ON THE ACTIVATION OF MOLECULAR HYDROGEN BY PROTEUS VULGARIS

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The activation of molecular hydrogen by suspensions of bacteria was discovered in 1931 by Stephenson and Stickland (1), who found that methylene blue was reduced by hydrogen in the presence of suspensions of *Bacillus coli*. An active cell-free extract has since been obtained by Bovarnick, through autolysis of *Bacillus coli* (2).

The microbiological activation of hydrogen has been investigated by two main methods: (a) the hydrogenation of various substrates (\( \text{H}_2 + X \rightarrow X\text{H}_2 \)) and (b) the exchange reaction between heavy hydrogen and normal water (\( \text{D}_2 + \text{H}_2\text{O} \rightarrow \text{HD} + \text{HDO} \)). Presumably in both types of reaction some kind of activation of the hydrogen molecule occurs, and it seems justified to reserve the name hydrogenase for that part of the bacterial enzyme complex which is responsible for the activation of molecular hydrogen. This activation may be accompanied or followed by further processes, catalyzed by other parts of the enzyme system. The exchange reaction (b) is simpler than the hydrogenation reaction, since it does not involve the activation of an acceptor molecule.

Catalytic hydrogenations in the presence of various species of bacteria have been reported, but no hydrogenase has as yet been isolated. The bacteria studied include *Bacillus coli* (1, 4–6), *Bacillus acidilactici* (7), *Proteus vulgaris* (8), *Bacillus lactis aerogenes* (8, 6), *Bacterium freundii*, *Bacterium dispers* (7), some luminiscent bacteria (9–11), *Clostridium sporogenes* (12), *Azolobacter* (13–17) (from which Wilson et al. obtained a cell-free preparation by means of a bacterial mill (14)), *Bacillus delbrueckii* (4), a species of methane-producing bacteria (18), and some purple bacteria (19). The exchange reaction has been investigated in the presence of the first three species mentioned above (20–22).

The acceptors used in the hydrogenation experiments included methylene blue, nitrate, sulfate, sulfite, thiosulfate, carbon monoxide (18), carbonate, hydrogen peroxide (24), oxygen, various amino acids (12), and, to a lesser extent, various aldehydes, alcohols, and carboxylic acids (12, 18).

1 The para-ortho conversion of hydrogen in the presence of *Bacillus coli* has also been investigated (3).

2 A preparation of succinic dehydrogenase has also been found to catalyze the exchange reaction (23).
Some colored bacteria, *e.g.* *Rhodovibrio*, and some algae, *e.g.* *Scenedesmus*, catalyze the photochemical reduction of carbon dioxide by hydrogen (25, 26).

Previous investigations have dealt either with the hydrogenation or with the exchange reaction. The experiments described in this paper were undertaken in order to compare the two processes when they occur in the presence of the same bacterium under comparable conditions.

**EXPERIMENTAL**

Suspensions of *Proteus vulgaris*, which Hoberman and Rittenberg have shown to be particularly active (8), were used throughout this work. The hydrogen acceptor used in the hydrogenation experiments was in general 0.05 M sodium fumarate. Light and heavy hydrogen were prepared by electrolysis of normal and heavy water respectively and were purified by diffusion through a hot palladium tube.

The broth used had the following composition (cf. Hoberman and Rittenberg (8)): 0.5 per cent concentrated yeast extract, 0.5 per cent glucose, 2 per cent Bacto-peptone (Difco), and 0.5 per cent NaCl. The stock culture was transferred to fresh broth about every 3 weeks. For each series of growth the bacteria were transferred three times through broth and then inoculated into 1 liter Erlenmeyer flasks containing broth and 2 per cent agar-agar. 24 hour cultures (37°) were washed out of the flasks with 0.85 per cent NaCl solution, washed thrice by centrifugation, and suspended in 1/15 neutral phosphate buffer. Each flask yielded about 10 to 35 X 10^10 cells. The suspensions were diluted to a concentration of about 10^10 cells per cc. (measured photometrically) and kept at 0°. Under these conditions they retained their activity for several weeks, irrespective of the ratio of viable to dead cells. In one case a decrease of only 30 per cent in activity was observed after 5 weeks storage. Similar results have been reported with some bacterial dehydrogenases (27, 28), with the exchange-catalyzing enzyme of *Bacillus acidithiogoli* (22), and with the *Knallgas* activity of *Azotobacter* (14). Experiments were generally carried out during the 1st week or 2 of storage.

