FORMIC DEHYDROGENASE AND THE HYDROGENLYASE ENZYME COMPLEX IN COLI-AEROGENES BACTERIA[1]

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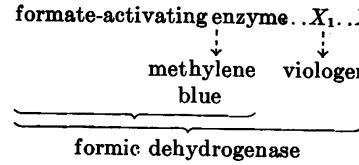
Escherichia coli and many other coli-aerogenes bacteria characteristically contain a formic dehydrogenase which is active with methylene blue as the hydrogen acceptor. This activity is primarily associated with the insoluble particulate fraction of such cells (Stickland, 1929; Gale, 1939; Gest, 1952) and consequently has been difficult to characterize from an enzymatic standpoint. Intact cells and cell-free extracts of anaerobically grown normal strains also catalyze the decomposition of formate to molecular hydrogen and carbon dioxide by the so-called hydrogenlyase reaction. From studies of intact cells of normal and non-gas producing (anaerogenic) variants, Ordal and Halvorson (1939) provided some evidence in support of an earlier suggestion (Stephenson and Stickland, 1931) that the hydrogenlyase reaction was catalyzed by formic dehydrogenase and hydrogenase functioning together as a coupled electron transfer system. This concept was further supported by investigations on the hydrogenlyase reaction in cell-free systems from *E. coli* (Gest, 1952; Gest and Gibbs, 1952).

The latter studies were recently amplified by investigation of the enzymatic constitution of six coli-aerogenes bacteria (anaerogenic variants) incapable of producing hydrogen from glucose (Gest and Peck, 1955). Cell-free extracts of one of the variants catalyzed the hydrogenlyase reaction and showed both formic dehydrogenase and hydrogenase activities; inability of this organism to produce hydrogen from glucose was consequently attributed to at least a partial block in the generation of formate from pyruvate via the phosphoroclastic reaction. Extracts from the other five organisms did not show the hydrogenlyase reaction. In three of these, hydrogenase activity could not be detected in the preparations and in another instance formic dehydrogenase was absent. In the extract of the sixth organism, hydrogenase and formic dehydrogenase (as tested with methylene blue) were present; absence of the hydrogenlyase reaction in this organism was attributed to the lack of an appropriate formic dehydrogenase component, active with the one-electron dye benzyl viologen ($E_0' = -0.359$ V) as the electron acceptor. Combination of extracts from variants containing formic dehydrogenase with extracts from organisms containing hydrogenase led, in one instance, to reconstruction of a very active hydrogenlyase reaction. Analysis of such reconstructed systems and of the system present in normal gas-producing cells led to the following formulation of the hydrogenlyase enzyme complex (Gest and Peck, 1955):



where X_1 and X_2 are unidentified electron transport carriers.

Of particular significance for the present paper was the conclusion that electrons can be transferred from formate to hydrogenase only if a formic dehydrogenase component capable of reducing benzyl viologen (through X_1) is present in the system. This report describes the properties of the formic dehydrogenases of coli-aerogenes bacteria and provides further evidence that the dehydrogenase active with viologen dyes is an integral component of the hydrogenlyase enzyme complex. Additional evidence supporting the general formulation given above is also presented.

MATERIALS AND METHODS

Cultivation of organisms and preparation of cell-free systems. E. coli strain Crookes (American

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Type Culture Collection 8739) was used for preparation of the normal hydrogenlyase complex (Gest, 1952). The organism was grown in stationary culture (10 L of medium in a 12-L Florence flask) at 37 C in a medium containing glucose, 1 per cent; yeast extract, 0.2 per cent; peptone, 0.2 per cent; nutrient broth, 0.8 per cent; KH_2PO_4, 1.4 per cent; and Na_2HPO_4, 1.4 per cent. The glucose was sterilized separately and added aseptically just before inoculation of the medium.

The culture and properties of the two anaerogenic strains WR 2 and WR 3 have been described previously (Gest and Peck, 1955). These organisms were recently further characterized through the kindness of Dr. P. R. Edwards of the United States Public Health Service Communicable Disease Center, Chamblee, Georgia. The strain referred to as WR 2 appears to be an anaerogenic *Escherichia coli* while WR 3 is an atypical Klebsiella. These organisms were generally grown in stationary culture in a medium containing 1 per cent glucose, 1 per cent tryptone, and 1 per cent yeast extract.

In some instances, WR 2 was grown in the medium specified above for *E. coli* in order to obtain greater cell yields. In preliminary tests it was found that when WR 3 was grown in this medium, the formic dehydrogenase was almost completely associated with the insoluble fraction of the cell-free extract. On the other hand, formic dehydrogenase activity was soluble in extracts from cells grown in the glucose-tryptone-yeast extract medium and the organism was consequently routinely cultivated in this manner. For aerobic growth experiments, 500 ml of medium in a 3-L Erlenmeyer flask were inoculated and the cultures incubated on a rotary shaker at 30 C for 12 hr.

Clostridium pasteurianum (strain W 5) was grown at 37 C in the complex glucose medium described by Wilson *et al.* (1948).

Cells were harvested in the usual manner and cell-free extracts prepared by grinding with Alumina A-301 as previously described (Gest and Peck, 1955).

Assays and determinations. Formic dehydrogenase and hydrogenlyase activities were determined by measuring rate of gas production from formate under an atmosphere of helium (at 30 C). For formic dehydrogenase assay, CO_2 formation in 0.0625 M phosphate pH 6.8 was measured in the presence of 100 μmoles sodium formate and either 16 μmoles of benzyl viologen or 8 μmoles methylene blue. When assaying purified enzyme, 10 mg of Armour crystalline bovine albumin were also added. Hydrogenlyase activity was assayed by measurement of either H_2 plus CO_2 evolution or H_2 formation (alkali in center well) in phosphate buffer (details given with results).

Hydrogenase activity was estimated (at 30 C) by measuring utilization of molecular hydrogen in the presence of methylene blue or benzyl viologen as described by Peck and Gest (1956).

Warburg vessels of approximately 10-ml capacity containing a final fluid volume of 1.2 ml were used for all manometric determinations.

Protein was determined by the trichloracetic acid method of Bücher (1947).

Ribonuclease (RNAase) was purchased from the Worthington Co. (Freehold, New Jersey).

RESULTS

The formic dehydrogenases. (1) Assay of formic dehydrogenase by coupling with hydrogenase:—Cell-free extracts of WR 3 display formic dehydrogenase activities with both methylene blue and benzyl viologen. For convenience, the former activity will be referred to as formic dehydrogenase (MB) and the latter as formic dehydrogenase (BV). The WR 3 extracts are virtually devoid of hydrogenase. Extracts of organism WR 2, on the other hand, show excellent hydrogenase activity but do not contain formic dehydrogenase. Neither WR 3 nor WR 2 extracts alone display hydrogenlyase activity, but a combination of the extracts rapidly decomposes formate to carbon dioxide and hydrogen (Gest and Peck, 1955). This coupling between formic dehydrogenase and hydrogenase provides a method for the quantitative assay of the former activity. Since ability to couple with hydrogenase is dependent on the presence of formic dehydrogenase (BV), the reconstituted hydrogenlyase reaction is considered to be a valid assay for formic dehydrogenase (BV) provided the WR 2 hydrogenase component is present in excess. Figure 1 shows that production of hydrogen from formate is proportional to amount of WR 3 extract under these conditions.

