PROPERTIES OF CELL-FREE HYDROGENASES OF ESCHERICHIA COLI AND RHODOSPIRILLUM RUBRUM[1]

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Received for publication July 24, 1951

The mechanism of conversion of substrate hydrogen to H_2 in microbial fermentations is still obscure. One of the enzymes presumably involved in the final phase of H_2 formation is hydrogenase, which catalyzes the reaction $H_2 \rightleftharpoons 2H^+ + 2e$ (Stephenson and Stickland, 1931). The present investigation was stimulated by our interest in the presumed participation of hydrogenase in photochemical production of H_2 by photosynthetic bacteria and the apparent relationship of this enzyme to fixation of N_2 in these organisms (Gest, Kamen, and Bregoff, 1950). Several properties of hydrogenase in cell-free preparations of *Rhodospirillum rubrum*, a photosynthetic N_2-fixing organism, have been studied and are compared with the corresponding enzyme of the heterotrophic bacterium, *Escherichia coli*, which has thus far not been shown to fix N_2 (Lindstrom, Lewis, and Pinsky, 1951).

METHODS AND RESULTS

Preparation of cell-free hydrogenases and partial purification of the enzyme. A. *Rhodospirillum hydrogenase. Rhodospirillum rubrum* (SI) was grown anaerobically in the light in the G3X medium described by Kohlmiller and Gest (1951). In some instances, this medium was supplemented with 0.1 per cent acid-hydrolyzed casein; this addition did not noticeably affect the properties of the organisms with respect to the present studies. Cell-free extracts containing active hydrogenase, as measured by H_2 uptake in the presence of methylene blue, were obtained by three procedures: (a) grinding with glass as described by Kalnitsky, Utter, and Werkman (1945), (b) grinding with "alumina A303" (Aluminum Company of America) according to the directions of McIlwain (1948), and (c) sonic disintegration of cells suspended in water or 0.5 per cent KCl. After centrifugation to remove abrasives and cell debris, clear deep-red extracts were obtained. Freezing and thawing of the extracts led to formation of a considerable amount of red precipitate (particularly in the more concentrated sonic extracts), which was removed by high speed centrifugation. The supernates, still intensely colored, were stored under H_2 at 5 C and used for the experiments described hereafter. Preliminary experiments with these preparations indicated that *Rhodospirillum* hydrogenase behaves very much like the hydrogenase of *E. coli* with respect to further purification.

[1] This investigation was performed under a contract (no. N6-ori-208, T.O.1) administered by the Office of Naval Research for the Atomic Energy Commission.

B. *Escherichia coli hydrogenase. Escherichia coli*, strain B, was grown for 8 to 24 hours in deep stationary culture at 37 C in the following medium: Difco peptone, 5 g; Difco beef extract, 3 g; glucose, 10 g; NaCl, 5 g; distilled water, 1 liter; pH adjusted to 7.5.

Acetone powders (prepared in the usual manner) of the cells contained active hydrogenase which could be extracted as indicated in table 1. From the results given, it appears that alumina grinding is the most effective means of extracting the enzyme.

Although acetone powders (and lyophilized cells) offer advantages as source material for preparation of the enzyme, our experience indicates that the use of fresh cells is preferable; the crude extracts prepared from fresh cells usually have considerably higher specific activity. The highest specific activity prepara-

TABLE 1

Extraction of Escherichia coli hydrogenase from acetone powder

EXTRACTING AGENT	MG PROTEIN/ML OF EXTRACT*	$Q_{30}^{H_2}C$†
A. Water	0.88	78
B. Phosphate buffer (0.05 M; pH 7)	2.29	63
C. KCl (1 per cent)	0.64	186
D. Alumina grinding	1.91	291

In A, B, and C, 50 mg of acetone powder were suspended in 5 ml of extracting agent for 30 minutes at 30 C. In D, 100 mg of powder plus 0.9 ml of water were ground with alumina and the paste extracted with 10 ml of 0.05 M phosphate pH 7. In all instances, insoluble debris, etc., was removed by centrifugation at 20,000 ×g for 20 minutes.

