XXVII. HYDROGENASE: A BACTERIAL ENZYME ACTIVATING MOLECULAR HYDROGEN.

I. THE PROPERTIES OF THE ENZYME.

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Biochemists are now accustomed to regard the transfer of hydrogen as an essential step in biological oxidations; such a view involves the conception of some enzymic mechanism for rendering active or unstable the molecule from which the hydrogen is transferred. No such enzyme acting on molecular hydrogen has so far been described, though several inorganic catalysts are known which function in this way. It is nevertheless almost certain that such an enzyme exists, as organisms producing molecular hydrogen presumably have such a catalyst; moreover bacteria have long been known which are capable of oxidising hydrogen gas by molecular oxygen and living autotrophically on the energy thus liberated. The earliest example of such an organism was *B. pantotrophus* (*Hydrogenomonas pantotropha*) isolated by Kaserer [1906]. Later other workers isolated a variety of species having the same characteristic [Niklewski, 1914; Grohmann, 1924]. It was subsequently discovered that in certain of these species nitrate can replace oxygen, so that an anaerobic autotrophic development occurs [Niklewski, 1914]. In all these cases the organisms are facultatively autotrophic, the oxidation of hydrogen playing an essential part in their metabolism.

A suggestion that these early observations come into line with recent work on the dehydrogenases of bacteria is contained in the recent work of Tausz and Donath [1930], who showed that a culture of *Bact. aliphaticum liquefaciens* grown autotrophically on hydrogen, oxygen and carbon dioxide is capable of reducing methylene blue when a stream of hydrogen is led through the apparatus, but not when nitrogen replaces the hydrogen.

Experimental.

During the study of anaerobic fermentations of fatty acids to methane by mixed cultures from river mud, a culture was obtained which (*a*) reduced sulphate to sulphide, and (*b*) decomposed formate quantitatively to methane, carbon dioxide and water. The same culture was also found to synthesise

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methane from a mixture of carbon dioxide and hydrogen and simultaneously to reduce sulphate to sulphide at the expense of hydrogen [*cf.* Söhngen, 1910]. This led to the conception that carbon dioxide and sulphate were acting as hydrogen acceptors in a system where molecular hydrogen was the hydrogen donator, and it seemed likely that bacteria were present in the mixed culture capable of activating molecular hydrogen. We therefore centrifuged a mixed culture which had been grown for some 20 subcultures on formate as sole source of carbon, and tested the washed suspension in Thunberg tubes with methylene blue and gaseous hydrogen. For this purpose hydrogen from a Kipp's apparatus was passed through three wash-bottles containing silver nitrate (for the removal of traces of volatile arsenic compounds), pyrogallol and water; the last bottle was connected with a 3-way tap (oblique bore) so that the Thunberg tube could be alternately evacuated and filled with hydrogen. The tube was evacuated thoroughly to remove dissolved oxygen, then filled with hydrogen and again evacuated and filled; finally the tap was closed and the tube was placed in a bath at 45° and shaken for 1 minute to ensure saturation of the liquid with the gas; the reduction time was measured.

Result:

	Reduction time (mins.)
1 cc. 1/25,000 methylene blue, 1 cc. phosphate buffer p_H 8·0, 1 cc. bacterial suspension, in hydrogen	15
1 cc. 1/25,000 methylene blue, 1 cc. phosphate buffer p_H 8·0, 1 cc. bacterial suspension, *in vacuo*	> 70
Control with boiled suspension in hydrogen	> 70

Having shown that our mixed culture contained organisms capable of reducing by means of molecular hydrogen, we diluted and plated on ordinary broth agar and incubated the plates anaerobically. We picked off a number of colonies, grew the corresponding cultures on broth, centrifuged and washed the organisms, and tested the suspensions as above with hydrogen and methylene blue. In these and all subsequent experiments 1 cc. of 1/5000 methylene blue in a total volume of 4 cc. was used, and the time was taken when reduction had proceeded to 75 %, because the rate of reduction appears to fall off after that point.

Result:

	Reduction time (mins.)	
Culture No.	With hydrogen	Without hydrogen
70	5½	> 120
74	> 120	> 120
79	> 120	> 120
80	5½	> 120

This shows that there is a clearly marked difference between organisms having the property of reducing by means of molecular hydrogen and those lacking the power. Culture 70 was re-plated and gave the strain known as 111.

Characteristics of organism 111. This organism is a short, motile, non-sporing, Gram-negative bacillus, growing both aerobically and anaerobically.

