Some Observations on the Enzyme, Hydrogenase*

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Hydrogenase is a member of a small class of enzymes that interact reversibly with diatomic gases (H₂, O₂, N₂, CO, and NO). These gases, with the exception of N₂, serve either as substrate or inhibitor of the enzyme, hydrogenase. Our knowledge of the nature of the enzyme is incomplete since it has been derived from studies on bacterial suspensions or partially purified extracts. The isolation of the enzyme in pure form has been hampered because in most organisms it appears to be in particulate form. The isolation of the enzyme in pure form has been derived from studies on bacterial suspensions or partially purified substrate or inhibitor of the enzyme, hydrogenase. Our knowledge of the nature of the enzyme is incomplete since it has been derived from studies on bacterial suspensions or partially purified extracts. The isolation of the enzyme in pure form has been hampered because in most organisms it appears to be in particulate form.

Soluble hydrogenases have been prepared from several organisms. The highest concentration of hydrogenase in whole cells is found in Desulfovibrio desulfuricans (1, 2). Various procedures for purifying the enzyme in this organism have been described by different investigators (1, 3-5). The activities of the purest fractions found by these investigators ranged from 2.5 x 10⁶ to 11.4 x 10⁶ liters of H₂ activated per mg of N per hour. Other investigators have obtained soluble hydrogenase preparations of similar activity from Clostridium pasteurianum (6) and Clostridium butylicum (7). Our experience with these methods as applied to D. desulfuricans is that none of them is reproducible. Apparently, the enzyme is partly in a soluble form and partly in particulate form. Most methods described will concentrate the soluble form; they fail if the enzyme is in particulate form.

We will describe here a method for solubilizing and concentrating the hydrogenase of D. desulfuricans and some properties of the preparations we obtain.

EXPERIMENTAL PROCEDURE

Methods—We have described previously the procedures for growth of Desulfovibrio desulfuricans, determination of nitrogen and protein concentration, and the test for enzyme activity (1). This is based on the ability of the enzyme to catalyze the exchange reaction

\[ \text{H}_2 + \text{HDO} \rightleftharpoons \text{HD} + \text{H}_2\text{O} \]

Preparation of Enzyme—An enzyme extract was prepared from D. desulfuricans by extraction in 1 M glycine solution according to the method previously reported (1). The active fraction which was obtained by 60 to 95% ammonium sulfate saturation was dialyzed against 0.01 M phosphate buffer, pH 7.0, for 8 hours and stored overnight at -20°C. Considerable activity was lost in this step. The dialyzed fraction was placed on a DEAE-cellulose column prepared according to Sober et al. (8). Five grams of the commercial powder (> 80 mesh) were washed with 0.01 M phosphate buffer, pH 7.0, poured as a slurry into a glass tube 15 mm in diameter fitted at the bottom with a glass wool plug and washed with 0.01 M phosphate buffer, pH 7.0.

Stepwise elution of the adsorbed protein was carried out by washing with buffer of increasing salt concentration and decreasing pH. The elution diagram obtained is shown in Fig. 1. The enzyme activity of each of the purified fractions was determined as soon as possible after obtaining the chromatographed enzyme fraction. Ammonium sulfate was added to 95% saturation to each fraction containing enzyme activity. Each fraction was then stirred for 30 minutes, centrifuged at 78,000 x g for 45 minutes, and the precipitate of each fraction was dissolved in 2 ml of 0.1 M phosphate buffer, pH 6.4. These fractions were stored overnight at -20°C. When they were thawed the next day, a second precipitate was removed by centrifugation. The clear enzyme solutions thus obtained were stable for several weeks at -20°C. The results of this purification procedure are summarized in Table 1. The fractions eluted by the 0.08 M phosphate buffer, pH 6.0, plus 0.02 M NaCl contained 0.92 mg of protein with QH₂ between 26 x 10⁶ and 55 x 10⁶.

Stability of Purified Enzyme—In the process of purifying the enzyme, it has been found that the fractions up to and including the 60 to 95% ammonium sulfate fractions are relatively stable for many weeks when kept at -20°C, although some loss of activity was observed in every freezing and thawing.

The enzyme activity of the pure fractions is lost rapidly if the enzyme protein is kept in dilute buffer solutions or if the enzyme protein concentration is very low. This was the cause of the loss of activity after dialysis of the 60 to 95% ammonium sulfate fraction. We avoided the loss of activity by carrying out the chromatography on DEAE-cellulose immediately after dialysis. The active fractions from the column were precipitated by 95% saturation with ammonium sulfate, and the precipitate was dissolved in a small volume of 0.1 M phosphate buffer. This sequence was carried out in 1 day.

Absorption Spectrum of Purified Enzyme—The absorption spectra of the various enzyme fractions were recorded on the Cary recording spectrophotometer in 10-cm cells 2 ml in volume. The glycine extract shows the absorption spectrum to be attributable to cytochrome c₅ (bands at 418, 525 and 553 mμ in the reduced state) and the green pigment, desulfovirdin (9).