* The proteins were precipitated with trichloroacetic acid and the precipitate analyzed for protein content by the procedure described by Sutherland *et al.* (1949) using crystalline serum albumin as the standard.

† $Q_{30}^{H_2}C$ = μl H_2/hr/mg protein. The rates given are based on the uptake observed during the first 20 minutes, using 0.7 ml of extract + 0.3 ml 0.5 M phosphate buffer pH 6.7 + 0.2 ml neutralized methylene blue (8 μM) in 10 ml Warburg vessels.

tion obtained thus far was made by the following procedure: 12 g (wet weight) of 8-hr old cells were washed once with 25 ml of water and then ground by hand with 30 g of "alumina A303" in a large mortar. The paste was extracted with 60 ml of water for 20 min and alumina, etc., removed by centrifugation at high speed. A considerable amount of nucleoprotein was precipitated from the brown colored extract by addition of one-twentieth volume of 1 M $MnCl_2$. After removal of the precipitate, the hydrogenase was precipitated by 50 per cent saturation with $(NH_4)_2SO_4$. This protein fraction was dissolved in water and centrifuged to remove residual insoluble particles. For most of the experiments described later, preparations at this stage of purification were used; in the best cases, the specific activity at this point was approximately 30,000 μl H_2 per hr per mg protein N at 37 C with methylene blue as the acceptor.

It has been observed that further purification can be achieved by a subsequent adsorption step using reagent grade MnO_2 (Merck) as follows: the solution is

adjusted to 0.05 M phosphate pH 5.5 to 5.9 and solid MnO_2 added (MnO_2/protein $\cong \frac{20\text{–}40}{1}$). After 10 minutes, the MnO_2 is centrifuged out, washed with water, and hydrogenase eluted from the adsorbent with 0.2 M phosphate buffer pH 7.3. Proper application of this adsorption step (i.e., fractional adsorption and elution) should facilitate further purification of the enzyme.

In the procedures previously outlined, all steps are conducted in the cold as rapidly as possible because of the lability of the enzyme with respect to oxidation. When storage periods greater than 2 or 3 hours were necessary, the preparations were kept under H_2 at 5 C.

Hydrogenase assay. Enzyme activity in the cell-free preparations was determined by measuring H_2 consumption at 30 C or 37 C with methylene blue as the acceptor. Methylene blue chloride National Aniline was used; the dye solution was neutralized to a pH between 6 and 7. It should be noted that at concentrations required for adequate manometric assay, methylene blue may cause protein precipitation, particularly in crude extracts.

Hydrogenase shows maximal activity over a rather broad pH range (Joklik, 1950*a*); in the present experiments, 0.125 M (final conc) phosphate buffer of pH 6.7 was generally used. Phosphate is not required for activity of the enzyme, and the concentration of this buffer does not affect the rate of H_2 uptake appreciably (0.06 to 0.25 M; *Rhodospirillum* hydrogenase). It is of interest that H_2 uptake has not been observed in the absence of methylene blue, even in crude extracts.

Using crude preparations, with relatively high protein content, the rate of H_2 consumption is ordinarily observed to be linear until most of the methylene blue is reduced to the leucoform. More purified preparations, on the other hand, may show marked instability during the activity assay. This point is illustrated in figure 1 for a case (*Escherichia* hydrogenase) in which the protein concentration was approximately 33 μg per ml in the reaction mixture. In phosphate buffer, the activity falls off rapidly. Better assay conditions are obtained when versene buffer is used in place of phosphate. Addition of crystalline serum albumin to phosphate buffer is most effective as indicated by curve A. The partial protection by versene buffer suggests that the progressive inactivation in phosphate is partly due to metal ions (possibly introduced as impurities in the methylene blue).