**Apparatus**

All experiments were carried out in vessels of 25 to 35 cc. capacity under hydrogen pressures of 50 to 250 mm. of Hg and at 37°, unless otherwise stated. 4 to 6 cc. of liquid were introduced into each vessel, made up of the suspension (1 to 3 cc.), buffer, and, in the appropriate cases, sodium fumarate and inhibitor solution. The vessels were thoroughly evacuated.

We are indebted to the Department of Bacteriology and Hygiene for rendering possible the preparation of the cultures.
through a liquid air trap. Hydrogen was then introduced at the desired pressure. Shaking took place at a frequency of 150 to 200 full strokes per minute through a 1 inch stroke. The bottom of each vessel was indented at several points in order to insure better mixing of the liquid with the gas.

Manometric measurements of the hydrogenation reaction were carried out simultaneously in seven reaction vessels, each of which was connected to a separate manometer dipping into a common mercury reservoir. The manometers were read by a cathetometer with an accuracy of 0.05 mm. The general arrangement is shown in Fig. 1, A, in which only one vessel is shown for the sake of simplicity. The pressure changes in the reaction vessels were obtained by comparing the readings of the respective manometers with that of a manometer connected to an eighth vessel containing 5 cc. of water.

Measurements of the exchange reaction were performed simultaneously in four reaction vessels. These were connected to mercury manometers and to the high vacuum line by spirals of narrow glass tubing in order to permit rapid shaking (Fig. 1, B).

The rate of exchange between heavy hydrogen and normal water was measured by determining the deuterium content of the gas from time to time. Samples of gas amounting to 0.1 per cent of the total volume of gas were withdrawn by means of a capillary lock L and analyzed by the micro conductivity method (29).

![Diagram of apparatus](http://www.jbc.org/)

**Fig. 1. A**, apparatus for measuring the rate of hydrogenation. **B**, apparatus for measuring the rate of the exchange reaction.
The heavy hydrogen used was in equilibrium with regard to the reaction \( \text{H}_2 + \text{D}_2 \rightleftharpoons 2\text{HD} \) and contained about 50 per cent deuterium.

Fig. 2. The rates of hydrogenation with suspensions which had been shaken at 37° for 2 hours with air (Vessel 1), with hydrogen (Vessel 2), and in vacuo (Vessel 3), prior to the commencement of the measurements.

Fig. 3. The decrease of the hydrogen pressure with time at various temperatures. Initial pressure, 250 mm. of Hg, 1.5 \( \times 10^{10} \) cells.

Results

No change in pressure was observed when suspensions of bacteria were shaken with hydrogen in the absence of any acceptor.

(a) Pressure-Time Graphs—During the hydrogenation experiments the pressure in each vessel was measured every 20 to 30 minutes during 2 to 3 hours. On plotting the decrease in pressure against time, straight lines were obtained (Figs. 2 and 3). If the slope of these lines, \( S \), is expressed in mm. of Hg per hour, then the volume of hydrogen at normal temperature and pressure taken up per hour is given by the expression \( V(S/760) \)
(273/310) cc. where \( V \) is the volume occupied by the gas. (The dissolved hydrogen constitutes only about 0.2 per cent of the total quantity of hydrogen present.) For the sake of brevity we shall denote this expression by \( R_n \).

The rates of hydrogenation in six vessels containing equal amounts of suspension are given in Table I.

If the straight lines obtained on plotting the pressure decrease against time are extended backwards, they cut the time axis at times \( t' \) varying between 5 and 60 minutes, the actual value of \( t' \) depending primarily on the treatment of the suspension previous to the commencement of the experiment. The normal value of 15 to 25 minutes was not affected by previous shaking of the suspension in vacuo. On the other hand, shaking with hydrogen caused an appreciable decrease in the value of \( t' \), even if the suspension was subsequently allowed to come into contact with air for a short time. Shaking with air increased the value of \( t' \) to about an hour, besides causing a decrease in the activity of the preparation (see \( j \) below). Fig. 2 shows the effect of the three treatments mentioned.

A rather slow transition of the enzyme system from aerobic to anaerobic conditions and vice versa may be responsible for this behavior. A somewhat similar phenomenon has been investigated in detail in the case of the hydrogenase system of green algae (26). Hoberman and Rittenberg assume that such a transition occurs in the case of the exchange reaction in the presence of Proteus vulgaris.

(b) Decrease of Deuterium Content of the Gas with Time—The experimental relation between the ratio of deuterium to total hydrogen in the gas \( D \) and time \( t \) was found to be

\[
\log_{10} (D) = -kt + c
\]

where \( k \) and \( c \) are constants (cf. Figs. 4 and 11). For reasons explained below, the product defined by \( R_p = 2.3 kV_\sigma \) (where \( V_\sigma \) denotes the total
quantity of gas present, expressed in cc. at N.T.P.) may serve as a measure of the rate of activation of hydrogen molecules on the catalyst.

