PURIFICATION AND PROPERTIES OF THE HYDROGENASE OF *DESULFOVIBRIO DESULFURICANS*

by

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The enzyme hydrogenase which catalyses the reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$ was discovered in *Bacterium formicum* by STEPHENSON AND STICKLAND[1] who observed that this organism could use molecular hydrogen to reduce a number of quite diverse substrates. The enzyme was later found in several autotrophic and heterotrophic bacteria[2–6] and in a few algae[7]. FARKAS, FARKAS AND YUDKIN[8] showed that the hydrogenase of *E.coli* was capable of catalysing the exchange reaction between D_2O and molecular hydrogen. This exchange reaction has been studied in detail by HOBERMAN AND RITTENBERG[9] and FARKAS AND FISCHER[3]. Hydrogenase appears to be involved in all biological reactions which consume, and probably also liberate, hydrogen.

The study of the properties of hydrogenase has been limited to relatively crude preparations. Cell-free extracts of the enzyme were prepared from *Escherichia coli*[10], *Rhodospirillum rubrum*[5], *Azotobacter vinelandii*[6,11] and *Hydrogenomonas facilis*[12]. Solubilisation of the enzyme was reported from a few organisms. The yield of the soluble enzyme and the specific activity of the purified enzyme $-Q_{H_2}$ (Methylene blue) = 30,000 μl per h per mg protein nitrogen) were usually low.

The present investigation was undertaken during the course of a study of the reduction of inorganic sulphate to hydrogen sulphide by the obligate anaerobe, *Desulfovibrio desulfuricans*. The presence of hydrogenase in sulfate-reducing bacteria was first reported by STEPHENSON AND STICKLAND[13] and later by WRIGHT AND STARKEY[14], BUTLIN *et al.*[15], POSTGATE[16], and SISLER AND ZOBELL[17]. These studies were made on suspensions of resting cells. Our preliminary studies indicated that *D. desulfuricans* (Hildenborough) was an unusually rich source of the enzyme. Extraction of acetone dried bacteria was found to give a hydrogenase which was both soluble and highly stable. Fractionation of the extract and isolation of hydrogenase of high specific activity [$-Q_{H_2}$ (methylene blue) = $2.5 \cdot 10^6$ μl per mg N] was briefly reported in an earlier communication (SANADA AND JAGANNATHAN[18]).

SHUG *et al.*[19] have recently made a notable contribution to the study of the hydrogenase of *Clostridium pasteurianum*. The enzyme was solubilized by sonic disruption of the cells and obtained in highly purified form [$-Q_{H_2}$ (methylene blue) = $7.5 \cdot 10^5$ μl per h per mg N] by protamine sulphate. The purified enzyme was shown to require flavin dinucleotide, molybdenum and inorganic phosphate for the reduction of cytochrome *c* but not of methylene blue.

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The purification and properties of hydrogenase from *D. desulfuricans* are described in the present communication. The purified enzyme has been shown to require ferrous or ferric salts for maximum activity.

MATERIALS AND METHODS

Desulfovibrio desulfuricans, strain "Hildenborough" No. NCIB 8303, kindly supplied by Dr. K. R. BUTLIN was used in these studies. The organism was maintained in BAARS[20] medium. The media used for growth contained: 0.5 g Na_2SO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.4 g sodium lactate, 0.08 g K_2HPO_4, 0.1 g yeast extract and 0.001 g $FeSO_4(NH_4)_2SO_4 \cdot 2H_2O$ in 100 ml distilled water with other additions as indicated below. For large scale culture, the medium was supplemented with 0.4% yeast extract and 0.5% peptone. The media were sterilised by autoclaving at 20 lbs per sq. inch for 30 minutes, the cysteine supplement of 1 to 1.5 μmole/ml used in subculturing being separately added after sterilisation through a bacterial filter. The final pH of the media was 7.4 to 7.6 and the temperature of incubation 34°.

The organism was initially sub-cultured 4 to 5 times on 15 ml lots of the above medium supplemented with 20 μmoles of cysteine and incubated each time for 48 h under H_2 in an anaerobic jar. The inoculum was transferred to ten volumes of the same medium (containing about 200 μmoles cysteine per 100 ml) and incubated for 48 h under H_2. The inoculum was now transferred to 10 to 15 volumes of the large scale culture medium (containing no cysteine) in 5-liter flasks, which were filled to the top. After incubation for 72 h the cells were harvested by centrifugation at 0° in the angle head attachment of the International refrigerated centrifuge at 3,500 r.p.m. for one hour. The cells were suspended in cold 0.8% KCl and re-centrifuged. The washed cells from 10 liters of medium were thoroughly mixed with about 50 ml of 0.8% KCl at 0° to form a homogeneous suspension and added with stirring to 15 to 20 volumes of acetone at 0° to 5°. After 40 minutes the precipitate was rapidly filtered under suction and washed 3 to 4 times with ice-cold acetone and dried at 0° in a vacuum desiccator over P_2O_5 and paraffin shavings. The material took about one to two days to dry completely. The average yield was 25 to 30 g of wet bacterial cells giving 5 to 6 g dry material from 10 liters of medium. The bacteria contained the contaminant described by POSTGATE[21] but it has no hydrogenase activity. No attempt was made to separate it off. Whether this organism has any effect on solubilisation of hydrogenase or on its purification has not been determined.