Stability of hydrogenase during storage: inactivation by O_2. Back, Lascelles, and Still (1946) reported that the activity of the cell-free hydrogenase of *E. coli* is retained for long periods if the preparations are stored anaerobically at 5 C. This result has been confirmed using both *Rhodospirillum* hydrogenase and *Escherichia* hydrogenase. The inactivation of hydrogenase by incubation in the presence of air and its preservation by anaerobic storage is indicated in figure 2. The curves represent the course of H_2 consumption by a crude *Rhodospirillum* extract after 15 days of storage under three different conditions. Immediately after preparation, the extract utilized H_2 at a rate identical with that shown by the top curve. The activity was completely preserved by storage under H_2

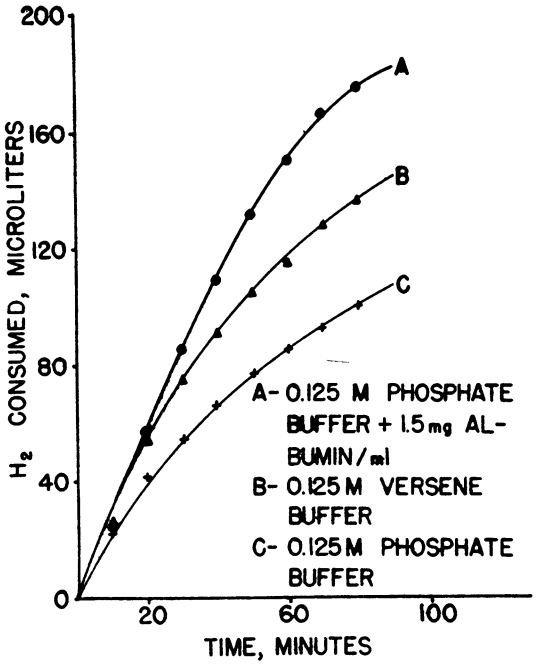

Figure 1. Stability of *Escherichia coli* hydrogenase during activity assay under three different conditions. At zero time, 0.2 ml of methylene blue (approximately 8 μM) was tipped into 1.0 ml of buffer-enzyme mixture containing 40 μg of enzyme protein. The pH in A and C was 6.65; in B, 6.3. Temperature, 37 C.

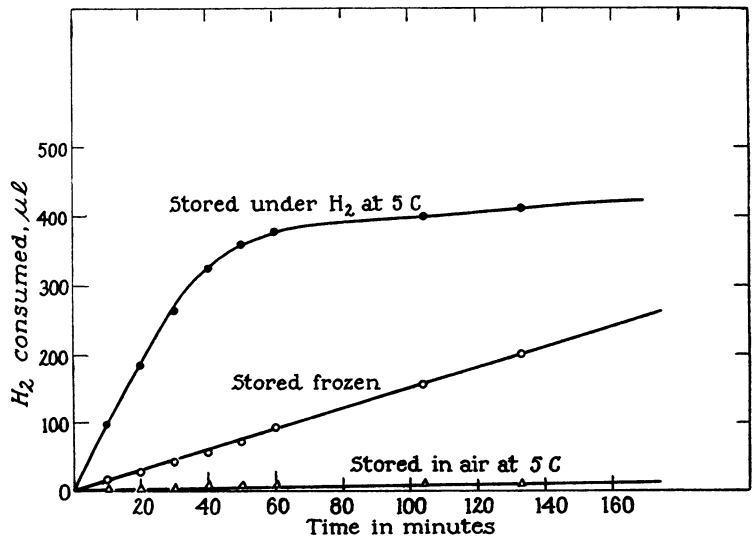

Figure 2. Activity of cell-free *Rhodospirillum rubrum* hydrogenase after storage under three different conditions. The curves represent the hydrogenase activity (at 37 C and pH 7) observed on the 15th day of storage under the conditions noted.

at 5 C. As is evident from the lower curves, considerable activity is lost if the preparation is maintained at 5 C in contact with air or in the frozen state. In view of the stability of the enzyme under H_2 at 5 C, all preparations of *Rhodospirillum* hydrogenase and *Escherichia* hydrogenase were stored in this manner.

