methylene blue has been employed most frequently for assays of hydrogenase, although the ability to reduce this dye is not necessarily an absolute criterion of hydrogenase activity (Curtis and Ordal, 1954).

In the course of fractionating extracts of *Micrococcos lactilyticus* (*Veillonella gazogenes*) prepared by sonic disintegration, pronounced variations in hydrogenase activity were found when different dyes were used as hydrogen acceptors. In particular, it was found that a fraction having the highest specific activity with benzyl viologen was either completely inactive, or had very low hydrogenase activity, when assayed with methylene blue. A logical explanation for this finding is that another factor, in addition to hydrogenase, is required for the reduction of methylene blue by molecular hydrogen. The present paper gives evidence supporting this interpretation.

METHODS

The methods employed for growing bacterial cultures, for preparation of extracts and fractions, and for concentration and assay of hydrogenase activity have been described (Whiteley and Ordal, 1955).

RESULTS

Hydrogenation of various substrates by sonicates and fractions of M. lactilyticus. Sonicates of *M. lactilyticus* were found to catalyze the hydrogenation of a variety of chemical compounds. The compounds tested, arranged in order of activity are as follows: benzyl viologen, methyl viologen, methylene blue, methyl violet, phenosafranin, uric acid, hydroxylamine, nitrate, nitrite, xanthine, 2,4 dinitrophenol, sodium hydrosulfite, sodium bisulfite, and sodium sulfite.[2] The hydrogenation of some of these compounds is reversible, i. e., hydrogen is evolved from reduced methyl viologen, benzyl viologen, and hypoxanthine in an atmosphere of nitrogen gas.

An uptake of hydrogen is sometimes obtained with fumarate, but this occurs regularly only if semicarbazide is added (0.02 M final concentration). In the absence of this compound, fumarate is rapidly degraded to acetate, propionate, carbon dioxide and hydrogen (Whiteley, 1955, unpublished data). No uptake of hydrogen was observed with ferricyanide, diphosphopyridine nucleotide, or lipoic acid in substrate amounts, even in the presence of catalytic quantities of methyl or benzyl viologen. It may be noted that riboflavin (Witter, 1953) and endogenous flavins (Peck and Gest, 1954) have been found to act as hydrogen acceptors with extracts of *M. lactilyticus*.

The relative rates of hydrogenation of dyes by an extract and by a preparation of hydrogenase are given in table 1. When a partly purified preparation of hydrogenase (referred to as "soluble hydrogenase" and derived from a fraction precipitated by 70 to 90 per cent saturation with ammonium sulfate; Whiteley and Ordal, 1955) was assayed with these dyes, it was found that there was no hydrogen uptake with methylene blue although there had been an increase in specific activity with the four other dyes used. Other preparations of soluble hydrogenase from *M. lactilyticus* behaved in a similar way or had

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[2] The latter three compounds were added as dry crystals in 10-mg amounts. With large quantities of freshly prepared extracts, there is a rapid enzymatic evolution of great amounts of gas from sodium hydrosulfite followed by a slower absorption and a net uptake of hydrogen.

TABLE 1

Hydrogenase activities of crude sonicate and "soluble hydrogenase" as measured with several dyes

Hydrogen Acceptor	Hydrogenase Activity*	
	Sonicate	"Soluble hydrogenase"†
Benzyl viologen	15.0	40.0
Methyl viologen	9.1	22.0
Methylene blue	7.5	0.0
Phenosafranin	3.0	7.5
Methyl violet	2.0	5.0

* μL H_2/min/mg protein.

† Fraction precipitated from supernatant after protamine precipitation, by 70 to 90 per cent saturation with ammonium sulfate. Reaction mixture contained: 0.2 ml sonicate or fraction, 20 μM dye, 20 μM cysteine, 20 mg purified plasma albumin, and 50 μM pH 7.0 phosphate buffer; total volume, 2.2 ml; KOH in center well, hydrogen atmosphere.

at most very small amounts of activity when tested with methylene blue.

An examination of fractions separated in the first stage of the fractionation procedures revealed (table 2) that those obtained with the higher concentrations of ammonium sulfate showed a marked increase in activity when assayed with benzyl viologen, but decreased sharply in activity when methylene blue was used as acceptor substance. On the other hand, the fraction obtained by 0 to 50 per cent saturation with ammonium sulfate showed comparable hydrogenase activity when assayed with both benzyl viologen and methylene blue.