Sown into peptone medium with 1 % of various sugars, according to the usual bacteriological practice, the following results were obtained:

Sugar	Acid	Gas
Glucose	+	+
Fructose	+	+
Mannitol	−	+
Sucrose	−	−
Maltose	−	+
Lactose	+	+
Salicin	−	−

Other characteristics are (1) acid clot in litmus milk, (2) no liquefaction of gelatin, (3) no production of indole, (4) negative Voges and Proskauer test. We are therefore assigning this organism to the *coli-typhosus* group; its properties as far as they have been investigated agree with those of *B. formicus* (*Escherichia formica*), but its identity cannot be considered proved.

Hydrogenase. It is clear from the experiments cited already that we are dealing with a bacterial enzyme comparable with the dehydrogenases, the substrate in this case being molecular hydrogen. We must assume that the hydrogen is in some way activated, and this activation can be conveniently expressed $H_2 \rightleftharpoons 2H$ without implying anything about the nature of the reaction. In order to conform with the accepted terminology we suggest that this enzyme be called "hydrogenase."

Distribution of hydrogenase. The presence of this enzyme in what appears to be a common bacterial species made us suspect that it might be found in other well-known organisms. Various strains were grown on broth medium, centrifuged and washed, and tested in vacuum tubes in the usual way, and the following results were obtained. (To get a rough comparison between the activities of the different species, the suspensions were diluted to the same opacity.)

	Reduction time (mins.)		
Strain	With H_2	Without H_2	Hydrogenase
Bact. coli (Escherich)	8½	> 120	+
Bact. coli (Houston 1)	11¼	75	+
Bact. coli (Houston 2)	8	> 120	+
Bact. coli (Houston 3)	8¼	> 120	+
Bact. acidi lactici	9¼	> 150	+
Pseudomon. pyocyanea	65	63	−
Chromobact. prodigiosum	> 60	> 60	−
Bac. megatherium	> 120	> 120	−
Bact. lactis aerogenes	9½	15½	?
Clostr. sporogenes	> 150	> 150	−
Bac. subtilis	> 120	> 120	−
Bact. alcaligenes	27½	28	−

The relation of these results to other known facts will be discussed later.

The unexpected discovery of hydrogenase in a number of widely differing bacterial species led us to look for it elsewhere; baker's yeast and sheep's heart muscle, both normally active with regard to other dehydrogenases, were completely lacking in hydrogenase.

Properties of hydrogenase.

(*a*) *Effect of pressure of hydrogen.* From electrode potential measurements it has been shown that methylene blue 50 % reduced would be in equilibrium with 10^{-15} atmospheres of hydrogen at p_H 7·0 and 30°; so that at pressures far too small for measurement we may expect methylene blue to be completely reduced provided the molecular hydrogen is activated, as it is by platinum black or by hydrogenase. This complete reduction at very low pressures naturally cannot be verified experimentally. Apart from the effect of pressure in deciding whether the methylene blue will be reduced or not, we should expect variation of pressure to affect the velocity of reduction, the upper limit of pressure at which this effect becomes noticeable being decided by the affinity of hydrogenase for hydrogen. Actually we have been unable to measure this affinity owing to other complications. In the first place, even at pressures still capable of manometric measurement, the amount of hydrogen in solution in the liquid is insufficient stoichiometrically to reduce the methylene blue present in the tube, so that the rate of diffusion of hydrogen into the liquid becomes the limiting factor; in the second place, at still lower pressures, the total amount of hydrogen in the vacuum tube is insufficient to effect the complete reduction of the methylene blue. By lowering the methylene blue concentration these difficulties could be to some extent removed, but a limit is imposed by the experimental difficulty of measuring the reduction time of very small quantities of methylene blue.

The rates of reduction of the dye were measured at different pressures of hydrogen, using the same apparatus as that described previously, except that a manometer was inserted between the 3-way tap and the pump, and a tap between the manometer and the pump. But this means the vacuum tubes could be filled with hydrogen at any desired pressure.

Two curves were obtained, one using a concentration of 1/20,000 methylene

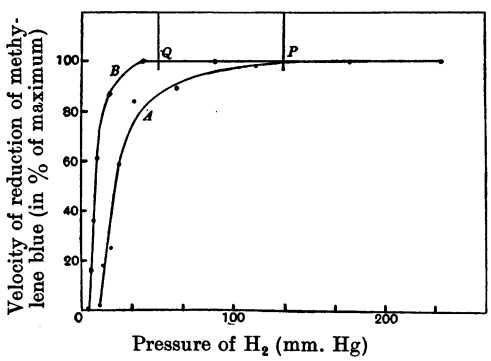

Fig. 1.

blue (Fig. 1, curve *A*), the other using 1/50,000 (curve *B*). From the solubility of hydrogen at 45° and the molecular weight of methylene blue it is calculated

that at a pressure of 130 mm. (*P*) just sufficient hydrogen is dissolved to reduce the higher concentration of methylene blue, and that at 50 mm. (*Q*) there is enough dissolved for the reduction of the lower concentration (*cf.* curves *A* and *B*). Hence the pressure-velocity curves give no measure of the affinity of the enzyme for its substrate, but serve to show that by working at a pressure of one atmosphere we are certain that the enzyme is saturated.