At high levels of WR 3 extract, the hydrogenase component (WR 2) is no longer in excess and there is, therefore, no further increase in activity with increasing amounts of the WR 3

preparation. For unknown reasons, the linear curve does not pass through the origin.

The WR 2 preparation tends to lose activity on storage and consequently must be frequently titrated with WR 3 to insure that an excess of hydrogenase is added to the assay mixture. It should also be noted that the WR 2 extract, as usually prepared, probably contains free formate which causes a considerable endogenous formation of gas. This can be eliminated by dialyzing the WR 2 preparation or, alternatively, by keeping the two extracts separate until exogenous formate is added. Since the reconstituted formic hydrogenlyase system often exhibits a substantial lag period it may be necessary to follow activity as long as one hour before a constant rate of gas evolution is attained.

(2) Assay of formic dehydrogenase activity with benzyl viologen:—When WR 3 extract and formate are incubated under nitrogen or helium with benzyl viologen, carbon dioxide is evolved at a rate proportional to the concentration of crude extract, as shown in figure 2. This activity is maximal at a pH of 6.8 in contrast with the formic hydrogenlyase reaction which has a pH optimum of 6.2 to 6.7. The rate of CO_2 production is not affected appreciably by altering the concentration of formate (5 to 100 μmoles) or benzyl viologen (2 to 16 μmoles). The assay is quite satisfactory with crude enzyme extracts but with fractionated preparations some inactivation of the enzyme apparently occurs during the assay

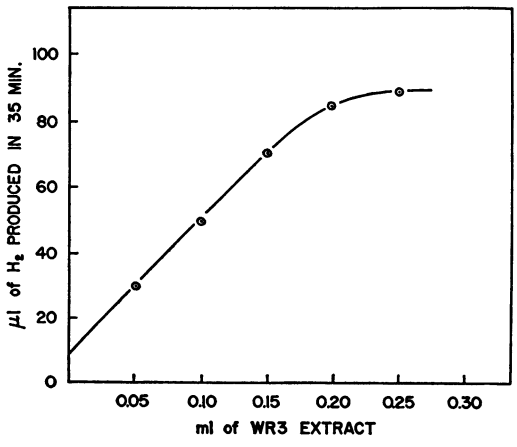


Figure 1. Assay of formic dehydrogenase (BV) activity in WR 3 extract by coupling with hydrogenase. Conditions: 0.0625 M phosphate pH 6.0, 50 μmoles sodium formate, 0.2 ml WR 2 extract.

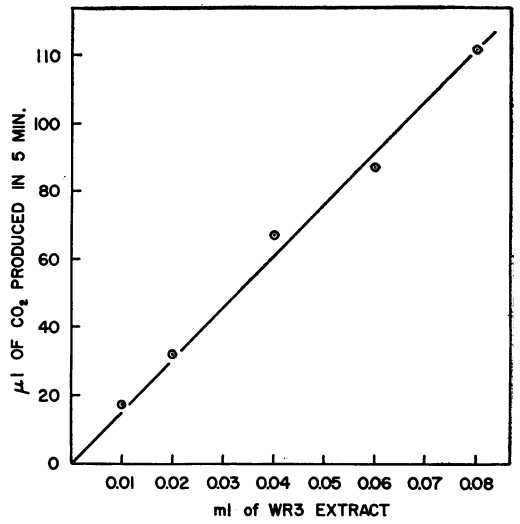


Figure 2. Relationship between enzyme concentration and formic dehydrogenase activity with benzyl viologen.

and, in addition, activity is not directly proportional to quantity of enzyme at low concentrations of the latter. It was observed that linear kinetics could be partially restored to the fractionated enzyme by addition of crystalline bovine albumin (10 mg) and glutathione (0.01 per cent) to the assay mixture. The rate of carbon dioxide formation during the first 5-min interval after addition of enzyme was usually taken as a measure of enzyme activity. In cases where a lag period occurred, the rate during the first two 5-min intervals through which the activity was constant were used for calculation of enzyme activity.

(3) Assay of formic dehydrogenase activity with methylene blue:—Evolution of carbon dioxide from formate upon addition of methylene blue has been traditionally used as a measure of the formic dehydrogenase present in various organisms. Activity with this acceptor was generally proportional to concentration of WR 3 extract, and did not show lag periods under any conditions (figure 3). The activity of formic dehydrogenase (MB) was usually calculated on the basis of carbon dioxide production observed during the first 10 min of assay. In some preparations, the activity was not proportional to enzyme concentration. This may be a reflection of differences between batches of extract with respect to content of a secondary component required for methylene blue reduction.

(4) Solubility of formic dehydrogenase activities:—It was already noted that the formic dehydrogenase of coli-aerogenes bacteria as measured with methylene blue (and oxygen) occurs predominantly in the insoluble particulate fractions of such cells. The formic dehydrogenase (MB) is reported to be partially solubilized by pretreatment of particles with butanol (Pinsent, 1954) but the soluble activity observed was apparently extremely labile and further characterization was not achieved. Gest (1952) and Wolin and Lichstein (1956) have also reported the apparent solubilization of this activity from particles of *E. coli*.

As a preliminary to fractionation of the formic dehydrogenase activities, their distribution between particulate and soluble fractions of the WR 3 extract was examined as follows. The crude extract prepared as noted above was first diluted with an equal volume of 0.1 M phosphate buffer pH 6.4, containing 0.1 per cent cysteine; 0.05 ml of an RNAase solution (5 mg/ml) was added per ml of diluted extract and the mixture incubated under helium for 1.5 hr at 37 C. The preparation was then centrifuged at 16,000 × G for 1 hr in a Servall SS-1 centrifuge and the residue discarded. The supernatant fluid

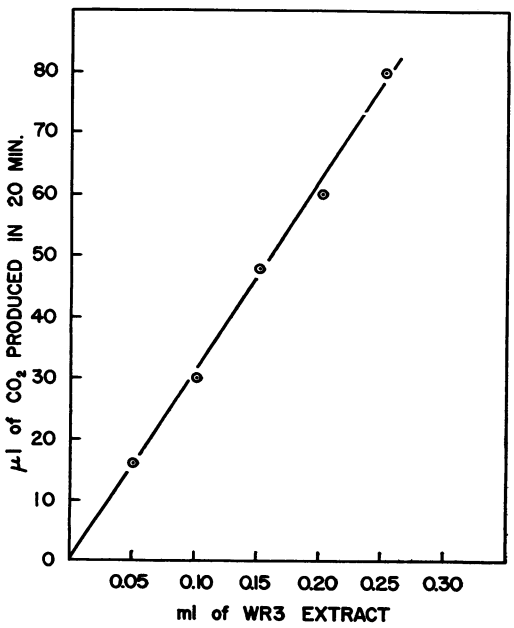


Figure 3. Relationship between enzyme concentration and formic dehydrogenase activity with methylene blue.