The methylene blue factor. The data given in table 2 suggest that some component which is needed for the reduction of methylene blue is present in the original sonicate and the first ammonium sulfate fraction, but is entirely absent in fractions obtained by precipitation with higher concentrations of ammonium sulfate. To test this hypothesis, various materials were added to preparations of "soluble hydrogenase" in order to determine whether methylene blue activity could be restored.

It may be noted from table 2 that considerable methylene blue activity was lost on precipitation with protamine. However, addition of heated protamine-precipitated material did not enhance methylene blue reduction with soluble hydrogenase. Addition of the fraction (after heating) obtained by 0 to 50 per cent saturation with ammonium sulfate was also without effect. Additions of boiled crude extracts of *M. lactilyticus*, yeast extract, small amounts of methyl viologen, pyruvate, acetyl phosphate, coenzyme A, molybdenum trioxide, ferrous am-

TABLE 2

Recovery of hydrogenase in fractions tested with methylene blue and benzyl viologen

Fraction	Total Protein	Benzyl Viologen		Methylene Blue	
		Specific activity	Total activity	Specific activity	Total activity
	mg				
(1) Sonicate	1,120	7.7	8,600	4.7	5,250
(2) Supernatant after protamine precipitation	320	25.0	8,000	11.8	3,780
(3) (2) fractionated with ammonium sulfate 0–50% saturation	200	13.6	2,720	12.0	2,400
(4) 50–70% saturation	115	30.0	3,440	2.0	230
(5) 70–90% saturation	20	72.2	1,450	1.0	20
Σ of ammonium sulfate fractions			7,610		2,650
Per cent of original activity found in ammonium sulfate fractions			88		51

Specific activity = μL H_2/min/mg protein.
Total activity = specific activity × total protein.
Conditions of assay as in table 1.

TABLE 3

Activity of "soluble hydrogenase" with two hydrogen acceptors in the presence of a second fraction

Source of Hydrogenase	μL H_2 Taken up After 30 min	
	Methylene blue	Benzyl viologen
(1) "Soluble hydrogenase"...	12	455
(2) Fraction 1.....	5	4
(3) (1) plus (2).....	102	450
(4) Diluted *Escherichia coli* extract.....	10	5
(5) (1) plus (4).....	120	448
(6) (1) plus boiled (2) or boiled (4).....	20	453

"Soluble hydrogenase" = supernatant after protamine treatment fractionated with ammonium sulfate, fraction precipitating between 70 and 90 per cent saturation with ammonium sulfate after treatment with calcium phosphate gel; 0.2 mg protein/vessel. Fraction 1 = material precipitated by protamine washed 2 × with 0.5 per cent protamine solution; 0.5 mg protein/vessel. *E. coli* extract prepared by grinding a cell paste with alumina, followed by centrifugation at 15,000 × G for 20 min; 0.5 mg protein/vessel.

Conditions of assay as for table 1.

monium sulfate, flavin adenine nucleotide, or diphosphopyridine nucleotide singly and in combination were also without effect.

On the other hand, the addition of a small amount of unheated protamine-precipitated material to a preparation of "soluble hydrogenase" greatly increased hydrogen uptake with methylene blue, although it had no effect on hydrogen uptake with benzyl viologen (table 3). If precipitation with protamine is omitted from the fractionation procedure, similar results are obtained on adding the material precipitated between 0 and 25 per cent saturation with ammonium sulfate. Also, the addition of a dilute extract of *Escherichia coli* greatly enhanced the reduction of methylene blue by "soluble hydrogenase" (table 3). The ability of these fractions to affect the reduction of methylene blue was destroyed by heating, but not by dialysis.

Hydrogenase activity of "supernatant" and "particulate" fractions of M. lactilyticus. These results recalled the work of Swim and Gest (1954) on the synergistic effect between "soluble" and "particulate" fractions of *E. coli* with respect to hydrogenase activity assayed with methylene blue. Consequently, "supernatant" and "particulate" fractions were prepared from both *E. coli* and *M. lactilyticus* (Whiteley and Ordal. 1955) and were tested for hydrogenase activity separately and in combination, using methylene blue and benzyl viologen as hydrogen acceptors. The results of these experiments are given in table 4.

A synergism between the two fractions of *E. coli* was obtained when methylene blue was used for measuring hydrogen, thus confirming the work of Swim and Gest (1954). A similar synergistic effect was found when the two fractions of *M. lactilyticus* were combined using methylene blue as hydrogen acceptor. However, no synergism was found when either benzyl viologen or methyl violet (not shown) were used in assays of hydrogenase activity.