In the fractionation on DEAE-cellulose a separation of the pigments from the enzyme was obtained. Cytochrome c₅ with its high isoelectric point (pH > 10) was not adsorbed on the column and was found in the breakthrough effluents. The green pigment remained stationary as a band at the top of the...
adsorbing column. When the eluting buffer concentration was increased to 0.1 M phosphate + 0.05 M NaCl, the pigment separated into two bands, a fast moving green band and a slow moving pink band. The latter was eluted by 0.1 M phosphate buffer, pH 5.6, + 0.1 M NaCl. The pink solution was found to contain protein and shows in the reduced state a general absorption spectrum in the visible. Its presence in *D. desulfuricans* has never been reported.

Presence of very small amounts of cytochrome c₃ could be detected in most of the active fractions. It was not present in some fractions with activities as great as \( Q_{H_2} = 24 \times 10^6 \). It is clear from the elution diagram that cytochrome c₃ can be separated from the purified hydrogenase. It is present in some of the active fractions as a contaminant, but cytochrome c₃ is not a cofactor of the hydrogen-activating enzyme. From 220 mµ to 800 mµ, no absorption bands are present that seem to be related to the enzyme activity.

Solubilization of Particulate Hydrogenase of *D. desulfuricans*

We have occasionally found that some batches of *D. desulfuricans* grown under our standard conditions yield cells from which the hydrogenase cannot be extracted by incubation with molar glycine. We are unaware of any differences which can account for this change in the property of the enzyme. Extended treatment with sonic vibrations does not bring the enzyme into solution. This suggests that the enzyme was in a different state of aggregation in the cell and would have to be released from its binding with other cellular constituents in order to be made soluble.

Treatment of such cells in 1 M glycine-0.2 M phosphate buffer, pH 7.5, with trypsin (trypsin concentration = 1% of total protein concentration) for 3 hours at 36° resulted in the solubilization of 73% of the activity; i.e., it was not sedimented at 105,000 x g for 80 minutes. No loss of activity was incurred by the trypsin treatment because the remainder of the activity was found in the sediment. Fractionation by ammonium sulfate now yielded the enzyme in the 30 to 60% saturation fraction (see Table II). Further purification was then carried out by chromatography on a DEAE-cellulose column.

The active fractions were eluted off the column in two different regions. The break-through volume, 90% of the enzyme, was not adsorbed on the cellulose and was located in the first few tubes. However, 10% of the activity was eluted by 0.06 M phosphate buffer, pH 6.2 + 0.02 M NaCl. This fraction was highly purified, with a \( Q_{H_2} \) of \( 27 \times 10^6 \).

The enzyme not adsorbed (Fractions 2, 3, and 4) had only a

### Table I

**Purification of hydrogenase by Method A (glycine extraction)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluting buffer*</th>
<th>Activity recovered</th>
<th>Total protein</th>
<th>Exchange ( Q_{H_2} \times 10^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphate</td>
<td>NaCl</td>
<td>pH</td>
<td>%</td>
</tr>
<tr>
<td>Whole cells</td>
<td>0.01-0.04</td>
<td>0</td>
<td>7.0-6.4</td>
<td>100</td>
</tr>
<tr>
<td>Glycine extract of cells</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>25.7</td>
</tr>
<tr>
<td>Dialysate of glycine extract</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>29.4</td>
</tr>
<tr>
<td>Supernatant of protamine precipitate</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate, 60 to 95%</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Dialysate of (NH₄)₂SO₄ precipitate</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>7.8</td>
</tr>
<tr>
<td>DEAE-cellulose fractions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-8</td>
<td>0.01-0.04</td>
<td>0</td>
<td>7.0-6.4</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>0.675</td>
</tr>
<tr>
<td>10</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>0.475</td>
</tr>
<tr>
<td>11</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.117</td>
</tr>
<tr>
<td>12</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.250</td>
</tr>
<tr>
<td>13</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.150</td>
</tr>
<tr>
<td>14</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.100</td>
</tr>
<tr>
<td>15</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.300</td>
</tr>
<tr>
<td>16</td>
<td>0.10</td>
<td>0.02</td>
<td>6.0</td>
<td>0.110</td>
</tr>
<tr>
<td>17</td>
<td>0.10</td>
<td>0.02</td>
<td>6.0</td>
<td>0.070</td>
</tr>
<tr>
<td>18</td>
<td>0.10</td>
<td>0.02</td>
<td>6.0</td>
<td>0.050</td>
</tr>
<tr>
<td>19</td>
<td>0.10</td>
<td>0.02</td>
<td>6.0</td>
<td>0.030</td>
</tr>
</tbody>
</table>

* Fractions 9 to 19 contain 3.5% of the original activity.
small increase in specific activity. When these fractions were
again treated with trypsin for 3 hours, and rechromatographed
with respect to its ability to catalyze the H2-D2O exchange
reaction, there was never any indication that a dialyzable or easily dissociable
cofactor was involved in the activity of the enzyme.