TABLE 1

Distribution of formic dehydrogenase activities between soluble and particulate fractions of WR 3 extract

Preparation	Formic Dehydrogenase Assay		
	Methylene blue	Benzyl viologen	Reconstructed hydrogenlyase*
	μL CO_2/10 min/ml	μL CO_2/10 min/ml	μL Gas/10 min/ml
Crude extract	1783	951	740
RNAase treated extract.	1900	770	440
Spinco supernatant fluid (SSF)	1250	580	320
SSF, frozen 5 days	—	145	—
SSF, frozen 7 days	285	125	—
Particles from Spinco centrifugation	500	0	0

* Production of H_2 plus CO_2 in the presence of excess WR 2; 0.0625 M phosphate buffer pH 6.4; 100 μmoles sodium formate.

was recentrifuged for 2 hr in a Spinco centrifuge at 120,000 × G. The residue from this centrifugation was dispersed in distilled water (one-half original extract volume). Formic dehydrogenase activities in the crude extract, RNAase treated extract, and in the final supernatant fluid and residue were determined using the three assays described above. The results shown in table 1 indicate that formic dehydrogenase (BV) and the enzymes required to activate formate for the hydrogenlyase reaction are soluble and therefore remain in the supernatant fraction. Formic dehydrogenase (MB), on the other hand, is found in both the insoluble and soluble fractions. The particles are unable to reduce benzyl viologen in the presence of formate and also do not stimulate the formic dehydrogenase (BV) activity of the supernatant. Although the insoluble fraction contains formic dehydrogenase (MB), it is unable to activate formate for the hydrogenlyase reaction in the coupled system. These results are in accord with the earlier conclusion that only preparations containing formic dehydrogenase (BV) are capable of coupling with WR 2 hydrogenase to form an active hydrogenlyase complex (Gest and Peck, 1955).

(5) pH optima:—The pH-activity curves of formic dehydrogenase (MB) and formic dehydrogenase (BV) are very similar, but the pH optimum of the latter is slightly higher (figure 4).

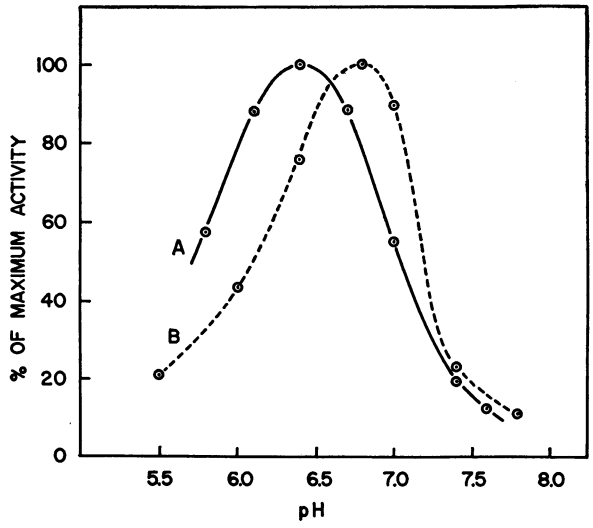


Figure 4. pH optima of formic dehydrogenase activities. Curve A: formic dehydrogenase (MB) Curve B: formic dehydrogenase (BV).

With both electron acceptors, activity rapidly decreases above pH 7.0. Essentially the same pH-activity curves were also obtained in experiments wherein acid was added to liberate bound carbon dioxide.

(6) Stability and other properties of fractionated formic dehydrogenase (BV):—A partially purified formic dehydrogenase fraction was prepared from the crude WR 3 extract as follows. Ten ml of 0.05 M phosphate pH 6.4 (containing 0.05 per cent cysteine) were added to 10 ml of crude extract and this solution was then supplemented with 1 ml (5 mg) of RNAase. After incubation at 37 C under helium for 2 hr, the solution was cooled and the precipitate removed by centrifugation in the Servall centrifuge at 16,000 × G for 1 hr. To the supernatant fluid, 0.8 vol of saturated (room temperature) ammonium sulfate pH 6.4 was added and the precipitate collected by centrifugation in the usual manner. The latter was dissolved in 15 ml of 0.05 M phosphate buffer pH 6.4, containing 250 μmoles sodium formate per ml, placed under helium and frozen at −20 C. Eight ml of this ammonium sulfate fraction were thawed and the pH adjusted to 4.7 with 0.25 M acetic acid. The precipitate which formed was collected and dissolved in 3 ml of the phosphate-formate solution described above. The results of the fractionation are summarized in table 2.

All of the fractions indicated usually contained formic dehydrogenase (MB) but this activity did not show the same fractionation behavior as formic dehydrogenase (BV). The procedure outlined effected approximately 14 fold purification of the latter activity. Assay of purified fractions was complicated by the occurrence of induction periods. This phenomenon, particularly characteristic of stored preparations, resulted in apparent loss of activity and non-proportionality between activity and enzyme concentration. It was found that induction periods could be experimentally caused by dialysis, by addition of chelating agents or by aging and was most pronounced at low enzyme concentration. The induction phase could not be abolished by change

TABLE 2

Fractionation of formic dehydrogenase (BV)

Fraction	Protein Conc.	Activity*	Specific Activity
	mg/ml	*μL CO_2/hr/ml*	*μL CO_2/hr/mg protein*
Crude extract	23.6	2400	102
RNAase treated extract	8.1	2277	282
0.8 Vol ammonium sulfate precipitate	5.1	2460	482
pH 4.7 Precipitate	1.4	1970	1410

* In the presence of 10 mg crystalline bovine albumin.

of pH or by addition of various sulfhydryl reducing agents, metals, boiled extracts, known coenzymes, or catalase. On the other hand, the lag period is eliminated or shortened by increasing the enzyme concentration as shown in figure 5.

Since activity eventually returned with autocatalytic kinetics even at low enzyme concentration, it appeared that the induction period reflected either the slow formation of an intermediate or required cofactor or, alternatively, that it signified gradual removal of an inhibitor from the system. Because preincubation with formate or the additions noted above did not eliminate the lag, it seemed more likely that an inhibitor, probably oxygen, was responsible for the induction phase. Oxygen inhibits formic dehydrogenase, hydrogenase, and hydrogenlyase activities (Gale, 1939; Gest, 1952; Gest, 1954; Fisher *et al.*, 1954). In the case of hydrogenase, the inactivation is due to both irreversible oxidation and reversible oxygenation (Joklik, 1950; Fisher *et al.*, 1954). It is noteworthy that lag periods similar to those shown by fractionated formic dehydrogenase (BV) are frequently observed with aged, diluted, or the ammonium sulfate-precipitated hydrogenlyase complex from extracts of *E. coli* (Gest, 1952). We have also observed induction periods under similar conditions with the reconstructed hydrogenlyase system (WR 3 plus WR 2).