It has been recently suggested by Joklik (1950*a*) that the inactivation of *Escherichia* hydrogenase by oxygen is due to oxidation of essential sulfhydryl groups. This view was based on the observations that the enzyme was inhibited

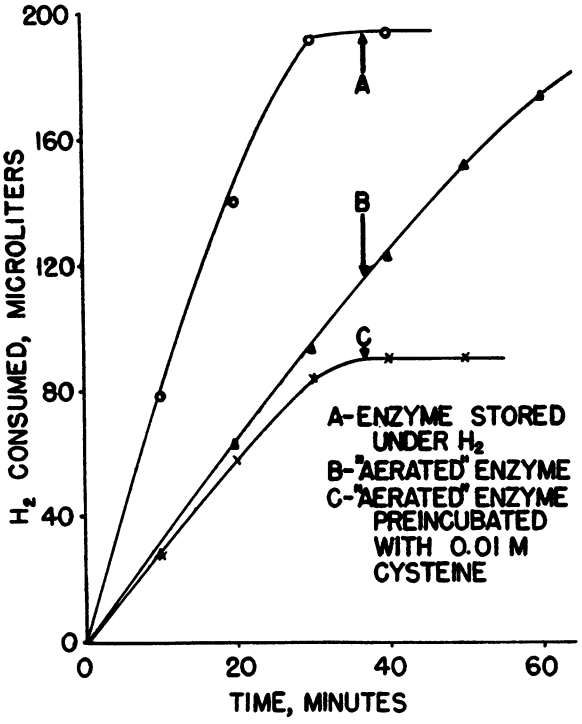

Figure 3. Attempted reactivation of "aerated" *Escherichia coli* hydrogenase by preincubation with cysteine. In each case, 120 μg of the enzyme preparation were assayed for activity in 0.125 M phosphate (pH 6.5) + 1.5 mg albumin per ml at 37 C. In C, the "aerated" enzyme was preincubated with approximately 0.01 M cysteine for 35 minutes (under H_2) before the methylene blue was tipped in.

by "sulfhydryl reagents" and that certain preparations could be activated by reducing agents such as cysteine, glutathione, etc. (Joklik, 1950*a*,*b*). Thus far we have been unable to demonstrate unambiguous reactivation by addition of such compounds. The results of an experiment designed to detect reactivation by cysteine of partially-inactivated *Escherichia* hydrogenase are shown in figure 3.

From curves A and B it is evident that considerable inactivation of *Escherichia* hydrogenase occurs during storage for 2 days in contact with air (at 5 C). Curve C illustrates typical results observed when the "aerated" enzyme is preincubated with cysteine (under H_2) for 35 minutes before addition of methylene blue.

There is no acceleration of H_2 uptake, and it is to be noted that the total amount of H_2 consumed is markedly smaller than that observed in the absence of cysteine. Addition of cysteine after methylene blue has been added to the reaction mixture does not cause reactivation, and the total H_2 uptake is again found to be decreased as compared with the controls. Diminution of the total H_2 consumption is also observed when other reducing agents such as glutathione, ascorbic acid, $FeSO_4$, or sodium hydrosulfite are added (also found with *Rhodospirillum* hydrogenase). In the presence of reducing substances, the cessation of H_2 uptake is coincident with decolorization of the methylene blue which indicates that poisoning of the enzyme is not the cause of the decreased total uptake. The diminished total uptake observed in these instances is undoubtedly due to nonenzymatic reduction of part of the methylene blue by the added reducing agents.

The failure to obtain a clearcut reactivation of "aerated" hydrogenase by cysteine, etc. suggests that the effect of O_2 cannot be attributed solely to oxidation of essential sulfhydryl groups on the enzyme. In this connection it is of interest to note that 10^{-3} M *p*-chloromercuribenzoate, which is considered to be one of the most specific inhibitors of —SH enzymes, does not appreciably inhibit *Rhodospirillum* hydrogenase in crude sonic extracts or purified *Escherichia* hydrogenase.

Partial reactivation of "aerated" *Rhodospirillum* hydrogenase has been obtained by storing preparations under H_2 at 5 C for several days. A similar reactivation of *Escherichia* hydrogenase, presumably oxidized during preparation of the enzyme, was reported by Back *et al.* (1946).

Attempts to demonstrate a cofactor in hydrogenase activity. The observations described hereafter indicate that an easily dissociable cofactor is not involved in hydrogenase activity (with methylene blue as acceptor):

(1) Anaerobic dialysis of *Rhodospirillum* hydrogenase against oxygen-free water (saturated with H_2) for periods as long as 18 hours does not cause appreciable loss of activity. Preparations dialyzed for this length of time frequently show an unexplained slight lag in H_2 uptake; this lag is usually of the order of 10 minutes. Addition of boiled juices or triphosphopyridine nucleotide to extracts dialyzed for 5 hours did not accelerate H_2 consumption.