Reduction Activity of Purified Enzyme—The enzyme was puri-
ified with respect to its ability to catalyze the H2-D2O exchange
reaction. Attempts to compare quantitatively the rates of
reduction of dyes with the rate of the exchange reactions were
not accurate. These difficulties were due to the inhibition of the
purified enzyme by traces of oxygen in the Warburg flasks (1).
Nevertheless, the ratios of the rate of reduction of dyes (methyl-
ene blue or benzyl viologen) to the exchange reaction in various
pure fractions show that they are not very different from these ratios in the whole cells. The ability to reduce the
substrates tested was not lost by the purification procedure.

During the course of purification of the enzyme with respect
to its ability to catalyze the H2-D2O exchange reaction, there
was never any indication that a dialyzable or easily dissociable
cofactor was involved in the activity of the enzyme.

Molecular Weight of Hydrogenase—Ultracentrifugal studies
were carried out on the proteins of the combined Fractions 11 to
15 of Table I, and of the combined Fractions 19 and 20 of Table
II. Two components were observed; a very slow moving protein
with a sedimentation constant of 1.1 and a somewhat faster component with a sedimentation constant of 3.0. About 90%
of the enzyme activity was found in the slow component. These
results indicate a molecular weight of about 9,000 for the com-
ponent containing the major part of the enzymatic activity.

We have also estimated the molecular weight of the enzyme
by measurement of its rate of diffusion (10). The diffusion of
hydrogenase activity and of human hemoglobin was measured
in the same solution. Assuming that both molecules have the
same shape and hydration the molecular weight (mol. wt.) of
hydrogenase can be calculated from the formula

\[ \text{Mol. wt. (hydrogenase)} = \left( \frac{D \text{ hemoglobin}}{D \text{ hydrogenase}} \right) \]

in which D hemoglobin is the diffusion constant of hemoglobin
and D hydrogenase is the diffusion constant of hydrogenase.
The results yield a value of 15,000 for the molecular weight of
hydrogenase. This value is not in disagreement with the value
found by ultracentrifugation.

Metal Content of Enzyme—The one point of general agreement
among investigators is that a metal is involved in the activity
of the enzyme hydrogenase. There is, however, no agreement
as to the nature of the metal involved.

The most purified fractions of Table I and of Table II were
analyzed qualitatively for the metal content by x-ray fluorescence
spectroscopy.2 Iron was the only heavy metal found in these
fractions. This is in agreement with the analyses for metal in
the hydrogenase of Proteus vulgaris (2).

SUMMARY

A procedure is described for the purification of hydrogenase
that increases its specific activity by over 300-fold to an activity
of Biochemistry, Graduate School of Public Health, University of
Pittsburgh, for the ultracentrifugal studies.

* We wish to thank Philips Electronics for the analyses.

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TABLE II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluting buffer*</th>
<th>Activity recovered</th>
<th>Total protein</th>
<th>Exchange constant ⋅ 10−10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphate</td>
<td>NaCl</td>
<td>pH</td>
<td>%</td>
</tr>
<tr>
<td>Whole cells</td>
<td>0.01</td>
<td>0</td>
<td>7.0</td>
<td>40</td>
</tr>
<tr>
<td>Glycine trypsin extract</td>
<td>0.01</td>
<td>0</td>
<td>7.0</td>
<td>16.4</td>
</tr>
<tr>
<td>Protamine supernatant</td>
<td>0.02-0.04</td>
<td>0</td>
<td>7.0-6.4</td>
<td>0.275</td>
</tr>
<tr>
<td>Ammonium sulfate, 30-60%</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>0.409</td>
</tr>
<tr>
<td>Dialyzed ammonium sulfate 30-60%</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>0.361</td>
</tr>
<tr>
<td>DEAE-cellulose fractions</td>
<td>0.06</td>
<td>0.02</td>
<td>6.2</td>
<td>0.89</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.225</td>
</tr>
<tr>
<td>3</td>
<td>0.08</td>
<td>0.02</td>
<td>6.0</td>
<td>0.301</td>
</tr>
</tbody>
</table>

* Fractions 2 to 4 contain 42% of the original activity, and Fractions 17 to 22 contain 3% of the original activity.
of $Q_H^{max}$ = $54 \times 10^4 \mu l$ of $H_2$ per hour per mg of N. Hydrogenase within the cell seems to be bound to large molecules and can be released from this aggregate by treatment with trypsin in glycine solution. The molecular weight of hydrogenase is about 10,000. The most active fractions contain iron but no other metal.

REFERENCES