Although hydrosulfite is probably the most effective nonenzymatic agent for removal of oxygen from hydrogenase preparations (Fisher *et al.*, 1954), this compound could not be used as a deoxygenation agent in the present instance because it markedly inhibits formic dehydrogenase (BV). The lag period is eliminated, however, when O_2 is removed enzymatically by addition of formate and extracts from aerobically-grown normal *E. coli*. The latter extracts possess formic dehydrogenase activity with methylene blue and oxygen but do not show appreciable formic dehydrogenase (BV) activity. Since the linear rate of CO_2 evolution attained by adding extract from aerobically-grown *E. coli* is the same as that observed after the induction period with unsupplemented formic dehydrogenase (BV), it appears that the function of the former preparation is to remove oxygen from the system. This interpretation is further supported by the observation that occurrence of

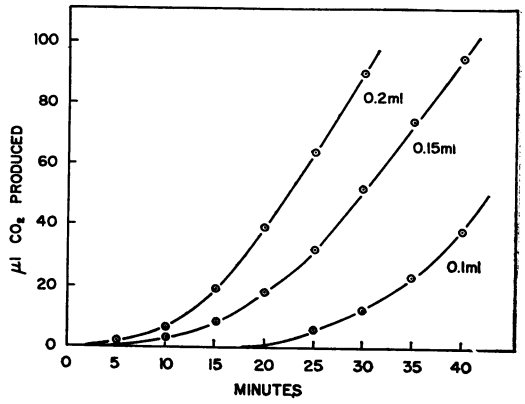


Figure 5. Effect of enzyme concentration on the induction period shown by fractionated formic dehydrogenase (BV).

induction periods in different preparations can be correlated with absence of formic dehydrogenase activity with oxygen as the acceptor. Restoration of hydrogenlyase activity in diluted and other preparations from *E. coli* occurs in the same way when "aerobic formic dehydrogenase" and formate are added (Gest, 1952).

In contrast with plant formic dehydrogenase (Mathews and Vennesland, 1950; Davison, 1951), formic dehydrogenase (BV) does not reduce pyridine nucleotides and, in addition, does not appear to require folic acid compounds for activity. Gale (1939) has provided evidence that the formic dehydrogenase (MB) of *E. coli* similarly does not reduce pyridine nucleotides. The plant and bacterial enzymes also differ in another important respect. The oxidation of formate by the former enzyme is irreversible, whereas the reaction catalyzed by bacterial formic dehydrogenase (BV) must be reversible since the over-all hydrogenlyase reaction is a reversible process (Woods, 1936).

The hydrogenase component of the hydrogenlyase complex. Extracts of WR 2 rapidly reduce methylene blue and benzyl viologen with molecular hydrogen. The extract also produces hydrogen from reduced methyl viologen (Peck and Gest, 1956), but does not display formic dehydrogenase or hydrogenlyase activities. As described previously, the formic dehydrogenase (BV) of organism WR 3 can serve as the electron donor system for the WR 2 hydrogenase. This coupling provides a method for quantitative assay of the hydrogenase system present in the WR 2

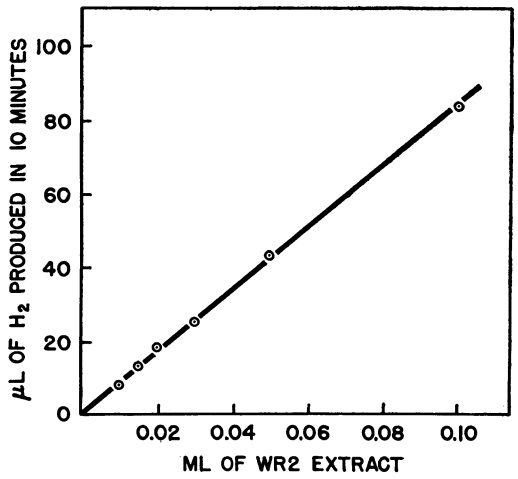


Figure 6. Assay of hydrogenase in WR 2 extract by coupling with formic dehydrogenase (BV). Conditions: 0.0625 M phosphate pH 6.1, 60 μmoles sodium formate, 0.2 ml WR 3 extract, 0.1 ml 20 per cent KOH in center well.

preparation. As shown in figure 6, formation of hydrogen is proportional to the amount of WR 2 extract added, provided the formic dehydrogenase (BV) is present in excess.

(1) Solubility of the hydrogenase system of WR 2:—The WR 2 extract was centrifuged for 1.5 hr in a Spinco centrifuge at 120,000 × G and the insoluble residue homogenized in distilled water (volume equal to original volume of extract). Hydrogenase activities in the supernatant fluid and the homogenized residue were determined using three different assays. As shown in table 3, the soluble portion of the extract (supernatant fluid) contains hydrogenase activity with both of the dyes, but it exhibits very little "coupling activity" with formic dehydrogenase (BV). The insoluble residue, on the other hand, shows high activity in the coupling test assay. A further slight increase in coupling activity is observed when both the soluble and insoluble WR 2 fractions are added to the formic dehydrogenase (BV). These data indicate that the components of WR 2 required for coupling are located almost entirely in the insoluble portion of the cell-free extract.

Both the soluble and insoluble fractions exhibit hydrogenase activity with methylene blue and benzyl viologen. When catalytic concentrations of methyl viologen are added to a mixture containing the soluble portion of the WR 2 extract and excess WR 3 formic dehydrogenase (i. e., as in 2, column 4 of table 3) formate is rapidly decomposed to hydrogen and carbon dioxide. This hydrogenlyase reaction is similar to a model hydrogenlyase system described by Gest and Peck (1955) which was composed of extract of WR 3, a soluble clostridial hydrogenase, and methyl viologen which served to transfer electrons between the two enzymes. It appears that the particles from WR 2 extract contain, in addition to hydrogenase, a component (designated as X_2) which is required for electron transfer from formic dehydrogenase to hydrogenase. This component, presumed to be an electron carrier, is apparently present only in small amount in the soluble fraction (and absent from clostridial extracts) and can be replaced by methyl viologen, thus reconstituting a "model" hydrogenlyase system.

(2) Effects of various treatments on the coupling activity of WR 2 particles:—In order to obtain further evidence regarding the nature of the natural electron carrier suggested above, attempts were made to remove or destroy the hydrogenase in WR 2 particles without inactivating the postulated carrier. Since the latter is presumably also required for transfer of electrons to clostridial hydrogenase (Gest and Peck, 1955), the assay used to detect the carrier was the ability to replace methyl viologen in the artificial

TABLE 3

Solubility of hydrogenase activities of WR 2

Fraction	Hydrogenase Assay		
	Methylene blue reduction*	Benzyl viologen reduction*	Coupling with formic dehydrogenase (BV)†
	μL H_2 utilized/ 20 min/ 0.1 ml	*μL H_2 utilized/ 20 min/ 0.1 ml*	*μL H_2 produced/ 20 min/ 0.1 ml*
1. Crude extract	—	—	166
2. Spinco supernatant fluid	156	46	10
3. Spinco residue	92	32	82
4. = 2 + 3	—	—	104

* Conditions: 0.0625 M phosphate pH 7, 8 μmoles methylene blue or 16 μmoles benzyl viologen, 0.1 ml 20 per cent KOH in center well, gas phase H_2.

† Conditions: 0.0625 M phosphate pH 6, 60 μmoles sodium formate, 0.2 ml WR 3 extract, 0.1 ml 20 per cent KOH in center well, gas phase helium.

hydrogenlyase system: formic dehydrogenase (BV) ... methyl viologen ... clostridial hydrogenase.

Because of the particulate character of the WR 2 system, classical protein fractionation procedures could not be employed to separate hydrogenase from "electron carrier activity." Various solvents were, therefore, examined for ability to effect differential inactivation.