Calcium phosphate gel was prepared according to KEILIN AND HARTREE[22] and was aged for nine months before use. The following substances were commercial samples: DPN*, 85% pure (Schwartz Laboratories); FAD, 80% pure; FMN (Sigma Chemical Co.); Crystalline bovine albumin (Armour & Co.); protamine sulphate (Light & Co.); benzyl viologen (Bios Laboratories); methylene blue (National Aniline); yeast extract and peptone (Oxo Ltd.).

Protein was determined by the turbidimetric method of STADTMAN *et al.*[23] and by the micro-kjeldahl method.

All optical measurements were carried out in a Beckman model DU spectrophotometer in 3 ml cuvettes or in Thunberg tubes (1 cm light path). Manometric experiments were carried out in the conventional Warburg apparatus and pH measurements were made with the glass electrode. Glass-distilled water was used in all experiments.

Enzyme assay

Enzyme activity was determined manometrically by measuring hydrogen uptake at 34° with methylene blue as hydrogen acceptor. The main compartment of a twin-armed Warburg vessel contained, except when otherwise indicated, 160 μM TRIS buffer, 20 μM cysteine, 1.5 μM freshly prepared $FeCl_2$, 2 mg crystalline bovine albumin** and enzyme in a final volume of 1.5 ml, pH 8.4. One side-arm contained 10 μM methylene blue in 0.2 ml H_2O. Alkaline pyrogallol was placed in the centre-well (containing a filter paper strip) and in the second side-arm. After filling the flasks with hydrogen and equilibrating in the bath for 15 minutes methylene blue was rapidly tipped in and measurements taken every minute for five minutes. The rate of hydrogen uptake remained linear for the first three minutes and then gradually decreased, possibly due to inacti-

* The following abbreviations have been used in the text: Diphosphopyridine nucleotide (DPN); Triphosphopyridine nucleotide (TPN); Flavin mononucleotide (FMN); Flavin adenine dinucleotide (FAD); Tris(hydroxymethyl) aminomethane (TRIS); Ethylenediaminetetraacetic acid (Versene); N–N-dihydroxyethyl glycine (Versene-Fe-3-specific); *p*-chloromercuribenzoate (PCMB).

** The amount of serum albumin for the test was incorrectly reported in the previous publication (SADANA AND JAGANNATHAN[18]) as 0.2 mg instead of 2.0 mg.

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vation of the enzyme by methylene blue. Controls run without enzyme and without methylene blue respectively gave negligible blanks. All experiments were carried out in duplicate. The amount of enzyme taken for the test was such that the rate of hydrogen uptake was about 10 to 30 μl per minute. Enzyme activity was calculated from the mean of the first three one-minute readings and expressed as μl H_2 absorbed per hour under these experimental conditions. Specific activity was expressed in terms of $-Q_{H_2}$ (methylene blue) (μl per h per mg protein N).

RESULTS

Purification of hydrogenase

The following operations were carried out at 0° to 4° unless otherwise indicated. The acetone-dried bacterial cells were ground with frozen 0.2 *M* phosphate buffer, pH 6.4 (10 ml for every gram of dried bacteria) and left at room temperature for 20 to 30 minutes. Cellular debris was then removed by centrifugation at 15,000 r.p.m. in the high-speed attachment of the International centrifuge for one hour. The cell-free supernatant was reddish-brown and contained 70 to 80% of the hydrogenase activity of the dried bacteria.

The extract was heated in a water-bath in 20 ml aliquots with constant stirring so that the temperature of the enzyme solution rose to 60° in 2 minutes. After keeping for 10 minutes at 60°, it was rapidly cooled to 0° and centrifuged to remove the coagulated impurities.

The supernatant was adjusted to pH 5.0 by dropwise addition of 0.1 *N* acetic acid and centrifuged after an hour. The inactive precipitate was discarded and the supernatant re-heated in a water-bath so that the temperature rose to 50° in 1.5 minutes. After maintaining at 50° for 2 minutes, the enzyme was cooled to 0° and the denatured proteins centrifuged off. A third inactive precipitate was removed by adding to the solution with stirring half its volume of acetone at —10° and centrifuging after 30 minutes. Hydrogenase was precipitated from the supernatant by the addition of 2.3 times its volume of acetone and collected by centrifugation after keeping for 60 minutes at —10°. The precipitate was dried overnight at 0° *in vacuo* over P_2O_5 and paraffin shavings. It was then dissolved in 0.1 *M* phosphate buffer, pH 6.0, to give an approximately 0.1% solution of protein.

Protamine sulfate (1% solution adjusted to pH 5.0 with dilute acetic acid) was then added to the enzyme with stirring till no further precipitate was formed. The precipitate was centrifuged off and discarded. Calcium phosphate gel was then added to the enzyme solution (about 1 ml of gel containing 10 mg dry weight for 1 ml enzyme), stirred for 10 minutes and then centrifuged. The gel, which contained all the hydrogenase, was washed four to five times with water and then eluted with 0.2 *M* phosphate buffer, pH 7.8 (0.5 ml of buffer for 1 ml of initial enzyme solution before the addition of gel). The elution was then repeated twice in a similar manner. The combined eluates contained hydrogenase of maximum purity. Protamine treatment and gel adsorption increased the ratio of light absorption at 280 mμ to that at 260 mμ from about 0.35 to 1.00 or more, indicating removal of nucleic acid and other coloured impurities.