The value of $R_H$ was measured with an accuracy somewhat less than that obtained for the value of $R_{sh}$ by the simple manometric method. Deviations between the rates of the exchange reaction measured in parallel vessels may reach 10 per cent.

(c) Influence of Growth Conditions—It was found that the hydrogenation activity of the bacteria could be increased by 50 to 200 per cent by adding 1.5 per cent fumarate to the broth through which the bacteria were transferred. This has been verified by comparing the activity of parallel growths, with and without the addition of fumarate. In different series of growths large fluctuations of the hydrogenation activity were observed (see the third column, Table IV). On the other hand the activity with regard to the exchange reaction was practically the same in all cases, and was not affected by the presence of fumarate during growth. This stimulation of the formation (or activation) of the fumarate-activating part of the enzyme system suggests that we have here a case of a so called semi-adaptive enzyme (30).

(d) Influence of Prolonged Contact with Hydrogen—It was found that storing the suspension in an atmosphere of hydrogen for 8 to 12 hours at $37^\circ$ prior to the commencement of the experiment caused an increase of 30 to 60 per cent in both the hydrogenation activity (Table I) and the exchange activity of the suspensions. This treatment had the same effect on cells which had been stored for a month at $0^\circ$. The addition of fumarate previous to the above treatment was without influence on the increase in activity. Storing the suspension in vacuo for 8 to 12 hours at $37^\circ$ did not affect its activity, suggesting that the rise in activity is not due to causes such as partial lysis or a change in the permeability of the cell walls. The turbidity of the suspensions (measured photometrically) did not change during this activation.

A somewhat similar increase of the reaction rate has been reported by van Niel in the case of the photochemical reduction of carbon dioxide by hydrogen in the presence of colored bacteria (25), p. 301.

(e) Effect of Temperature—For reasons explained below ("Kinetics"), these experiments were carried out with relatively high hydrogen pressures (200 to 250 mm. of Hg), and at each temperature simultaneous experiments were carried out with varying numbers of cells. The velocity constants at various temperatures were calculated from the slopes of the graphs of the decrease of pressure or deuterium content against time (Figs. 3 and 4). The apparent energies of activation of both reactions were calculated from the slope of the log $R_H$ or log $R_{sh}$ against $1/T$ lines (Figs. 5 and 6; Tables II and III). In these experiments the reaction rate was measured
first at the lowest temperature and then at progressively higher temperatures, the same bacteria being used at all temperatures. Values of about

![Graph](image1.png)

**FIG. 4.** The decrease of the deuterium concentration in the gas, \(D\), with time at various temperatures. Initial pressure, 150 mm. of Hg, \(1.5 \times 10^{10}\) cells.

![Graph](image2.png)

**FIG. 5.** The effect of temperature on the rate of hydrogenation. The numbers of cells used were: \(1 \times 10^{10}\) in Vessel 1, \(1.5 \times 10^{10}\) in Vessels 2 and 3, and \(2 \times 10^{10}\) in Vessel 4.

**FIG. 6.** The effect of temperature on the rate of exchange \(R_E\).

15.0 kilocalories were thus obtained both for the hydrogenation and for the exchange reactions. In other experiments, in which a fresh suspension
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(but from the same batch) was used at each temperature, a value of only
about 14.0 kilocalories was found for the hydrogenation reaction. The
difference between this value and the one mentioned above is probably due
to the effect described in (d).

Table II

<table>
<thead>
<tr>
<th>Vessel No.</th>
<th>( R_H ) at 40°</th>
<th>No. of cells ( \times 10^{-10} )</th>
<th>Apparent energy of activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cc. per hr.†</td>
<td></td>
<td>kilocalories</td>
</tr>
<tr>
<td>1</td>
<td>0.43</td>
<td>1.0</td>
<td>15.5</td>
</tr>
<tr>
<td>2</td>
<td>0.44</td>
<td>1.0</td>
<td>14.4</td>
</tr>
<tr>
<td>3</td>
<td>0.69</td>
<td>1.5</td>
<td>15.4</td>
</tr>
<tr>
<td>4</td>
<td>0.67</td>
<td>1.5</td>
<td>14.9</td>
</tr>
<tr>
<td>5</td>
<td>0.94</td>
<td>2.0</td>
<td>15.6</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>2.0</td>
<td>15.5</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>15.2</td>
</tr>
</tbody>
</table>

* These reaction rates are given in order to show that the proportionality of the
\( R_H \) to the cell number is maintained even at the highest temperature.
† Normal temperature and pressure.