Ethyl alcohol inactivated both activities while *n*-butanol had little effect. The effect of acetone treatment on the particles is shown in table 4. The WR 2 particles were prepared by centrifuging crude WR 2 extract at 40,000 × G for 1.5 hr. The sediment was washed once with distilled water and resuspended in distilled water (one fourth original extract volume). This suspension was then poured into 20 vol of cold acetone (−10 C) and the solids allowed to settle. Most of the acetone was poured off and the remainder removed by centrifugation at −5 C for 5 min. The treated particles were then washed with cold acetone and subsequently with cold anhydrous ether. After drying in air, the acetone powder was stored at −20 C.

It can be seen from table 4 that untreated WR 2 particles couple with WR 3 formic dehydrogenase to form an active hydrogenlyase complex, whereas this ability to couple is destroyed by acetone treatment. In separate tests it was found that the untreated WR 2 extract utilized 324 μL of hydrogen per 20 min with methylene blue and 140 μL of hydrogen in the same time period with benzyl viologen as electron acceptor. Under the same conditions, the acetone powder of WR 2 particles utilized 109 μL of hydrogen with methylene blue and 164 μL with benzyl viologen. Thus the inability of acetone treated particles to couple with formic dehydrogenase appears to be due to destruction of the coupling factor X_2 rather than to inactivation of the hydrogenase. It is also evident from table 4 that acetone treated WR 2 particles cannot replace methyl viologen in the model hydrogenlyase system using clostridial hydrogenase.

Hydrogenase can be irreversibly inactivated by oxygen and consequently an attempt was made to specifically destroy the hydrogenase of WR 2 particles by vigorous aeration. Particles were shaken aerobically in a shallow layer at 37 C for several hours and then tested for the two activities in question. Both hydrogenase and coupling activities were inactivated as a result of this treatment.

TABLE 4

Effect of acetone treatment on the coupling activity of WR 2 particles

Enzymatic Components	Hydrogenlyase Activity
	μL H_2 produced/20 min
WR 2	1
WR 3 + WR 2	185
WR 3 + WR 2 acetone powder	3
WR 3 + *Clostridium pasteurianum* extract	15
WR 3 + WR 2 acetone powder + *C. pasteurianum* extract	13
WR 2 acetone powder + *C. pasteurianum* extract	7

Conditions: 0.083 M phosphate pH 6, 50 μmoles sodium formate, 0.25 ml WR 2 extract, 0.25 ml WR 3 extract, 0.5 mg WR 2 acetone powder, 0.2 ml *C. pasteurianum* extract, 0.1 ml 20 per cent KOH in center well.

Although these attempts to demonstrate a clear cut separation of hydrogenase and carrier X_2 activities were not entirely successful, the available data are consistent with the hypothesis (Gest and Peck, 1955) that WR 2 particles contain a specific labile electron carrier required for coupling of formic dehydrogenase (BV) with the hydrogenase enzyme.

Formic dehydrogenase (benzyl viologen) and its relation to the hydrogenlyase reaction. (1) Comparison of hydrogenlyase systems:—Identity of the hydrogenlyase system present in normal *E. coli* with that reconstructed by mixing extracts of WR 3 and WR 2 is indicated by close similarity of properties. Both systems manifest formic dehydrogenase activities with methylene blue and benzyl viologen as well as hydrogenase determined by a number of procedures. The hydrogenlyase reaction in cell-free extracts of *E. coli* exhibits a marked dilution effect (Gest, 1952). When cell-free extracts of WR 3 and WR 2 were mixed in equal proportions and the hydrogenlyase activity determined using progressively smaller amounts of mixture, a similar dilution effect or nonlinear relationship between enzyme activity and enzyme concentration was observed.

The hydrogenlyase activity in cell-free preparations of *E. coli* strain Crookes has a broad pH optimum from pH 6.2 to 6.8 as shown in figure 7

(Curve A). A striking feature is the great decrease in activity above pH 7 (Gest, 1952). The pH activity curve of the formic hydrogenlyase system reconstructed by mixing WR 3 and WR 2 is also shown in figure 7 (Curve B). In this particular mixture the formic dehydrogenase (BV) component was limiting and the overall reaction shows an optimum at pH 6.4 to 6.6, very similar to the pH activity curve of formic dehydrogenase (BV) (figure 4). The relatively broad pH optimum of the hydrogenlyase complex of *E. coli* suggests the possibility that a component(s) other than the formate-activating enzyme may be limiting in this system. Differences have been observed in pH optima of different formic hydrogenlyase preparations and this appears to be due to variation in the relative levels of the several components required for the reaction. Thus, experimental alteration of the pH activity curve can be readily demonstrated in hydrogenlyase preparations made with different mixtures of WR 3 and WR 2.

The cell-free hydrogenlyase of *E. coli* is inhibited by a large number of different compounds (Gest, 1952). Effects of some of these inhibitors on the normal and reconstructed hydrogenlyase systems are shown in table 5. The increase in activity of the *E. coli* system in the presence of "Tiron" (sodium catechol disulfonate) is not considered significant. The metal chelating agents, 8-hydroxyquinoline and *o*-phenanthroline, as well as α,α'-dipyridyl (not shown), markedly inhibit the hydrogenlyase activity of both systems. Formaldehyde also strongly inhibits, whereas KSCN has little or no effect on either enzyme complex. Gest (1952) reported that KSCN caused a two-fold stimulation of hydrogenlyase activity in *E. coli* extracts and suggested that this compound complexed inhibitory cations. It is presumed that such cations were absent from the extracts used in the present studies. Nitrate prevents formation of the hydrogenlyase system in growing cells (Yudkin, 1932) and also inhibits its activity in cell-free preparations (Gest, 1952) and intact cells containing the enzyme complex (Stephenson and Stickland, 1932). It has been suggested that the actual inhibitory agent is nitrite produced by reduction of nitrate. As shown in table 5, nitrate and nitrite inhibit both hydrogenlyase systems to approximately the same extent. It was observed that inhibition of

TABLE 5

Effects of various compounds on the normal and reconstructed hydrogenlyase systems

Compound	Conc	Hydrogenlyase Activity	
		Escherichia coli system	Reconstructed system (WR 3 + WR 2)
	M	% of control	
Tiron*	5.0×10^{-3}	125	100
8-Hydroxyquinoline	7.5×10^{-3}	4	5
o-Phenanthroline	6.3×10^{-3}	2	14
HCHO	1.2×10^{-3}	0	3
KSCN	4.2×10^{-3}	110	113
KNO_3	6.3×10^{-3}	5	1
$NaNO_2$	8.3×10^{-4}	3	1
$Na_2S_2O_4$	6.7×10^{-3}	16	17

Conditions: 0.0625 M phosphate pH 6.4, 100 μmoles sodium formate, 0.2 ml *E. coli* extract, 0.2 ml WR 3 extract, 0.2 ml WR 2 extract, 0.1 ml 20 per cent KOH in center well. Each compound was incubated with extracts for 15 min before addition of formate.

* Sodium catechol disulfonate.