The results of a typical experiment with the above procedure are summarised in Table I. The purification was fairly reproducible and the specific activity of the purified enzyme obtained from different batches of bacteria varied from $2.2 \cdot 10^6$ to $2.6 \cdot 10^6$. The final product with a fifty-fold increase in activity is the purest specimen

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of hydrogenase obtained hitherto. The amount of enzyme available was however insufficient to attempt further purification or to carry out detailed studies on homogeneity.

TABLE I

PURIFICATION OF *D. desulfuricans* HYDROGENASE

5 g acetone-dried bacterial cells

Enzyme preparation	*Volume of solution ml*	*Total activity (μl H_2 absorbed per hour)*	*Total protein mg*	*Specific activity μl H_2 per h per mg N*	*Yield %*
1. Non-proliferating washed bacterial cells	—	15 to $20 \cdot 10^6$	—		—
2. Acetone-dried cells	50	$12.5 \cdot 10^6$	1800	$4.2 \cdot 10^4$	100
3. Phosphate extract, centrifuged at 18,000 *g* for 1 h	40	$11.7 \cdot 10^6$	347	$20 \cdot 10^4$	94
4. Extract (3) heated at 60° for 10 minutes	45	$11.2 \cdot 10^6$	216	$31 \cdot 10^4$	89
5. pH 5.0 supernatant reheated at 50° for 2 minutes	90	$9.3 \cdot 10^6$	63	$92 \cdot 10^4$	74
6. Acetone precipitate	50	$8.9 \cdot 10^6$	45	$118 \cdot 10^4$	71
7. Eluate from gel	100	$8.3 \cdot 10^6$	20	$235 \cdot 10^4$	66

A different procedure, which was described earlier (SADANA AND JAGANNATHAN[18]) was found to give enzyme of the same activity. The supernatant obtained after precipitation of impurities at pH 5.0 was brought to about pH 4.0 with dilute acetic acid and centrifuged. The precipitate containing hydrogenase was dissolved in 0.1 *M* TRIS, final pH 5.0, and heated at 50° for 2 minutes. The precipitate was centrifuged off and discarded and the supernatant treated with protamine sulfate and calcium phosphate gel as described above to remove nucleic acid and coloured impurities. This method had the disadvantage that the pH at which hydrogenase was completely precipitated varied with different extracts. The purified enzyme was also less suitable for the activation studies described below, since some of the preparations obtained by this procedure failed to show activation by $FeCl_2$, which is discussed in a later section.

Absorption spectrum

The absorption spectrum of purified hydrogenase with oxidising and reducing agents is shown in Fig. 1. In air the enzyme showed an absorption maximum at 409 mμ. On reduction with hydrogen, an absorption band appeared between 500 to 580 mμ with a peak at 555 mμ and the intense γ- or Soret band at 409 mμ was shifted to 419 mμ. On shaking with air for a few seconds, the spectrum reverted to that of the oxidised state with the disappearance of the 555 mμ band and the shift of the band at 419 mμ to 409 mμ. Similar results were obtained when reduced hydrogenase was oxidised by FMN or methylene blue (Fig. 1). In the presence of excess H_2, the spectrum of reduced hydrogenase reappeared when all the FMN or dye had been reduced. The absorption bands of the oxidised and reduced enzyme are characteristic of iron-porphyrin respiratory enzymes and resemble those of cytochrome *c*.

Hydrogenase also showed a decrease in absorption at 450 mμ and 390 mμ on reduction, which was reversed on oxidation. The difference in absorption of the

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oxidised and reduced enzyme at these two wavelengths suggests the presence of flavin in hydrogenase, as has been previously indicated by SHUG *et al.*[19] on the basis of similar observations with the hydrogenase of *Cl. pasteurianum*. We have, however, been unable to confirm their observation that the absorption spectrum of the reduced enzyme reverted to that of the oxidised state when H_2 was evacuated from the tube, indicating reversibility of flavin reduction at low H_2 tensions. Prolonged incubation of reduced enzyme from *D. desulfuricans* at room temperature after evacuation of H_2 failed to show any change in absorption.

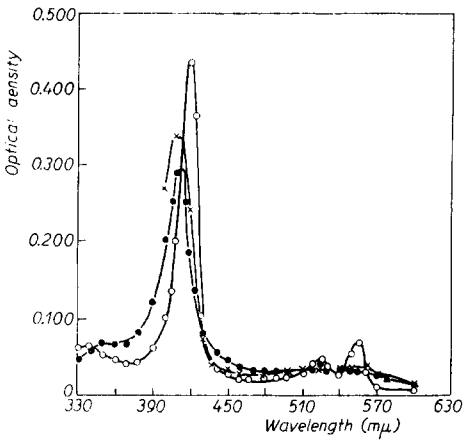

Fig. 1. Absorption spectrum of purified hydrogenase. ●—● A. Enzyme in 0.10 *M* TRIS, 0.007 *M* cysteine, pH 7.4; in air. O—O B. As in A, after 24 hours incubation in H_2 at 0°. ×—× C. H_2 evacuated from B and 0.2 μM FMN added; readings corrected for absorption of FMN.