Table III

Effect of Temperature on Rate of Exchange, \( R_E \), with Varying Cell Numbers, at Hydrogen
Pressure of 160 Mm. of Hg

<table>
<thead>
<tr>
<th>Temperature</th>
<th>( R_E ) with 1.5 ( \times 10^{10} ) cells</th>
<th>( R_E ) with 3.0 ( \times 10^{10} ) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C.</td>
<td>cc. per hr.*</td>
<td>cc. per hr.*</td>
</tr>
<tr>
<td>26.0</td>
<td>0.14</td>
<td>0.28</td>
</tr>
<tr>
<td>31.0</td>
<td>0.20</td>
<td>0.38</td>
</tr>
<tr>
<td>36.4</td>
<td>0.35</td>
<td>0.63</td>
</tr>
<tr>
<td>41.9</td>
<td>0.43</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Apparent energy of activation

<table>
<thead>
<tr>
<th>Kilocalories</th>
<th>Kilocalories</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.5</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* Normal temperature and pressure.

(j) Effect of Hydrogen Pressure—Experiments were carried out in parallel
vessels at different pressures under otherwise identical conditions. The
values of \( R_H \) and of \( R_E \) were found to rise with increasing pressures of total
hydrogen, \( P \), in accordance with the equations

\[
R_H = CP^n
\]

\[(2, a)\]

\[
R_E = C'P^n
\]
where \( C, C', n, \) and \( n' \) are constants. The values of \( n \) and \( n' \) are obtained from the slopes of the graphs of \( \log R_H \) and \( \log R_E \) against \( \log P \); such graphs are shown in Figs. 7 and 8. Equation 2, \( a \) is seen to hold only for pressures above about 50 mm. of Hg. Table IV gives the values found for

\[ \text{Fig. 7} \]

The dependence of the rate of hydrogenation \( R_H \) on the hydrogen pressure \( P \).

\[ \text{Fig. 8} \]

The dependence of the rate of exchange \( R_E \) on the hydrogen pressure \( P \). At 30 mm. of Hg \( R_H = 0.015 \text{ cc. at N.T.P. per hour.} \)

**Table IV**

*Effect of Hydrogen Pressure and Cyanide on Rate of Hydrogenation with Bacteria Differing in Hydrogenation Activity (the Latter Represented by \( R_H \) at 100 mm. of Hg)*

<table>
<thead>
<tr>
<th>Series of growth</th>
<th>No. of cells ( \times 10^{-12} )</th>
<th>( R_H ) at 100 mm. Hg*</th>
<th>( n ) (Equation 2)</th>
<th>( R_H ) after aerobic incubation with cyanide, 0.005 m. per cent of original ( R_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cc. per hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.02</td>
<td>0.10</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.08</td>
<td>0.15</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.14</td>
<td>0.18</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.17</td>
<td>0.41</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>0.33</td>
<td>0.37</td>
<td></td>
</tr>
</tbody>
</table>

* Normal temperature and pressure.

\( n \) in experiments with bacteria from different series of growth, having different hydrogenation activities. A hydrogen pressure of 100 mm. of Hg was chosen as the standard pressure for comparing the various activities. The values of \( R_H \) at this pressure were obtained from the log \( R_H \) versus log \( P \) lines.

In the experiment presented in Fig. 8 the value of \( R_H \) at 30 mm. of Hg was as low as 0.015 cc. per hour at N.T.P. From this and similar sets of experiments values of 0.4 to 0.5 were found for \( n' \). Polanyi and coworkers,
using Bacillus acidi lactici, showed that \( n' \) had a value of 0.5 over a wide range of pressures (22).

(g) Effect of Fumarate Concentration on Rate of Hydrogenation—\( R_R \) is independent of the fumarate concentration above \( m/80 \) (Fig. 9).

(h) End-Point of Hydrogen Uptake—The total hydrogen uptake did not amount to more than 60 to 65 per cent of the fumarate initially present, even when most of the cells had lost their viability (after a month of storage at 0°). The reason may be reduction of the fumarate by cell material or fermentation of the fumarate according to the scheme:

\[
\text{7 fumarate}^{-} + 2\text{H}_2\text{O} = 6\text{succinate}^{-} + 2\text{H}_2\text{CO}_3 + 2\text{CO}_2
\]

Both reactions have been observed (1, 9, 5, 31). The formation of small quantities of carbon dioxide would not interfere with the manometric measurements, since the solution is strongly buffered at pH 7.