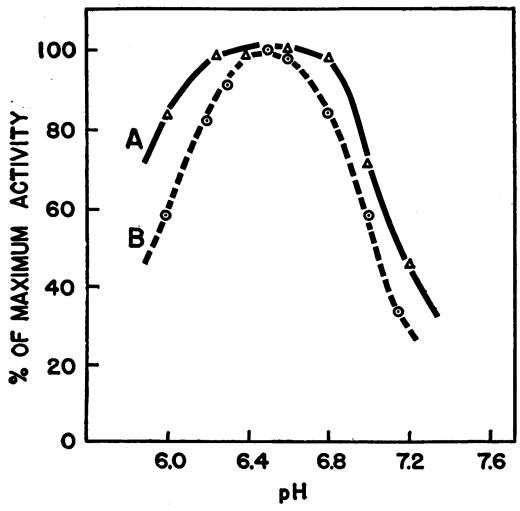


Figure 7. pH optima of normal and reconstructed hydrogenlyase systems. Curve A: hydrogenlyase complex of *Escherichia coli*. Curve B: hydrogenlyase complex reconstructed by mixing WR 3 and WR 2 extracts. Conditions: 0.083 M phosphate, 100 μmoles sodium formate, 0.2 ml *E. coli* extract, 0.2 ml WR 3 extract, 0.2 ml WR 2 extract, 0.1 ml 20 per cent KOH in center well.

the reconstructed hydrogenlyase reaction diminished after incubation longer than 2 hr. This could well be due to the removal of nitrate and nitrite by enzymatic reduction to ammonia. Sodium hydrosulfite similarly inhibits both hydrogenlyase complexes and inhibition is relieved with time in the reconstructed system (possibly due to metabolic removal of the inhibitor). The close similarities described above indicate that the hydrogenlyase complex from *E. coli* and that reconstructed by mixing cell-free extracts of WR 3 and WR 2 are identical multienzyme systems.

(2) Comparison of formic dehydrogenase and hydrogenlyase activities:—The nutritional studies of Waring and Werkman (1944) and Pinsent (1954) have implicated iron, molybdate and selenite in the formation of bacterial formic dehydrogenase (assayed with methylene blue and oxygen as electron acceptors). Participation of metal cofactors in hydrogenlyase activity is also indicated by the effects of chelating agents (table 5). Inhibition by α,α'-dipyridyl is completely relieved by ferrous ion but only partially by manganous ion and the latter metal also stimulates the activity of the ammonium sulfate-precipitated complex from *E. coli* (Gest, 1952). From table 6 it can be seen that the reconstructed hydrogenlyase system is almost completely inhibited by *o*-phenanthroline, and that some relief of the inhibition can be achieved by addition of Fe^{++}. Similar results have also been observed using dipyridyl.

In preliminary experiments, the activity of formic dehydrogenase (BV) was observed to be inhibited erratically by dipyridyl. This was later found to be due to the fact that added complexing agents may induce a lag period, particularly when small amounts of enzyme are used. With large amounts of enzyme there is no induction period and the degree of inhibition by complexing agents can then be readily determined. As shown in table 6, under these conditions *o*-phenanthroline causes the same degree of inhibition of formic dehydrogenase (BV) and formic dehydrogenase (MB) activities and it is evident that ferrous ion completely reverses the effects of this chelating agent. Of interest in this connection is a recent report (Wolin and Lichstein, 1956) that iron stimulates the activity of a partially solubilized formic dehydrogenase (MB) from *E. coli*.

Cyanide markedly inhibits hydrogenlyase activity in intact cells (Stephenson and Stickland, 1932) and in cell-free extracts from *E. coli* (Gest, 1952). Table 7 summarizes the effects of cyanide on both formic dehydrogenase activities (see also Gale, 1939). Approximately the same degree of inhibition was observed with both electron acceptors. From these results and those of table 6, it may be concluded that the formic dehydrogenase(s) of coli-aerogenes bacteria is a metalloenzyme, but the identity of the metal is still uncertain. It is noteworthy that plant formic dehydrogenase also appears to be a metalloenzyme since its activity is inhibited by cyanide and other chelating agents (Davison, 1951; Yamamoto, 1954).

The hydrogenlyase reaction is more sensitive to inhibition by metal chelating agents than the formic dehydrogenase activities. The data of table 6 show that this inhibition of the hydrogenlyase system can be completely reversed by adding small quantities of methyl viologen. Since methyl viologen is capable of transporting electrons between formic dehydrogenase and hydrogenase (Gest and Peck, 1955), this observation suggests that the formic hydrogenlyase component most sensitive to metal complexing agents is the natural electron carrier functioning between formic dehydrogenase (BV) and hydrogenase (i. e., carrier X_2).

Tiron (sodium catechol disulfonate) does not

TABLE 6

Inhibition of formic dehydrogenase and hydrogenlyase activities by o-phenanthroline

Additions*	Formic Dehydrogenase (MB) Activity	Formic Dehydrogenase (BV) Activity	Reconstructed Hydrogenlyase Activity
	per cent inhibition		
o-Phenanthroline (6.3×10^{-3} M)	30	36	93
o-Phenanthroline (1×10^{-2} M)	70	74	—
o-Phenanthroline (6.3×10^{-3} M) + Fe^{++}(5×10^{-4}M)	0	0	69
o-Phenanthroline (6.3×10^{-3} M) + 1 μmole methyl viologen	—	—	(77)†

* Preincubated with extracts for 15 min before assay.

† Per cent stimulation.

TABLE 7

Inhibition of formic dehydrogenase activities by cyanide

Additions	Formic Dehydrogenase Activity	
	With methylene blue	With benzyl viologen
	μL CO_2/10 min	
None	84	44
KCN (8.3×10^{-4} M)	47	22
KCN (3.3×10^{-3} M)	30	14

Enzyme preparation: 0.05 ml of the 0.8 vol ammonium sulfate fraction of table 2.

KCN was preincubated with enzyme for 5 min before addition of dye.

affect hydrogenlyase or formic dehydrogenase (BV) activities but this chelating agent does inhibit formic dehydrogenase (MB) activity. This is the only differential inhibition found thus far for the two formic dehydrogenase activities (see below).

It was also found that sodium hydrosulfite and formaldehyde, which markedly inhibit hydrogenlyase activity (table 5), also strongly inhibit formic dehydrogenase (BV).

The closely comparable effects of inhibitors on formic dehydrogenase (BV) and hydrogenlyase activities provide further support for the conclusion that this formic dehydrogenase is in fact the primary component of the hydrogenlyase enzyme complex. In 1951 Grunberg-Manago, *et al.* reported that sodium hypophosphite inhibited the formic dehydrogenase (MB) activity of intact *E. coli* markedly, but had relatively little effect on hydrogenlyase activity and these results were interpreted as favoring the view that the latter activity is catalyzed by a single unique enzyme. The present results also indicate that formic dehydrogenase (MB) as such is not required for hydrogenlyase activity and it is now clear that this fact cannot be used as an argument against the multienzyme concept of the hydrogenlyase system. Using the same inhibitor, Gest and Peck (1955) found that the formic dehydrogenase (MB) activity in cell-free extracts of *E. coli* was inhibited 57 per cent while the hydrogenlyase activity was depressed by only 30 per cent. Similar results have been observed with respect to formic dehydrogenase (BV). The quantitative discrepancy in this effect on the formic dehydrogenase (BV) and hydrogenlyase systems can be readily explained as follows. In reconstructed hydrogenlyase systems made by mixing WR 3 and WR 2 extracts, the inhibitory effect of hypophosphite shows an inverse relationship to the amount of formic dehydrogenase (BV) (i. e., WR 3 extract) added. In other words, it appears that the inhibitor will affect both formic dehydrogenase (BV) and hydrogenlyase activities to the same extent only when the dehydrogenase is the limiting component of the enzyme complex.