It is uncertain whether the observed absorption changes characteristic of a cytochrome *c*-like component are due to hydrogenase or to an associated impurity. During the purification of hydrogenase two other "cytochrome" pigments were obtained, which had no hydrogenase activity but showed absorption in the oxidised and reduced states which was identical with that of purified hydrogenase. One pigment was isolated by precipitation at pH 2.8 from the supernatant obtained after removal of hydrogenase at pH 4.0 according to the second procedure for purification. This pigment was reduced by cysteine anaerobically and was reoxidised by air. Since it was reduced by cysteine, which is necessary for hydrogenase activity, it was not possible to determine whether this pigment could be reduced by H_2 and hydrogenase and we have not studied its properties further. The second pigment was present in the supernatant obtained after adsorption of hydrogenase by gel. This pigment was not reduced by cysteine and H_2, but was reduced when it was incubated with cysteine, H_2 and a small amount of purified hydrogenase, and was re-oxidised by O_2, FMN or methylene blue. The pigment was thermostable, since its capacity for reduction by H_2 and hydrogenase and re-oxidation by air *etc.* was unchanged by heating at 80° for 5 minutes, which destroyed hydrogenase activity completely. Since its absorption spectrum was identical with that of hydrogenase in the oxidised and reduced states respectively, it is possible that the absorption curves shown in Fig. 1 are due partly or wholly to a residual impurity of this "cytochrome" pigment. The ratio of hydrogenase activity ($\mu l H_2$ oxidised per hour) to the optical density of the enzyme at 409 mμ (1 cm cell, in air) varied considerably for different enzyme preparations of the same specific activity. The initial extract had a ratio of about 42,000 while the purified enzyme had a ratio varying from 80,000 to 182,000 indicating the presence of a coloured impurity in some of the final preparations. We have not attempted further purification of the enzyme and it remains to be determined whether hydrogenase can be obtained entirely free of "cytochrome" pigment.

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General properties

Hydrogenase solutions could be kept at 0° for a week or at —20° for several months with no loss in activity. The enzyme was unaffected by repeated freezing and thawing or lyophilisation. Unlike the hydrogenase of several other bacteria, which required storage under H_2 or anaerobically [5, 24], our preparations retained their activity even when kept in contact with air for long periods. If any inactivation of the enzyme occurs during storage, the short incubation with cysteine and H_2 during the test is probably sufficient for reversing the inhibition. The enzyme could be dialysed at 0° for three days against neutral phosphate or TRIS at pH 6.3 to 7.4 with no significant loss in activity. The enzyme was not sedimented by centrifugation for one hour at 80,000 *g*. Hydrogenase was completely inactivated at pH 2.0 in a few minutes at 0° or at pH 6.0 in 5 minutes at 70° or in 1 minute at 80°. It was however unaffected by heating for 10 minutes at 60° at pH 6.3 to 7.8. The enzyme was also stable when heated for 2 minutes at pH 5.0.

Purified hydrogenase was analysed for lipoic acid by Mr. W. E. RAZZELL through the kind courtesy of Dr. I. C. GUNSALUS and was found to contain 11.0 millimicrograms of lipoic acid per mg of protein. If the molecular weight of hydrogenase is assumed to be 100,000, it should contain at least 2 micrograms of lipoic acid per mg protein. Unless the enzyme is still grossly impure and requires a further 200-fold purification, it may be concluded that hydrogenase is not a lipoic acid enzyme. Crude extracts contained FAD and diaphorase but on purification hydrogenase contained no diaphorase.

After dialysis for 72 hours at 0° against TRIS buffer, pH 7.4, hydrogenase contained one μg of iron per mg of protein. But since the enzyme was partially resolved into metal and protein, as shown by activation with $FeCl_2$, and contained moreover "cytochrome" impurities it is uncertain what fraction of the total iron was in combination with hydrogenase.

Hydrogen acceptors

The following substances were reduced by hydrogen and hydrogenase: methylene blue and benzyl viologen, FAD, FMN and riboflavin, and the "cytochrome" of *D. desulfuricans*. The rate of hydrogen uptake was highest with methylene blue and benzyl viologen and several different enzyme preparations showed identical activities with equivalent concentrations of the two dyes. The addition of DPN, TPN, FMN, FAD, lipoic acid, lipothiamide, coenzyme A or co-carboxylase had no effect on enzyme activity with methylene blue. The rate of H_2 uptake was the same when the amount of methylene blue was varied between 5 to 20 μmoles.

Hydrogen uptake with FMN was $2.2 \cdot 10^4$ μl per h per mg protein N, which is about one-hundredth of the rate with methylene blue. The reduction of FMN, FAD and riboflavin was also measured spectrophotometrically at 450 mμ in Thunberg tubes containing 0.2 μM flavin (side-arm), 20 μM cysteine, 100 μM TRIS, and enzyme in a final volume of 3.0 ml at pH 7.5. After incubation with H_2 for a few hours, the flavin was tipped in and the decrease in optical density at 450 mμ was determined. The rate of reduction of the three flavins was nearly the same, but the optimum rate could not be determined owing to the high optical density of the solution at higher flavin concentrations. It was necessary to reduce the enzyme initially with H_2 before

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the addition of flavin to obtain maximum rates of reduction, possibly due to the presence of traces of O_2.

Sulfate, ferricyanide, DPN and TPN were not reduced by the enzyme. Cytochrome *c* reduction could not be studied owing to the reduction of cytochrome *c* by cysteine, which was necessary for hydrogenase activity. The reduction of the "cytochrome" of *D. desulfuricans* has been described in a previous section.

pH optimum

Hydrogenase showed a broad pH optimum between 7.4 and 8.6 with a maximum at 8.4 (Fig. 2). The enzyme was inactive below pH 5.0 or above pH 10.0. Hydrogenase activity was lower with phosphate than with TRIS at corresponding pH values.