(i) Hydrogenation of Other Substrates—Only a few substrates of particular interest in connection with the present work were investigated. Maleate is hydrogenated at least 20 times more slowly than fumarate, possibly via a slow conversion to fumarate. The rate of hydrogenation of freshly prepared solutions of malate does not differ from that of fumarate, indicating the presence of fumarase which rapidly dehydrates the malate to fumarate. Similar results have been reported with other bacteria (9). Carbonate is hydrogenated at about one-fifth the rate of fumarate.

The Knallgas reaction was carried out under partial pressures of oxygen amounting to 1.6 to 4.8 per cent of the hydrogen pressures. In all cases the reaction rate fell off with time, in accordance with the results reported with other bacteria (1, 9), and with algae (26). As the partial pressure of
oxygen is raised, the reaction rate rises up to a constant value, which is reached at a partial pressure of oxygen of about 8 mm. of Hg (Fig. 10).

(j) Effect of Various Inhibitors—Shaking the buffered suspensions with air at 37° for 2 hours prior to the commencement of the experiments lowered their catalytic activity with regard to both reactions to about half its original value (Fig. 2). The activity could be partly restored by the addition of hydrosulfite or by prolonged shaking with hydrogen. The addition of succinate had no restoring effect.

Hoberman and Rittenberg found that hydrosulfite, succinate, and other reducing anions restored the exchange activity of suspensions which had been previously inactivated by shaking with oxygen for 24 hours (8).

The usual technique adopted for testing the effect of inhibitors was to incubate the buffered suspensions aerobically or anaerobically (i.e. in an atmosphere of hydrogen) with the respective inhibitor solution for 30 to 120 minutes at 37°. In the first case allowance was made for the inactivation due to the aerobic incubation itself by making simultaneous blank experiments. The results of the relevant hydrogenation experiments are summarized in Table V.

The retarding effect of cyanide presents some special features. When added under anaerobic conditions, no effect was observed even after several hours at concentrations as high as M/60. On the other hand, if added aerobically, a retarding effect was observed with both reactions. The $R_b$ was always reduced to about 15 per cent of its original value (at a cyanide concentration of 0.005 M), whereas the extent of the retardation of the hydrogenation reaction depended markedly on the hydrogenation activity of the bacteria (cf. the last column of Table IV). Hoberman and Rittenberg (8) reported a complete inhibition of the exchange reaction by aerobic cyanide at 0.001 M, and no effect of anaerobic cyanide at 0.01 M. The photochemical hydrogenation of carbon dioxide in the presence of colored bacteria has also been shown to be inhibited only by cyanide added aerobically (25). Gaffron, working with algae, found that the adaptation of the enzyme system to anaerobic conditions was inhibited by concentrations of cyanide too small to influence the photochemical hydrogenation appreciably (26).

Aerobic incubation with 0.2 M fluoride reduced the $R_b$ to 60 per cent of its original value. Lower concentrations had no effect. Hydroxylamine, added aerobically at 0.1 and 0.05 M reduced the $R_b$ to 15 and 20 per cent respectively. Wilson and Wilson report similar results with Azotobacter for the Knallgas reaction even at 0.01 M (17). Gaffron, investigating the photohydrogenation of carbon dioxide in the presence of algae, found that hydroxylamine added anaerobically had only a comparatively slight inhibiting effect even at about 0.02 M, whereas when added aerobically it caused total inhibition even at 0.001 M (26).
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(\(k\)) *Experiments on Simultaneous Hydrogenation and Exchange*—These experiments were carried out in four parallel vessels; two of the vessels contained fumarate and all four contained the same quantity of buffered suspension. The rate of decrease of the deuterium content in all four vessels and the rate of hydrogenation in the vessels containing fumarate were measured. Bacteria from different series of growth, differing in their hydrogenation activity, were examined. In all cases the value of \(R_s\) was lowered by the addition of fumarate (Fig. 11). The results are summarized in Table VI, from which it can be seen that as the hydrogenation activity of the bacteria decreases the effect of the addition of fumarate on the value of \(R_s\) diminishes, until for very low values of the rate of hydrogenation the \(R_s\) is no longer affected by the addition of fumarate.

The presence of succinate in the above experiments had no effect on the results described.