(3) The hydrogenlyase system of *E. coli*:—Partial separation of the components of this system was achieved by high speed centrifugation. Fifteen ml of crude *E. coli* extract were centrifuged at 120,000 × G for 2 hr and to the supernatant fluid 4 ml of 0.25 M phosphate buffer pH 6.4 and 3.75 mg of ribonuclease were added. After incubation for 1 hr at 30 C under helium, the extract was recentrifuged at 120,000 × G for 2 hr providing a soluble extract and an insoluble residue. The latter was dispersed in 5 ml of 0.02 M phosphate buffer pH 6.4 and recentrifuged at 10,000 × G for 15 min. The pellet obtained was resuspended in 0.02 M phosphate pH 6.4 (5 ml) and this fraction designated as the Spinco residue. From table 8 it is evident that the soluble extract possessed some hydrogenlyase activity while the Spinco residue was devoid of this activity. In confirmation of earlier experiments (Swim and Gest, 1954), addition of Spinco residue to the soluble extract caused an appreciable increase in hydrogenlyase

TABLE 8

Partial separation of the formate-activating and hydrogenase components of the Escherichia coli hydrogenlyase complex

Enzymatic Components*	Hydrogenlyase Activity
	μL H_2/10 min
Soluble extract	32
plus Spinco residue	50
plus WR 2 extract	49
plus WR 3 extract	31
Spinco residue	0
plus WR 2 extract	0
plus WR 3 extract	24

Conditions: 0.083 M phosphate pH 6, 50 μmoles sodium formate, 0.1 ml 20 per cent KOH in center well.

* Soluble extract, 0.3 ml; other components, 0.2 ml.

activity. This result indicates the presence of complementary components required for hydrogenlyase activity in both fractions. By adding "external" preparations of hydrogenase (WR 2) and formic dehydrogenase (BV) (WR 3), it was possible to determine the relative distribution of the corresponding components in the two fractions from the *E. coli* extract. As shown in table 8, the soluble extract contains an excess of the formate activating component (i. e., formic dehydrogenase) but is deficient in hydrogenase, i. e., hydrogenlyase activity was stimulated by WR 2 but not by WR 3. The "residual" hydrogenlyase activity observed in the soluble extract is conditioned either by the presence of very small hydrogenase containing particles which were not removed by the centrifugation or may reflect the presence of solubilized hydrogenase (and X_2).

The Spinco residue alone shows no hydrogenlyase activity, but does when supplemented with WR 3 formic dehydrogenase (BV). Addition of hydrogenase (i. e., WR 2) to the *E. coli* particles, on the other hand, did not restore activity. Accordingly, the particulate fraction, which contains hydrogenase (Swim and Gest, 1954), is deficient in formic dehydrogenase (BV). These experiments supply further evidence supporting the conclusion that the hydrogenlyase complex present in *E. coli* is identical with that reconstituted by combining WR 2 and WR 3 extracts.

(4) Separation of formic dehydrogenase (MB) and formic dehydrogenase (BV) activities:—For the experiments described above the WR 3 extracts used were obtained from cells grown in deep stationary culture, i. e., under essentially anaerobic conditions. As indicated in table 1 such extracts contain both formic dehydrogenase (MB) and (BV) activities. The particulate fraction obtained from the crude extract displays only formic dehydrogenase (MB) activity, while the soluble fraction shows both activities and these appeared to have the same stability characteristics with regard to storage at −20 C. These observations suggest the possibility that a common component is required for reduction of both electron acceptors and that the particles may be deficient in an accessory factor necessary for reduction of benzyl viologen. Of interest in this connection is Davison's (1951) observation that partially purified plant formic dehydrogenase reduces benzyl viologen at a very low rate (in the presence of diphosphopyridine nucleotide) which is significantly increased upon addition of a heart diaphorase preparation.

TABLE 9

Separation of formic dehydrogenase (BV) activity from formic dehydrogenase (MB)

Fraction	Formic Dehydrogenase Activity*	
	With methylene blue	With benzyl viologen
	μL CO_2/10 min/ml	
RNAase treated extract	1680	440
0.8 Vol ammonium sulfate fraction	9	515

* In the presence of 10 mg crystalline bovine albumin.

The probability of different electron transfer sequences for reduction of the two dyes by formate is also indicated by the observation that Tiron inhibits methylene blue reduction but has no effect on formic dehydrogenase (BV) activity. A distinct separation of the two activities has been achieved as indicated in table 9. The ammonium sulfate fraction indicated was obtained by the same procedure outlined earlier, except that the supernatant fluid after the RNAase treatment was recentrifuged for 2 hr at 120,000 × G before proceeding with the ammonium sulfate precipitation. In contrast with the crude particulate fraction, which shows only methylene blue reducing activity (table 1), the soluble ammonium sulfate fraction shows appreciable activity only with benzyl viologen. As would be expected, this soluble fraction also can couple with WR 2 hydrogenase to reconstitute an active hydrogenlyase system.

In contrast with anaerobically-grown cells, extracts made from WR 3 grown with aeration exhibit high formic dehydrogenase (MB) activity whereas formic dehydrogenase (BV) activity is present in negligible quantity. Due to the absence of the latter activity, such extracts cannot couple with WR 2 hydrogenase. Comparable results have been observed when normal *E. coli* is grown aerobically, i. e., absence of formic dehydrogenase (BV) and hydrogenlyase (also hydrogenase). These observations indicate that the components of the electron transport sequence required for reduction of benzyl viologen are produced only under conditions of anaerobic growth, which is also a requirement for formation

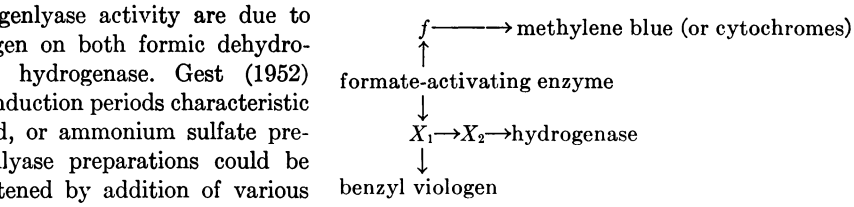
of the hydrogenlyase enzyme complex in coli-aerogenes bacteria (see discussion by Gest, 1954).

DISCUSSION

The hydrogenlyase system reconstructed by mixing WR 3 formic dehydrogenase (BV) with WR 2 hydrogenase shows the same behavior as the natural system present in *E. coli* extracts with regard to all properties examined thus far (e. g., pH activity curve, effects of inhibitors, etc.) Further, there is a striking parallelism between the properties of formic dehydrogenase (BV) and those of the hydrogenlyase complex. The present experiments demonstrate, in addition, that partially purified formic dehydrogenase (BV) can activate formate for electron transport to hydrogenase. These results considered together with those of earlier studies (Gest, 1952; Gest and Peck, 1955) establish formic dehydrogenase (BV) as an integral component of the hydrogenlyase complex.