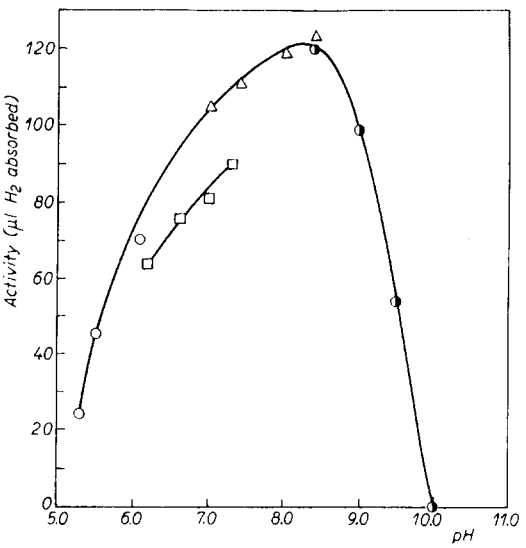

Fig. 2. pH activity curve of hydrogenase. (Activity expressed as μl H_2 absorbed in 3 minutes in the standard assay system). O—O pH 5.3 to 6.1 in acetate buffer, 0.01 *M*; □—□ pH 6.2 to 7.3 in phosphate buffer, 0.01 *M*; △—△ pH 7.0 to 8.6 in TRIS-HCl buffer, 0.1 *M*; ◑—◑ pH 8.4 to 10.0 in glycine-NaOH buffer, 0.1 *M*.

Activators

Hydrogenase was inactive in the absence of cysteine, even when it was incubated with H_2 for several hours. The activity was maximum when cysteine concentration was between 0.002 *M* and 0.02 *M*. Cysteine could be replaced by glutathione. Sodium sulfide and hydrosulfite were found to activate crude enzyme preparations, but gave somewhat lower activities than cysteine and were not routinely used.

In the absence of serum albumin, enzyme activity was not proportional to enzyme concentration and fell off rapidly at high dilutions. This was particularly noticeable with enzyme of maximum purity, which showed little or no activity at high dilutions in the absence of serum albumin. Erratic results were also obtained with purified enzyme when alkaline pyrogallol was omitted from the Warburg flasks, since traces of oxygen were inhibitory. In the presence of cysteine, serum albumin and pyrogallol, a linear relation was obtained between enzyme concentration and activity.

Several samples of purified hydrogenase obtained by the second purification procedure described previously showed little or no activation by $FeCl_2$ while the enzyme obtained by the procedure outlined in Table I showed an increase in activity of 30 to 80% when tested with 10^{-3} *M* ferrous or ferric chloride. Of several methods tried for obtaining greater activation by $FeCl_2$ the following gave the best results. Purified hydrogenase was adjusted to pH 4.5 and left at 0° for 24 hours. It was then precipitated with four volumes of acetone at 0° and dried *in vacuo*. Adsorption on calcium phosphate gel and elution with phosphate buffer were repeated as described previously. The recovery of activity (when tested with $FeCl_2$) was quantitative, but

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the activation by $FeCl_2$ increased from 70 to 250%. The same increase in activation was also observed when benzyl viologen was used in place of methylene blue. $FeCl_2$ could be replaced by $FeCl_3$, but no activation was observed with varying concentrations of $CoCl_2$, $CoCl_3$, MoO_3, ammonium molybdate or $CuCl_2$.

The optimum concentration of $FeCl_2$ was 1.5 μM in a total volume of 1.5 ml (Table II) though the actual concentration of Fe^{++} was much lower owing to the presence of excess of cysteine. No hydrogen uptake was observed with $FeCl_2$ or $FeCl_3$ in the absence of methylene blue. Cysteine forms a complex with the added metal and prevents precipitation of the metallic hydroxide. It is improbable that activation by $FeCl_2$ could be due to the action of this ferro-cysteine complex as an intermediate in methylene blue reduction, since different enzyme preparations with the same activity in the absence of $FeCl_2$ showed activation varying between 20 to 200% with $FeCl_2$. Activation of the enzyme due to removal of traces of oxygen by ferro-cysteine is also unlikely since prolonged shaking of the flasks for one or two hours to remove O_2 by cysteine and alkaline pyrogallol resulted in no increase in activity in the absence of $FeCl_2$. In a few experiments it was noted that the initially higher rate with $FeCl_2$ decreased after two to three minutes to the rate observed without activating metal, the reason for which is obscure.

TABLE II

EFFECT OF FERROUS CHLORIDE CONCENTRATION ON HYDROGENASE ACTIVITY

Hydrogenase preparation	*$FeCl_2$ added µmoles*	*Hydrogenase activity (µl H_2 absorbed per hour)*	*Per cent activation*
I	—	2040	. . .
	0.8	3900	91
	1.6	4440	117
	2.4	4440	117
II	—	1040	—
	0.4	1580	51
	0.8	3000	188
	1.2	3300	227
	2.0	3250	212

Inhibitors

The effect of inhibitors on hydrogenase was determined with the usual test system, unless otherwise indicated in the tables, and the inhibitor was added after pre-incubation of the enzyme with cysteine and H_2 to maintain it in the reduced rate. In some experiments the inhibitor was incubated in air at 34° for 15 minutes with the buffered enzyme solution containing serum albumin but no cysteine, so as to maintain the enzyme in the oxidised state, and the activity was then determined as usual after the addition of cysteine. When a lower pH was used for some inhibitors, as shown in the tables, the controls were also run at the same pH without the inhibitor. The results have been corrected for the blanks, if any, in the absence of enzyme. When cyanide was used, the centre-well of the flasks contained pyrogallol-alkali-cyanide mixture in which the concentration of free HCN was equal to that in the reaction mixture in order to prevent distillation of HCN into the alkali (KREBS[25]).