(2) Attempts to resolve *Escherichia* hydrogenase by precipitation of the apoenzyme with HCl—$(NH_4)_2SO_4$ according to the procedure of Warburg and Christian (1938) were not successful. Some inactivation occurred as a result of this treatment, and it could not be reversed by addition of supplements such as boiled juices and yeast extract.

(3) Exposure of *Escherichia* hydrogenase to the action of trypsin or alkaline phosphatase (which have been reported to be effective for resolution of certain other enzymes) under appropriate conditions did not cause any diminution in hydrogenase activity as compared with controls incubated in the absence of these enzymes.

(4) Metal complexing agents such as 1,10 phenanthroline, α,α'-dipyridyl, and diethyldithiocarbamate in concentrations up to 0.01 M do not inhibit *Escherichia* hydrogenase or *Rhodospirillum* hydrogenase appreciably even after pro-

longed contact. This indicates that heavy metal cations do not participate in hydrogenase activity.

(5) Attempts to demonstrate reduction of diphosphopyridine nucleotide by *Escherichia* hydrogenase in the presence of H_2 in the Beckman spectrophotometer have given negative results. Diphosphopyridine nucleotide in substrate quantities did not serve as an acceptor for H_2 in the presence of *Rhodospirillum* hydrogenase; similar results for *Escherichia* hydrogenase were reported by Joklik (1950*a*). We have also attempted to couple hydrogenase with (a) the diphosphopyridine nucleotide enzyme, formic dehydrogenase (prepared from peas)—no H_2 was evolved from reaction mixtures containing formate, diphosphopyridine nucleotide, and the two enzymes under an atmosphere of helium, and with (b) the triphosphopyridine nucleotide Zwischenferment enzyme system—with similar results.

H_2 utilization with acceptors other than methylene blue. It was previously reported (Gest, 1950, 1951) that intact cells of *R. rubrum* rapidly oxidize H_2 in the dark with $K_3Fe(CN)_6$ as the acceptor. This reaction can be readily demonstrated by suspending cells in a solution of 0.2 M phosphate pH 6.8 which is 0.1 M with respect to $K_3Fe(CN)_6$; KOH must be placed in the center well to absorb the relatively large amount of CO_2 produced from endogenous substrates when $K_3Fe(CN)_6$ is present. The ability of the latter compound to act as an acceptor appears to explain satisfactorily the observation that photoevolution of H_2 from malate or pyruvate does not occur when $K_3Fe(CN)_6$ is added (Gest, 1950).[2]

Intact cells of *E. coli* also can use $K_3Fe(CN)_6$ as an acceptor for oxidation of H_2. In contrast to the intact cells, cell-free *Escherichia* hydrogenase and *Rhodospirillum* hydrogenase have not been found to consistently oxidize H_2 in the presence of $K_3Fe(CN)_6$. A slight H_2 uptake is usually noted just after $K_3Fe(CN)_6$ is added, but the total amount consumed is far below the theoretically expected quantity. When present at a concentration of 0.01 M, $K_3Fe(CN)_6$ inhibits the consumption of H_2 with methylene blue (0.001 M $K_3Fe(CN)_6$ may cause a slight lag but does not affect the subsequent rate as compared with the controls). If reducing agents such as cysteine (0.02 M) are added to the inhibited system, immediate consumption of H_2 is observed, but the total quantity utilized is again well below the expected amount. Interpretation of these inhibition experiments is difficult because of the occurrence of nonenzymatic interaction between $K_3Fe(CN)_6$ and methylene blue; nonenzymatic reduction of ferricyanide to ferrocyanide by the added reducing agent can account for the "reactivation" observed. In view of the sensitivity of *Escherichia* hydrogenase and *Rhodospirillum* hydrogenase in the cell-free state to oxidizing agents, one would expect that $K_3Fe(CN)_6$ could not be a satisfactory acceptor. Cell-free *Azotobacter* hydrogenase appears to differ from *Escherichia* hydrogenase (and *Rhodospirillum* hydrogenase) in that $K_3Fe(CN)_6$ can act as an acceptor for the former enzyme

[2] We have found that intense illumination has no effect on hydrogenase activity of intact cells or extracts of *R. rubrum* using $K_3Fe(CN)_6$ or methylene blue as the respective acceptors; this result supports the notion that the activation of H_2 as a hydrogen donor in photosynthetic reactions and photoevolution of H_2 is not directly light-dependent.