---

**Table V**

*Effect of Various Inhibitors on Rate of Hydrogenation*

The time and type of incubation with the inhibitor (aerobic or anaerobic) had no influence on the inhibiting effect, unless stated otherwise.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final inhibitor concentration in liquid</th>
<th>Per cent of original (R_s)</th>
<th>Inhibitor</th>
<th>Final inhibitor concentration in liquid</th>
<th>Per cent of original (R_s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malonate</td>
<td>0.1</td>
<td>100</td>
<td>Bromine*</td>
<td>(4 \times 10^{-6} \text{ M})</td>
<td>0</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.1</td>
<td>100</td>
<td>&quot;</td>
<td>(8 \times 10^{-4} )</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>80</td>
<td>&quot;</td>
<td>(2 \times 10^{-4} )</td>
<td>70</td>
</tr>
<tr>
<td>Fluoride, 2 hrs. aerobic incubation</td>
<td>0.03</td>
<td>80</td>
<td>Urethane</td>
<td>(0.4 \text{ M})</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.2 &quot;</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.1 &quot;</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>70</td>
<td>2,4-Dinitrophenol†</td>
<td>(3 \times 10^{-3} \text{ M})</td>
<td>70</td>
</tr>
<tr>
<td>Fluoride, no incubation</td>
<td>0.06</td>
<td>90</td>
<td>&quot;</td>
<td>(1.5 \times 10^{-3} \text{ M})</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxylamine, Series 4‡</td>
<td>0.05</td>
<td>45</td>
<td>AgNO₃</td>
<td>(1 \times 10^{-3} \text{ M})</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>45</td>
<td>Carbon monoxide in dark</td>
<td>70 %‡</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>50 %</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>30 %</td>
<td>95</td>
</tr>
</tbody>
</table>

* The suspension was incubated with the bromine solution for 20 minutes. The bromine was then neutralized with thiosulfate. The blank experiments were incubated with neutralized bromine solution.

† This substance strongly inhibits the photoreduction of CO₂ by H₂ in the presence of algae (26).

‡ The respective activities of the bacteria in the two series are given in Table IV.

§ Concentrations are expressed as per cent of the total gas pressure (i.e. H₂ + CO).
Kinetics

The hydrogenation and exchange processes consist of two successive stages, the solution of the gaseous hydrogen in the liquid and the reaction between this dissolved hydrogen and the substrate or water molecules. Either or both of these processes may determine the rate of reaction, \( R \). If the number of cells present is small, \( R \) will also be small, so that we can assume that the solution is practically saturated with hydrogen for the particular hydrogen pressure of the experiment. \( R \) will then be given by

\[
R = k'N(H_2)^\alpha
\]

where \( k' \) is a constant, \( N \) is the number of cells present, \( (H_2) \) is the concentration of dissolved hydrogen irrespective of its isotopic composition, and \( \alpha \) is the order of the reaction with respect to hydrogen. Since \( (H_2) \) is proportional to the partial pressure of the hydrogen, \( P \), the above equation becomes

\[
R = k''NP^\alpha
\]

where \( k'' \) is a constant.

For a larger number of cells, the rate of reaction will be determined by both the solution and the hydrogenation processes, while in the presence of a still larger number of cells \( R \) will be so high that the concentration of the dissolved hydrogen (or deuterium in the case of the exchange reaction) will be very low. Under these circumstances, the rate at which the dis-
solved hydrogen (or deuterium) leaves the aqueous solution and returns to the gaseous phase can be neglected. The rate of solution of the hydrogen (or deuterium) will thus be the rate-determining process, so that the rate of reaction will be independent of the number of cells present and will be given by

\[ R = KP \]

where \( K \) is a constant.

The rate of reaction is thus seen to be proportional to the cell number, \( N \), for low cell numbers (Equation 4) and to become less and less affected by the cell number as the latter rises, until it finally becomes independent of the cell number (Equation 5). For certain values of \( \alpha \) it is possible to deduce a formula giving \( R \) in terms of \( k'' \), \( K \), \( N \), and \( P \) for the general case in which both the solution and hydrogenation processes determine the reaction rate. Such a formula can be obtained by a method similar to that employed by Roughton (32), based on the liquid film theory of diffusion.

If under a certain range of experimental conditions the rate of reaction is found to be proportional to the number of cells, then this can be taken as sufficient evidence that Equation 4 is valid under these conditions. Only then can the exponents \( n \) and \( n' \) in the empirical Equations 2 and 2, \( a \) be identified with \( \alpha \) in Equation 4. In this way \( \alpha \) was always found to have a value less than 0.5. For higher cell numbers the value of \( n \) would be expected to rise and eventually to reach the value of unity according to Equation 5.

Since \( \alpha < 1 \), the higher the hydrogen pressure, the higher the limiting cell number up to which Equation 4 holds good (Figs. 12 and 13).