It should be noted that although the "supernatant" and "particulate" fractions of *E. coli* and *M. lactilyticus* were prepared in the same way, there was a difference in the distribution of hydrogenase with the two organisms. With *E. coli* the greater part of the hydrogenase activity, determined either by methylene blue or by benzyl viologen, was in the "particulate" fraction; whereas with *M. lactilyticus*, the "supernatant" fraction had the higher specific activity.

Methylene blue reduction by "soluble hydro-

TABLE 4

Hydrogenase activity of "supernatant" and "particulate" fractions of M. lactilyticus and E. coli

	Specific Activity*	
	Benzyl viologen	Methylene blue
Micrococcus lactilyticus		
Supernatant.....	28.0	4.4
Particles.....	0.75	2.5
Supernatant and particles..	29.0	13.0
Escherichia coli		
Supernatant.....	5.0	2.0
Particles.....	18.0	17.0
Supernatant and particles..	22.0	40.0

Fractions prepared according to Whiteley and Ordal (1955); particles washed 2 × in water.

Conditions of assay as for table 1.

* μL H_2/min/mg protein.

genase" combined with "particulate" fractions. The increase in activity noted when "particulate" and "supernatant" fractions are combined may be interpreted to mean a coupling between hydrogenase and some component or components present in the "particulate" fractions of both organisms. If this hypothesis is correct, it would be expected that the activity of "soluble hydrogenase" with methylene blue would be restored upon addition of the "particulate" fraction. That this is so, is clearly shown in table 5. It is of interest that "particulate" fractions of *E. coli* were as effective as those from *M. lactilyticus* in this regard.

It had been suggested by Peck and Gest (1954) that the failure of extracts of *Clostridium butylicum* to reduce methylene blue was the result of a deficiency in a carrier required for the transfer of hydrogen to methylene blue, since they found hydrogenase present as measured by the catalytic hydrogenation of benzyl viologen. Although earlier attempts to demonstrate participation of an easily dissociable cofactor in the reduction of methylene blue by hydrogenase from *E. coli* were unsuccessful (Gest, 1952), the present work and the experiments of Swim and Gest on the synergism between "supernatant" and "particulate" fractions suggest that a dissociable component is required for the reduction of methylene blue. Although it has been postulated that such a component may be concerned with electron transport, it may equally well be involved in the detoxification of methylene blue.

TABLE 5

Activity of "soluble hydrogenase" in presence of "particulate" fractions

Source of Hydrogenase	Specific Activity*	
	Methylene blue	Benzyl viologen
(1) Crude extract	1.5	3.0
(2) "Soluble hydrogenase"	0.0	75.0
(3) *Escherichia coli* particles	17.0	—
(4) (2) plus (3)	54.0	—
(5) *Micrococcus lactilyticus* particles	2.5	—
(6) (2) plus (5)	39.5	—

* μL H_2/min/mg protein.

"Soluble hydrogenase" prepared as in the experiment reported in table 1; *E. coli* and *M. lactilyticus* "particle fraction" used in experiment reported in table 4; crude extract of *M. lactilyticus* prepared by sonic disintegration.

TABLE 6

Inhibition of "soluble hydrogenase" by methylene blue

Amount of Methylene Blue Added	Delay in Onset of Hydrogen Uptake	Specific Activity*
μM	*min*	
0	—	100
1.25	8	94
2.5	12	90
5.0	>40	—
10.0	<40	—

* μL H_2/min/mg protein.

Methylene blue added with benzyl viologen. Assay conditions as for table 1.

Methylene blue inhibition of "soluble hydrogenase." It has been demonstrated by Curtis and Ordal (1954) that methylene blue in the oxidized form inhibits the deuterium exchange reaction and also the hydrogenation of benzyl viologen and methyl violet by *Micrococcus aerogenes*. As seen from table 6, methylene blue also inhibits the hydrogenation of benzyl viologen by "soluble hydrogenase" from *M. lactilyticus*. The onset of hydrogen uptake is delayed and the rate of hydrogen uptake is somewhat decreased when small amounts of methylene blue are added simultaneously with benzyl viologen. With larger amounts of methylene blue, inhibition was complete. However, when small concentrations of methylene blue were added after half the benzyl viologen had undergone reduction, there was no effect on either the rate or the total uptake of hydrogen. Hence, with respect to the methylene blue effect, the "soluble hydrogenase" preparation from sonicates of *M. lactilyticus* behaves like the natural enzyme present in *M. aerogenes*. In view of these results, it is not certain whether the second component provided by the "particulate" fraction of *M. lactilyticus* or *E. coli* is involved in the transport of electrons from "soluble hydrogenase" to methylene blue, or whether it merely serves to detoxify methylene blue.