Formic dehydrogenase (BV) shows an induction period in its activity under certain circumstances and it appears that this is due to an inhibitory effect of oxygen. This inhibition is reversible and the gradual removal of oxygen leads to an apparently autocatalytic restoration of enzyme activity. Oxygen also inactivates hydrogenase, both by oxidation and reversible oxygenation (Fisher *et al.*, 1954). Accordingly, it seems very likely that the lag periods frequently observed in hydrogenlyase activity are due to the effects of oxygen on both formic dehydrogenase (BV) and hydrogenase. Gest (1952) observed that the induction periods characteristic of diluted, dialyzed, or ammonium sulfate precipitated hydrogenlyase preparations could be eliminated or shortened by addition of various compounds (mostly intermediates in carbohydrate metabolism), in some cases only if supplementary enzyme preparations were added. It was suggested (Gest, 1952; Barkulis and Gest, 1953; Gest, 1954) that these "activations" might reflect a cyclical mechanism for formate decomposition in which C_2 compounds act as coenzymes. The present results indicate, however, that the effects observed were no doubt indirect and can be attributed to removal of inhibitory oxygen by providing substrates for oxygen utilizing systems already present in the *E. coli* extracts or in the supplementary enzyme systems sometimes added. The possibility that oxygen inhibition might have been responsible for the induction period was appreciated during the earlier studies (Barkulis and Gest, 1953; Gest, 1954), but this was at the time considered an unlikely explanation because sulfhydryl compounds such as cysteine did not abolish the lag. In this connection it may be noted that oxygen, in remarkably small amounts, can effectively inhibit enzymes such as hydrogenase (Fisher *et al.*, 1954) and in some instances only very powerful reducing agents such as hydrosulfite are capable of restoring activity (Joklik, 1950). Reversal of oxygen inhibition can also account for a variety of other observations describing "activation" of the hydrogenlyase system in aged and other types of intact cell preparations (e. g., see Lascelles, 1948; Wolf *et al.*, 1954).

Detailed comparison of formic dehydrogenase (MB) with formic dehydrogenase (BV) indicates that these activities have many common properties, but that they distinctly differ in several respects. Of particular significance is the fact that while formic dehydrogenase (BV) can couple with hydrogenase to reconstitute hydrogenlyase activity, formic dehydrogenase (MB) cannot do so. In addition, the present experiments demonstrate that the two formic dehydrogenase activities can be separated from each other. These observations can be accounted for by the following scheme, where the arrows indicate possible directions of electron transport.



According to this representation, formate is initially activated by the same protein for the three activities considered, viz., formic dehydrogenase (MB), formic dehydrogenase (BV), and transport to hydrogenase (i. e., hydrogenlyase). A common protein activator could explain the many similarities observed in behavior of the three activities toward inhibitors.

Reduction of the two-electron dye methylene blue is visualized as requiring a factor *f*, perhaps of the nature of a diaphorase, which may also be the site of action of the specific Tiron inhibition. Presumably this pathway would also be involved in reduction of oxygen through the cytochrome

system. Loss of factor f during fractionation could account for the separation of a formic dehydrogenase incapable of reducing methylene blue, but still able to reduce benzyl viologen.

The alternative possibility that coli-aerogenes bacteria contain two different formic dehydrogenases cannot be conclusively eliminated at the present time. According to this interpretation, one of the formic dehydrogenases would be the initial catalyst for the pathway leading to methylene blue (or cytochromes) while the second enzyme, capable of reducing benzyl viologen, would funnel electrons to X_2. In this case postulation of the separate carriers X_1 and f would be unnecessary.

Carrier X_1 (or a specific formic dehydrogenase (BV)) is assumed to be absent from formic dehydrogenase preparations incapable of reducing viologen or coupling with hydrogenase, e. g., preparations from aerobically grown cells and the particulate formic dehydrogenase (MB) fraction described in table 1. Formation of this carrier (or formic dehydrogenase (BV)) and subsequent components of the hydrogenlyase complex during growth apparently cannot occur in the presence of oxygen and adaptive synthesis of the hydrogenlyase complex therefore requires anaerobic conditions (Stephenson, 1937; Pinsky and Stokes, 1952). In order to further establish the general validity of the requirement of formic dehydrogenase (BV) for hydrogenlyase activity we recently examined the enzymatic constitution of six non-gas producing organisms classified as *E. coli* 0 group (kindly supplied by Dr. P. R. Edwards). All of these showed formic dehydrogenase (MB) activity and all but one contained an active hydrogenase (MB). Formic dehydrogenase (BV) activity, however, was absent from all six strains. In view of these and earlier results (Gest and Peck, 1955) there is little question that presence of formic dehydrogenase (BV) is essential for formic hydrogenlyase activity in any type of coli-aerogenes organism. A recent study (Stokes, 1956) of five anaerogenic species of *Salmonella* showed that all contained hydrogenase, while formic dehydrogenase activity (with oxygen) was present in only three strains. Unfortunately, no tests were made for the presence or absence of formic dehydrogenase (BV).

The present results and the formulation given above can readily account for all of the physiological and biochemical observations available on the mechanism of the hydrogenlyase reaction including those which have been interpreted to support the view that the reaction is catalyzed by a single unique enzyme (see discussion by Gest, 1954; Gest and Peck, 1955). In large measure, these latter misinterpretations can be attributed to the incorrect assumption that formic dehydrogenase activity with methylene blue was a valid measure of any dehydrogenase component likely to be required for the overall reaction.

Establishment of the fact that hydrogenlyase activity requires at least two enzymes (formic dehydrogenase (BV) and hydrogenase) provides further support for the general principle that biological formation of molecular hydrogen is usually the result of a sequence of reactions catalyzed by a specific primary dehydrogenase, intermediate carriers, and hydrogenase which is the terminal component of the electron transfer chain.

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SUMMARY

The mechanism of formate decomposition to H_2 and CO_2 (hydrogenlyase activity) in coli-aerogenes bacteria was investigated and further direct evidence obtained that the reaction is catalyzed by a multienzyme system consisting of formic dehydrogenase, hydrogenase, and one or two intermediate factors.

The formic dehydrogenase component required is a soluble metalloenzyme capable of reducing one-electron dyes of low redox potential (viologens) in the presence of formate and is produced only during anaerobic growth. This dehydrogenase activity was separated from a second dehydrogenase activity measurable with two-electron dyes of the methylene blue type and the former partially purified from extracts of a non-gas producing coli-aerogenes variant. From a comparison of the properties of both dehydrogenase activities it appears that they may share a common protein component but the possibility that

there may be two distinct enzymes has not been eliminated.

The partially purified formic dehydrogenase (viologen) readily couples with particulate hydrogenase preparations, obtained from another non-gas producing variant, to reconstitute an active hydrogenlyase system. Formic dehydrogenase (methylene blue) is incapable of coupling in this manner. Further evidence is presented that the particles contain, in addition to hydrogenase, an intermediate carrier (X_2) necessary for electron transport from formic dehydrogenase (viologen) to hydrogenase.

Comparison of the hydrogenlyase system reconstructed by mixing "complementary" preparations from the two non-gas producers with the system present in extracts of normal *Escherichia coli* indicates that they are identical in all respects. It was also found that many properties of over all hydrogenlyase activity are referable to effects on formic dehydrogenase (viologen), particularly in systems wherein the latter is a limiting component. The present results are discussed in relation to the enzymatic constitution of normal and non-gas producing bacteria of the coli-aerogenes group.

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