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Sulfhydryl reagents

The effect of iodoacetate and PCMB, which combine with free SH groups of proteins, was tested with different cysteine concentrations (Table III). Inhibition by iodoacetate was low and at a concentration of $4 \cdot 10^{-2} M$ was only 17% even with the lowest permissible concentration ($2 \cdot 10^{-3} M$) of cysteine. PCMB was however strongly inhibitory and completely suppressed hydrogenase activity when the concentration of cysteine and inhibitor were nearly equal. The inhibition was reversible by the addition of excess cysteine or glutathione. The effect of the inhibitor was the same whether the enzyme was pre-incubated with PCMB aerobically or in the presence of cysteine and H_2. GEST[5] reported that $10^{-3} M$ PCMB does not appreciably inhibit crude preparations of hydrogenase from *R. rubrum* or *E. coli*. Our results indicate the presence of sulfhydryl groups in hydrogenase which are essential for enzymic activity.

TABLE III

EFFECT OF IODOACETATE AND *p*-CHLOROMERCURIBENZOATE ON HYDROGENASE ACTIVITY

Inhibitor	*Inhibitor concentration M*	*Reducing agent M*	*Reducing agent concentration M*	*Inhibition*	
				Inhibitor mixed in air %	*Inhibitor mixed under hydrogen* %
Iodoacetate	$3 \cdot 10^{-2}$	cysteine	$2 \cdot 10^{-3}$	12	13
	$4 \cdot 10^{-2}$	cysteine	$2 \cdot 10^{-3}$		17
	$3 \cdot 10^{-2}$	cysteine	$14 \cdot 10^{-3}$		8
p-Chloro-mercuribenzoate	$5 \cdot 10^{-4}$	cysteine	$2 \cdot 10^{-3}$		48
	$1.2 \cdot 10^{-3}$	cysteine	$2 \cdot 10^{-3}$	91	90
	$2 \cdot 10^{-3}$	cysteine	$2 \cdot 10^{-3}$	98	100
	$1.2 \cdot 10^{-3}$	cysteine	$28 \cdot 10^{-3}$		12
	$1.2 \cdot 10^{-3}$	glutathione	$28 \cdot 10^{-3}$		8

Metal complexing agents

Hydrogenase was inhibited by several metal-binding agents of which cyanide was the most active (Table IV). At about $10^{-3} M$ cyanide the enzyme was completely inhibited. Significant inhibition was also observed with α,α'-dipyridyl, *O*-phenanthroline and 8-hydroxyquinoline. "Versene", "Versene-Fe-3-specific" and azide showed only slight inhibition at much higher concentrations, while pyrophosphate had no inhibitory activity. Several workers have noted that reduced hydrogenase was inhibited less than the oxidised enzyme by cyanide or PCMB (FARKAS AND FISCHER[3]; HOBERMAN AND RITTENBERG[9]; GREEN AND WILSON[6]; HYNDMAN *et al.*[11]; ATKINSON AND MCFADDEN[12]; LASCELLES *et al.*[26]), while JOKLIK[27] was unable to confirm this observation. We have been unable to find any significant difference in inhibition by cyanide and PCMB, whether the inhibitor was mixed with hydrogenase aerobically or under reducing conditions. The discrepancy between the findings of the different workers is possibly due to differences in the purity and source of the enzyme preparations which were used.

Miscellaneous inhibitors

Among several other substances which were tested, hydroxylamine, nitrite and

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TABLE IV

EFFECT OF METAL BINDING AGENTS ON HYDROGENASE ACTIVITY

Inhibitor	*Inhibitor concentration M*	*pH*	*Inhibition*	
			Inhibitor mixed in air %	*Inhibitor mixed under hydrogen* %
Cyanide	$2.5 \cdot 10^{-4}$	8.4	80	82
	$5 \cdot 10^{-4}$	8.4	97	95
	$1 \cdot 10^{-3}$	8.4	100	100
8-Hydroxyquinoline	$4.0 \cdot 10^{-3}$	8.4	11	
$\alpha\alpha'$-Dipyridyl	$3 \cdot 10^{-3}$	8.4	11–15	
	$5 \cdot 10^{-3}$	8.4	25	30
o-Phenanthroline	$3 \cdot 10^{-3}$	6.4	10–15	
	$5 \cdot 10^{-3}$	6.4	20–30	25–35
"Versene (regular)"	$6 \cdot 10^{-3}$	8.4	10	
"Versene-Fe-3-specific"	$4 \cdot 10^{-2}$	6.4	12	
Sodium azide	$1 \cdot 10^{-2}$	7.4	7	5
Pyrophosphate	$2 \cdot 10^{-2}$	8.4	0	0

copper salts were the only compounds which showed marked inhibition at low concentrations (Table V). Copper salts showed extremely high inhibition and completely suppressed hydrogenase activity at $5 \cdot 10^{-4} M$. Fluoride, urethan and arsenite showed negligible inhibition. Phosphate and sulfate were inhibitory at high concentrations ($0.1 M$), but we have not determined whether the effect was due to trace metal impurities.