DISCUSSION

It is evident from these studies that the reduction of methylene blue by molecular hydrogen requires hydrogenase and an additional com-

ponent or components which are not required for the reduction of benzyl viologen. The demonstration by Curtis and Ordal (1954) of deuterium exchange and hydrogenation of benzyl viologen (a one-electron acceptor of low E_0) by *Micrococcus aerogenes* in the absence of methylene blue reduction clearly indicates that methylene blue reduction is not an adequate criterion for hydrogenase assay. In this connection, it may be noted that solubilization of a particle-bound enzyme may result in the loss of methylene blue activity. Thus, Singer and Kearney (1954) found that soluble preparations of succinic dehydrogenase were able to reduce phenazine methyl sulfonate, a one-electron dye, but were unable to reduce methylene blue. The hydrogenase of *M. lactilyticus* appears to be a soluble enzyme which can be concentrated and separated from the component necessary for methylene blue reduction. In another study, it has been shown that this soluble hydrogenase is an iron containing molybdoflavoprotein which can mobilize hydrogen to reduce purines in the presence of appropriate enzymes and carriers (Whiteley and Ordal, 1955).

Other workers have obtained preparations of soluble hydrogenase which may be contrasted with the enzyme from *M. lactilyticus* in that they actively reduce methylene blue. Sadana and Jagannathan (1954) obtained such an enzyme from *Desulfovibrio desulfuricans* and Shug *et al.* (1954) obtained a soluble hydrogenase from *Clostridium pasteurianum* which showed very high activity when assayed with methylene blue. This suggests that if a second component is required for methylene blue reduction, it apparently is not readily separated from hydrogenase in these bacteria. In some organisms, hydrogenase appears to be associated largely with insoluble particles. This was found to be the case with hydrogenase in *Azotobacter vinelandii* (Hyndman *et al.*, 1953), and in *E. coli*. A marked synergism was found between particulate and supernatant fractions of *E. coli* in the reduction of methylene blue, thus confirming the findings of Swim and Gest (1954). However, no synergism was found when benzyl viologen was used as acceptor. Synergism between particles and supernatant fractions of *M. lactilyticus* was also found with respect to methylene blue reduction but not benzyl viologen reduction. Here the "particulate" fraction had relatively little hydrogenase activity, suggesting that its main contribution was to provide the second component necessary for the reduction of methylene blue. Apparently this component can be solubilized by sonic disintegration and then recovered by the precipitation procedures used in enzyme fractionation. Although little has been done to characterize the second component, it is heat-labile and nondialyzable and is either a protein or is bound to a protein.

SUMMARY

Sonicates of *Micrococcus lactilyticus* were found to catalyse the hydrogenation of a wide variety of chemical compounds. The hydrogenation of some of these compounds is reversible, i. e., molecular hydrogen is evolved from reduced methyl viologen, reduced benzyl viologen and hypoxanthine in an atmosphere of nitrogen gas.

By fractionation of sonicates of *M. lactilyticus*, preparations of "soluble hydrogenase" were obtained which exhibited high specific activity when assayed with benzyl viologen and certain other dyes, but which showed little or no hydrogen uptake with methylene blue. The hydrogenation of benzyl viologen by "soluble hydrogenase" was inhibited by methylene blue in the oxidized form.

Evidence has been obtained that a component, in addition to hydrogenase, is required for the reduction of methylene blue by molecular hydrogen. Methylene blue activity was restored to preparations of "soluble hydrogenase" from *M. lactilyticus* by the addition of various heat-labile materials. These included a particulate fraction from *M. lactilyticus*, itself low in hydrogenase activity; protamine-precipitated material from *M. lactilyticus*; a particulate fraction from *Escherichia coli*; and a dilute extract of *E. coli*. In addition, marked synergism was found between particulate and supernatant fractions of *M. lactilyticus* and *E. coli* with respect to methylen blue reduction, but not benzyl viologen reduction.

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