Further evidence for the validity of Equation 4 is afforded by the fact that the reaction rate was found to be independent of the rate of shaking provided this was above a certain minimum. The minimum rate of shaking

<table>
<thead>
<tr>
<th>Series No.</th>
<th>Pressure (mm. Hg)</th>
<th>( R_2 ) in absence of fumarate</th>
<th>( R_2 ) in presence of fumarate</th>
<th>( R_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>60</td>
<td>6.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>6.1</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>1.4</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>1.4</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>1.0</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*The \( R_2 \) in the presence of fumarate was calculated by using a mean gas volume.
depends on the hydrogen pressure (e.g., 100 full strokes per minute at a pressure of 60 mm. of Hg).

The interpretation of the experimentally found temperature coefficient depends on the number of cells present. For those cell numbers for which Equation 4 is valid, the temperature coefficient measured is that of the constant \( k'' \). On the other hand for sufficiently high cell numbers the temperature coefficient of the constant \( K \) of Equation 5 will be obtained. Hoberman and Rittenberg have shown that this latter coefficient is comparatively small (8). The temperature coefficient of the reaction rate would thus be expected to fall as the cell number is increased (cf. Table III).

![Fig. 13. The effect of the number of cells on the rate of the exchange reaction at a total hydrogen pressure of 80 mm. of Hg.](image)

**Kinetics of Exchange Reaction**—The exchange reaction may be assumed to comprise the activation of the molecules of dissolved hydrogen, followed by a partial or complete exchange reaction between the activated hydrogen and water. We shall assume that the rate of activation of the dissolved hydrogen molecules, \( u \) cc. at N.T.P. per hour, is independent of the isotopic composition of the hydrogen. \( u \) will be given by expressions similar to Equations 4 and 5 for small and large cell numbers respectively. The connection between \( u \) and the observed rate of decrease of the deuterium content of the gas can be derived as follows:

Differentiating Equation 1, we obtain

\[
\frac{d(D)}{dt} = -2.3k(D)
\]

If the gas consists entirely of deuterium, i.e. when \( (D) = 1 \), then the rate at which deuterium disappears from the gas will give a lower limit for the value of \( u \). On the other hand, if we assume that Equation 6 holds also
under these conditions, then this rate will be given by the expression
\[ V_0 \frac{d(D)}{dt} = 2.3k \, V_0 \] where \( V_0 \) is the total quantity of gas present, expressed in cc. at N.T.P.

We shall denote the value of \( 2.3kV_0 \) by \( R \) and, in the absence of further information as to the value of \( u \), we shall consider \( R \) to be a measure of the hydrogen-activating activity of the bacteria.

Example—For \( V_0 = 2.5 \) cc. (N.T.P.) and \( k = 0.02 \) hour\(^{-1} \) we obtain \( R = 2.3 \times 2.5 \times 0.02 = 0.115 \) cc. per hour at N.T.P.

The theoretical deduction of an equation similar to our Equation 1 by Hoberman and Rittenberg (8) is not altogether convincing, as the use of a single constant \( f \) for giving the degree of equilibration of a volume element of gas with the water appears insufficient. According to their definition of \( \beta \) no molecules of HD should be formed as a result of the exchange reaction between \( \text{H}_2 \) and a large excess of heavy water whatever the value of \( \beta \); yet their experimental results clearly show the formation of such molecules.

DISCUSSION

The experimental results so far obtained are not yet sufficient to justify any definite conclusions concerning the mechanism of the hydrogenation and exchange reactions, and the mechanism outlined below, although it explains these results fairly satisfactorily, must not be considered to be the only possible mechanism capable of so doing.

The existence of two types of inhibitors, exemplified by cyanide and urethane respectively, and the fact that only the hydrogenation activity is affected by the conditions of growth suggest that the hydrogenation is brought about by the combined action of two different parts of the bacterial enzyme system, activating the hydrogen and the fumarate respectively, and that the presence of only the first of these is necessary for the exchange reaction. The experimental results already given are in accordance with the assumption that only the hydrogen-activating part is affected by hydrogen pressure and by cyanide and that the conditions of growth and addition of urethane influence only the fumarate-activating part. Some of the other inhibitors examined appear to behave similarly to cyanide.

If the number of hydrogen molecules activated in unit time is considerably larger than the corresponding number of fumarate molecules, and if we further assume that under such circumstances the rate of hydrogenation is nearly equal to the rate of activation of the fumarate, then those factors which influence the rate of activation of the hydrogen will have but little effect on the rate of hydrogenation. As the rate of activation of the fumarate rises, the effect of such factors will become more and more predominant. These considerations provide an explanation of the results given in Table IV.

The fact that the rate of exchange is almost the same with bacteria from
different series of growth indicates that the rate of activation of hydrogen is practically independent of the conditions of growth, so that the rate of hydrogenation can be taken as a rough measure of the rate of activation of the fumarate.