TABLE V

EFFECT OF INHIBITORS ON HYDROGENASE ACTIVITY

Inhibitor	*Inhibitor concentration M*	*Inhibition*	
		Inhibitor mixed in air %	*Inhibitor mixed under hydrogen* %
Hydroxylamine	$3 \cdot 10^{-3}$	12	
	$5 \cdot 10^{-3}$	33	
	$2 \cdot 10^{-2}$	42	
	$4 \cdot 10^{-2}$	77	
Urethan	$1 \cdot 10^{-2}$	0	0
Arsenite	$5 \cdot 10^{-2}$	5	6
Potassium nitrite	$2 \cdot 10^{-3}$	40	
	$3 \cdot 10^{-3}$	92	
	$4 \cdot 10^{-3}$	100	
Sodium fluoride	$8 \cdot 10^{-2}$	5	
Cupric chloride	$5 \cdot 10^{-4}$	100	100

DISCUSSION

From studies using cell suspensions and crude extracts a certain amount of indirect evidence has already been obtained to indicate that hydrogenase is an iron-containing enzyme. WARING AND WERKMAN[28] found that hydrogenase activity was markedly reduced by iron deficiency in *Aerobacter indologenes*. Inhibition of hydrogenase by cyanide when the enzyme is in the oxidised state, by carbon monoxide (HOBERMAN AND RITTENBERG[9], HYNDMAN *et al.*[11]) and by nitric oxide (KRASNA AND RITTENBERG[29]) suggests the presence of iron as the prosthetic group in the enzyme.

The finding now described of the inhibition of purified hydrogenase by metal

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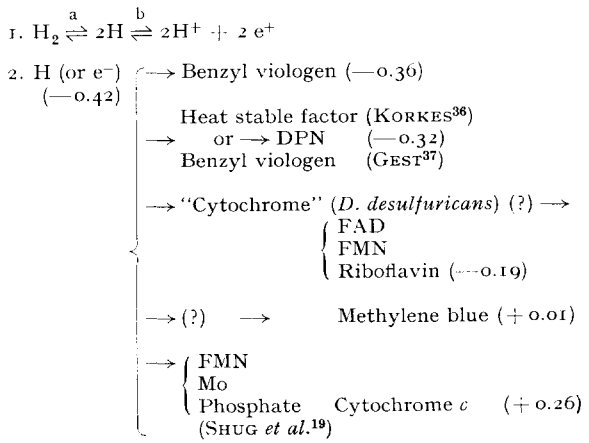
binding agents and the activation of the enzyme by $FeCl_2$ provides more direct evidence for this view. The activation was specific for iron salts and was observed only during the later stages of purification. The increase in activation by $FeCl_2$, which was observed when the enzyme was reprecipitated at pH 4.5 with acetone and adsorbed on gel, indicates partial splitting of metal from enzyme. The actual activation by $FeCl_2$ is likely to be higher than the observed value since the test system was not entirely free from iron (ca. 1 μg Fe). It is considered improbable that the effect of $FeCl_2$ is due to competitive reversal of inhibition by trace metals, such as copper, since no activation was observed with equivalent or higher amounts of other metals (Mo, Co *etc.*) or with different concentrations of metal-complexing agents (cysteine, ethylenediaminetetra-acetic acid).

Though there is considerable evidence for the presence of iron in hydrogenase, little can be said of the form in which it is present. From the photochemical reversal of CO inhibition of the hydrogenase of *P. vulgaris* HOBERMAN AND RITTENBERG[9] postulated the existence of an iron-porphyrin complex in analogy with Warburg's respiratory ferment. But other workers were unable to obtain reversal by light of CO inhibition of hydrogenase from other bacteria (HYNDMAN *et al.*[11], FARKAS AND FISCHER[3], LASCELLES AND STILL[26], JOKLIK[27], WILSON AND WILSON[30]). WARBURG[31] concluded that the prosthetic group of the hydrogen-liberating ferment of butyric acid bacteria, which is probably hydrogenase, contained iron but not haem. The presence of cytochrome-like pigments in *A. vinelandii* (HYNDMAN *et al.*[11]) and in sulfate-reducing bacteria (POSTGATE[32,33]; ISHIMOTO *et al.*[34]) has been previously reported. Though these pigments are undoubtedly important for electron-transport in these organisms, they are devoid of hydrogenase activity. To establish the nature of the iron in hydrogenase complete removal of these pigments becomes necessary.

The number of co-factors required for hydrogen uptake with hydrogenase is dependent on the oxidant used in the test system, and it is necessary to distinguish activation of hydrogenase from that of secondary transport systems which link hydrogenase with the terminal hydrogen acceptor. The co-factors required for the reduction of several different oxidants by H_2 and hydrogenase are indicated below. The approximate oxidation-reduction potentials (E_0' in volts at pH 7.0) of the respective hydrogen acceptors are shown in parenthesis (LARDY[35]).