(Hyndman and Wilson, 1951). The explanation for this difference is not apparent at the present time.

The ability of various other substances to act as acceptors for *Rhodospirillum* hydrogenase has been tested. Negative results were observed with fumarate, pyruvate, diphosphopyridine nucleotide (see before), $SO_4^=$, NO_2^-, and riboflavin. Intact cells of *Rhodospirillum* also do not reduce fumarate, malate, or diphosphopyridine nucleotide with H_2. Joklik (1950*a*) has reported that partially purified *Escherichia* hydrogenase reduces only dyes of the type of methylene blue.

Spectroscopic observations. Concentrated "solutions" of the 50 per cent $(NH_4)_2SO_4$ fraction from *E. coli* have been visually examined for absorption bands characteristic of porphyrin proteins with a Hilger angular spectroscope. A weak band can be observed in such preparations at approximately 560 mμ after incubation with H_2 or reduction by sodium hydrosulfite. Incubation under helium does not cause appearance of the band. The band decreases in intensity in the presence of air and can be restored again by incubation with H_2. The same phenomena can also be demonstrated in thick suspensions of intact cells. Although it is possible that the prosthetic group of hydrogenase itself is a porphyrin, these observations can also be interpreted on the basis that other known porphyrin enzymes may act as *carriers* in the utilization of H_2.

DISCUSSION

Inhibitor studies with O_2, CO, and KCN using intact bacteria have led to the suggestion that hydrogenase is an iron porphyrin protein which is active only in the reduced state. The results reported with CO and KCN are still somewhat contradictory. The "Knallgas" reaction, $H_2 + \frac{1}{2}O_2 \rightarrow H_2O$, in *Azotobacter* (Wilson and Wilson, 1943) and the reduction of methylene blue by *E. coli* (Lascelles and Still, 1946*a*) are inhibited by CO, but the inhibitions could not be reversed by light. Catalysis of the exchange reaction between water and H_2 by the hydrogenase of *Proteus vulgaris* is also inhibited by CO, and in this instance a partial reversal by light has been reported, thus indicating participation of an iron porphyrin enzyme (Hoberman and Rittenberg, 1943). One of the enzymes involved in H_2 evolution from sugars by *Clostridium butyricum*, presumably hydrogenase, is sensitive to CO, and the inhibition has been shown to be reversed by high light intensities; the "absorption spectrum" of the hydrogen-evolving enzyme in intact cells as determined by photochemical means, however, suggests an iron enzyme which does not appear to be a typical iron porphyrin (Kempner and Kubowitz, 1933). Experiments with the cell-free hydrogenase of *E. coli* by Joklik (1950*b*) showed that the reduction of methylene blue in the partially purified system is also CO sensitive, but attempts to reverse the inhibition by high light intensities were unsuccessful.

Similar ambiguities have been found with regard to cyanide inhibition. The "Knallgas" reaction and the reduction of methylene blue and certain other acceptors in various intact organisms are markedly inhibited by cyanide (Wilson and Wilson, 1943; Lascelles and Still, 1946*a*,*b*). In contrast to these cases the "Knallgas" reaction in *Lactobacillus delbrueckii*, which apparently metabolizes

mainly by means of flavin enzymes, is not sensitive to cyanide (Yamagata and Nakamura, 1938). Recent studies with the cell-free hydrogenase of *E. coli* have shown that the reduction of methylene blue in this system is also not markedly suppressed by cyanide (Joklik, 1950*b*).