The nature of the enzyme activating the fumarate is not clear. The absence of inhibition by malonate, succinate, and fluoride seems to indicate that it is not succinic dehydrogenase. It should be remembered, however, that the formation of succinate is inhibited by malonate far less than the reverse reaction (33). Moreover, the activity of succinic dehydrogenase bound to the intact cells is lowered by the inhibitors mentioned above to a lesser extent than that of cell-free preparations or even of cells treated with toluene (34). Another possibility is that the fumarate is activated by "fumaric hydrogenase," an enzyme which, like succinic dehydrogenase, catalyzes the reduction of fumarate by leuco dyestuffs, but which is not inhibited by succinate, malonate, or fluoride (35).

It is somewhat surprising that both the hydrogenation and the exchange reaction have almost the same apparent energies of activation. At the moment it seems more likely that this agreement is fortuitous rather than that it is due to the step common to both reactions (the activation of hydrogen), since the rate of hydrogenation is believed to be largely dependent on the rate of activation of the fumarate.

The influence of the concentration of fumarate on the rate of hydrogenation can be explained by assuming that the formation of activated fumarate takes place in two stages: adsorption on the fumarate-activating portion of the catalyst, followed by activation of the adsorbed fumarate. The fact that the rate of hydrogenation is independent of the concentration of fumarate (when this is above a certain value) can then be interpreted as being due to saturation of the catalyst with adsorbed fumarate.

The results of our hydrogenation experiments in the presence of carbon monoxide (Table V) are in fair agreement with those reported by Hoberman and Rittenberg (8) for the exchange reaction in the presence of the same inhibitor. The rapid decrease of the inhibitory effect with decreasing concentrations of carbon monoxide is remarkable, as also is the inhibition by very low concentrations of bromine. Bromine has been reported to inhibit various bacterial dehydrogenases (36).

We shall now consider the effect of the addition of fumarate on the rate of exchange and shall make the simplifying assumption that the velocity constants of all the processes concerned, in which hydrogen is involved, are independent of the isotopic composition of the hydrogen. Owing to the exchange reaction, the ratio of deuterium to total hydrogen in the activated hydrogen on the catalyst will be less than in the gaseous phase. The addition of fumarate will have the following three effects: (1) The concentration of the total activated hydrogen on the catalyst will be reduced.
(2) The ratio of deuterium to total hydrogen in the activated hydrogen on
the catalyst will be raised, but will still be less than that in the gaseous
phase. (3) Light and heavy hydrogen will be removed by combination
with fumarate in the same proportion as that in which they exist in the
activated hydrogen on the catalyst.

All three effects will increase with the rate of hydrogenation. Effects
(1) and (3) tend to reduce the rate of decrease of the ratio of deuterium to
total hydrogen in the gaseous phase, while effect (2) will tend to increase
this rate.

The experimental data summarized in Table VI indicate that effects
(1) and (3) predominate.

Mention should also be made of experiments performed in this laboratory
in which the isotopic composition of the succinate formed during the
hydrogenation of fumarate with heavy hydrogen in the presence of Bacillus
coli was measured (37).

In these experiments it was found by isotopic analysis of the succinate
that the concentration of deuterium in the hydrogen which combined with
the fumarate was about one-fifth of that in the gaseous phase. This
comparatively low deuterium content of the succinate may be due to a
similar deuterium content of the adsorbed hydrogen as described above.4

The authors are indebted to Mr. H. J. G. Hayman for his valued assist-
ance.

SUMMARY

1. The hydrogenation of fumarate is preceded by an induction period
of varying length, owing possibly to a slow reduction of the enzyme.
2. The specific hydrogenation activity of Proteus vulgaris towards fumar-
ate is increased by 50 to 200 per cent if fumarate is added to the broth
during growth. However, the exchange activity of the bacteria is not
affected by such an addition of fumarate.
3. The rates of both hydrogenation and exchange are increased by
shaking the buffered suspensions with hydrogen.
4. The apparent energies of activation of both reactions are about 14
kilocalories.
5. The effect of various inhibitors on both reactions has been studied.
6. The influence of factors, such as hydrogen pressure and certain
inhibitors, on the rate of hydrogenation, increases with increasing hydro-
genation activity of the bacteria.
7. Fumarate retards the rate of the exchange reaction, the retardation

The reactions mentioned in (h) also tend to decrease the deuterium content of
the succinate formed.
being the more marked the greater the hydrogenation activity of the bacteria.

8. The nature and kinetics of the reactions concerned are discussed.

Addendum—Recently a series of articles has been published in Australia, dealing with the activation of molecular hydrogen by bacteria (38).

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L. Farkas and E. Fischer


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