It is not known at present whether hydrogenase catalyses both the reactions I(a) and I(b) or whether two enzymes are involved. It is also uncertain whether hydrogenase and the co-factors indicated above are sufficient for reduction of cytochrome *c etc.* or whether they require, in addition, one or more other enzymes. The test system is however less complex with oxidants of low E_0'. Benzyl viologen has the lowest oxidation-reduction potential



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among substances which are rapidly reduced by purified hydrogenase and is likely to require the least number of factors other than hydrogenase. Purified hydrogenase preparations showed the same activity with benzyl viologen or methylene blue and the degree of activation by $FeCl_2$ was the same with either of the dyes. It appears probable that the effect of $FeCl_2$ is due to activation of hydrogenase itself. In view of the reported presence of flavin in hydrogenase (SHUG *et al.*[19]) the possibility that hydrogenase is a ferro-flavoprotein merits further study. It would also be desirable to confirm the findings on the activation of hydrogenase by $FeCl_2$ by means of a different test system, preferably the exchange reaction between H_2 and D_2O, which requires no extraneous hydrogen acceptor.

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SUMMARY

Hydrogenase from *Desulfovibrio desulfuricans* has been purified about fifty-fold. At the highest level of purity 1 mg of protein N catalyses the oxidation of $2.6 \cdot 10^6$ μl of H_2 per hour by methylene blue at 34°. Purified hydrogenase was found to be activated by $FeCl_2$ or $FeCl_3$, the increase in activity being the same with benzyl viologen or methylene blue as oxidant. A thermostable cytochrome-like component was obtained during the purification, which has no hydrogenase activity, but could be reduced by hydrogen and hydrogenase and reoxidised by O_2, methylene blue or FMN.

Methylene blue and benzyl viologen were reduced rapidly and at the same rate by hydrogen in the presence of purified hydrogenase; FMN, FAD and riboflavin were reduced at about one-hundredth of this rate, while DPN, TPN, sulfate and ferricyanide were not reduced. The optimum pH for methylene blue reduction was 8.4, cysteine or glutathione was essential for enzyme activity and serum albumin had a protective action at high enzyme dilutions. Hydrogenase was inhibited by *p*-chloromercuribenzoate and the inhibition was reversed by cysteine or glutathione. Cyanide and other metal-binding agents, hydroxylamine and nitrite were also inhibitory.

RÉSUMÉ

L'hydrogénase de *Desulfovibrio desulfuricans* a été purifiée environ cinquante fois. Dans l'état de pureté le plus élevé, 1 mg d'N protéique catalyse l'oxydation par le bleu de méthylène, à 34°, de $2.6 \cdot 10^6$ μl d'H_2 par heure. L'hydrogénase purifiée est activée par $FeCl_2$ ou $FeCl_3$, l'augmentation d'activité étant la même que l'oxydant soit le benzyl viologène ou le bleu de méthylène. Au cours de la purification, les auteurs ont obtenu un composé thermostable, analogue aux cytochromes, qui n'a pas d'activité hydrogénasique, mais qui peut être réduit par l'hydrogène et l'hydrogénase et réoxydé par O_2, le bleu de méthylène ou le FMN.

Le bleu de méthylène et le benzyl viologène sont réduits rapidement et à la même vitesse par l'hydrogène en présence d'hydrogénase purifiée; le FMN, le FAD et la riboflavine sont réduits à une vitesse environ cent fois inférieure, tandis que le DPN, le TPN, le sulfate et le ferricyanure ne sont pas réduits. Le pH optimum de la réduction du bleu de méthylène est de 8.4. La cystéine ou le glutathion sont essentiels à l'activité enzymatique et la sérumalbumine possède une action protectrice sur les fortes dilutions de l'enzyme.

L'hydrogénase est inhibée par le *p*-chloromercuribenzoate et l'inhibition est réversible en présence de cystéine ou de glutathion. Le cyanure et les autres agents complexants des métaux, l'hydroxylamine et le nitrite sont également inhibiteurs.

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ZUSAMMENFASSUNG

Aus *Desulfovibrio desulfuricans* gewonnene Hydrogenase wurde etwa fünfzigfach gereinigt. Auf dem höchsten Reinheitsgrade katalysiert 1 mg Protein-Stickstoff die Oxydation von $2.6 \cdot 10^6$ µl H_2 pro Stunde durch Methylenblau, bei 34°. Es wurde festgestellt, dass gereinigte Hydrogenase durch $FeCl_2$ oder $FeCl_3$ aktiviert wird. Benzylviologen oder Methylenblau wurden als Oxydierungsagenten verwendet: in beiden Fällen fand man die gleiche Aktivitätssteigerung. Während der Purifikation wurde eine thermostabile Zytochrom-ähnliche Komponente erhalten, welche keine Hydrogenasetätigkeit zeigte, jedoch durch Wasserstoff und Hydrogenase reduziert, und durch O_2, Methylenblau oder FMN reoxydiert werden konnte.

In Anwesenheit von gereinigter Hydrogenase, wurden Methylenblau und Benzylviologen schnell und mit der gleichen Geschwindigkeit durch Wasserstoff reduziert; FMN, FAD und Riboflavin wurden mit einer ungefähr hundertmal geringeren Geschwindigkeit reduziert, während DPN, TPN, Sulfat und Ferricyanid nicht reduziert wurden. Der optimale pH-Wert für die Methylenblau-Reduktion war 8.4. Cystein oder Glutathion waren für die enzymatische Tätigkeit unerlässlich; Serumalbumin hatte bei stark verdünnten Enzymen eine Schutzwirkung.

Hydrogenase wurde durch *p*-Chloromerkuribenzoat gehemmt, und diese Wirkung konnte durch Cystein oder Glutathion rückgängig gemacht werden. Cyanid und andere metallbindende Substanzen, sowie Hydroxylamin und Nitrit wirkten gleichfalls hemmend.

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