Many of the foregoing observations can be adequately rationalized if it is assumed that the oxidation of H_2 by bacteria involves a complex system of the type proposed by Yamagata and Nakamura (1938), viz.

$$\begin{array}{ccccc} H_2 & \longrightarrow & \text{Intermediary carriers} & \rightarrow & \text{Acceptor} \\ \uparrow & & & & \uparrow \\ \text{Hydrogenase} & & & & \text{Specific} \\ (+ \text{ other enzymes?}) & & & & \text{"acceptor" enzymes} \end{array}$$

Thus depending on the nature of the acceptor, and consequently on the specific enzymes concerned with the acceptor, the utilization of H_2 in a particular case may or may not be inhibited by agents such as CO and KCN. Inhibition would be expected when the "acceptor-enzymes" are typical iron porphyrins; for example, in *E. coli* when the acceptor is O_2 or nitrate.[3] The participation of porphyrin carriers in certain types of hydrogenase activity in *E. coli* therefore appears likely. This view is supported to some extent by the spectroscopic observations previously described and by the dramatic effect of iron nutrition on the level of hydrogenase activity in intact cells (Waring and Werkman, 1944).

The fact that hydrogenase activity in intact cells is inhibited by incubation with oxygen and can be reactivated by reducing agents has also been used in support of the hypothesis that the enzyme itself possesses an iron porphyrin prosthetic group. It is evident that these effects may well be indirect; in order to determine the nature of the hydrogenase prosthetic group conclusively, further purification of the enzyme is required. The suggestion that oxygen inhibition is due to oxidation of essential sulfhydryl groups (Joklik, 1950*a*) of the enzyme is not supported by the present studies. In this connection, it should be noted that Joklik found "reactivation" of the cell-free enzyme by reducing agents to be greater under H_2 than under N_2, and he concludes that "it has not yet been definitely determined whether H_2 is necessary for the action of —SH reagents or not."

In considering the apparent contradictions between effects of inhibitors on intact cells and cell-free systems, it is evident that corresponding results would not necessarily be expected. Observation of differences in this respect obviously does not justify the conclusion that the inherent properties of hydrogenase are significantly altered by extraction and purification procedures as has been suggested by Joklik (1950*b*).

The present results indicate that the cell-free hydrogenases (defined on the basis of H_2 utilization with methylene blue) of *E. coli* and *R. rubrum* are very similar in all respects. Both of these organisms, as grown in the present instance, *produce* H_2 as a major metabolic product. There is little question that hydro-

[3] The nitrate reductase of *E. coli* appears to be an iron porphyrin enzyme of the cytochrome b type (Sato and Egami, 1949).

genase is essential in the enzyme complex responsible for H_2 formation in these organisms. Thus far there is no information available on the nature of the carriers involved, and it is conceivable that they may be different from those participating in reduction of various terminal acceptors by H_2. Although direct or indirect reduction of known dissociable coenzymes as a result of H_2 oxidation has not yet been demonstrated, it seems reasonable to presume that such reactions occur since numerous organisms are capable of fulfilling all their necessary metabolic requirements with H_2 as the primary hydrogen donor.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Drs. L. O. Krampitz, J. O Lampen, and S. J. Cooperstein for their interest and advice during this investigation. The technical assistance of Mrs. M. A. Lasoski and Mrs. S. J. Corn is gratefully acknowledged.

SUMMARY

Cell-free hydrogenases have been prepared from *Escherichia coli* and *Rhodospirillum rubrum* by a variety of methods and the enzyme partially purified. A survey of properties indicates that the enzymes from both sources are essentially similar. Attempts to demonstrate participation of a dissociable cofactor in the reaction: H_2 + methylene blue $\rightarrow$ leucomethylene blue, have given negative results. Evidence suggesting participation of iron porphyrin enzymes in hydrogenase activity has been obtained by visual spectroscopic observations on concentrated solutions of the enzyme from *E. coli* and on intact cells; these observations have disclosed an absorption band (at about 560 mμ) which is accentuated by incubation with H_2 and discharged by aeration. Some of the factors involved in assay of hydrogenase and in the lability of the enzyme with respect to oxygen have been studied; oxidation of essential sulfhydryl groups on the enzyme does not appear to explain the inactivation by air. The present results are discussed in relation to the mechanisms of various metabolic reactions involving H_2.

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