(*b*) *Effect of hydrogen ion concentration.* The relation between hydrogen ion concentration and velocity of reduction of methylene blue has been determined in the case of two organisms, viz. *Bact. coli* (Escherich) and strain 111. The results are given in Figs. 2 and 3, each compiled from the results of two separate experiments.

Alcock and Cook [personal communication] have determined the p_H curves of several bacterial dehydrogenases, and they find that after the characteristic increase of velocity up to a maximum at p_H 6·0 the velocity remains constant up to p_H 9·0, *i.e.* until irreversible destruction of the enzyme takes place in

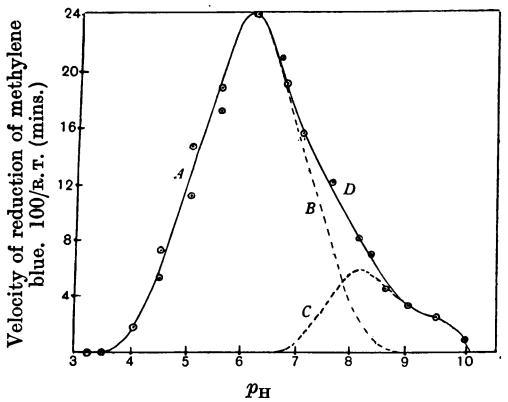

Fig. 2. *Bact. coli* (Escherich).

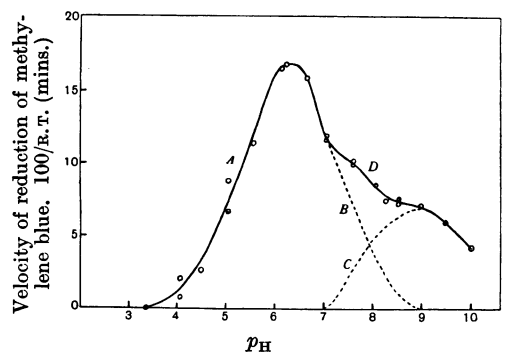

Fig. 3. Strain 111.

the alkaline region. In this respect hydrogenase differs very greatly from these dehydrogenases. Bernheim however [1928] described an aldehyde oxidase

from potato whose p_H-activity curve with respect to methylene blue showed a very clear peak with its optimum at p_H 6·8.

The shoulder on the descending part of the curve is difficult to explain; it is possible to analyse it into two dissociation residue curves, as shown by the dotted lines in the diagram. The ascending part of the curve *A* is identical in the case of the two organisms; the descending line *B* is obtained by drawing a curve symmetrical with *A*; the points on the line *C* are obtained by subtracting the values on the hypothetical curve *B* from the corresponding points on the experimental curve *D*. Whether this may be regarded as evidence for the existence of two hydrogenases having different p_H optima is a matter we are not prepared to discuss; it is noteworthy, however, that the optimum on the first peak is at about p_H 6·3 and that on the second at about p_H 8·5 in both organisms.

(*c*) *Effect of cyanide.* There is obviously an analogy between the activation of hydrogen by hydrogenase and that of the hydrogen atoms of carbon compounds by dehydrogenases. This similarity is borne out by the action of potassium cyanide, as the following experiment shows.

In order to show the effect of cyanide on the rate of reduction of methylene blue in a vacuum tube it is necessary to guard against the loss of hydrogen cyanide during the evacuation. For this purpose vacuum tubes with hollow stoppers were employed, the potassium cyanide solution being placed in the stopper at p_H 12; the contents of the tubes themselves were strongly buffered at p_H 6·0, and it was found by trial that when the contents of the stopper were mixed with those of the tube the p_H of the resulting mixture was not above 6·5, *i.e.* was still within the optimum range.

Results:

Concentration of KCN	Reduction time (mins.)
0	7
M/800	5
M/240	4¼
M/ 80	4
M/ 24	8

(*d*) *Effect of narcotics.* Keilin [1929] has shown that whereas the inhibition of respiration produced by cyanide is due to inhibition of the indophenol oxidase, that produced by urethane is to be attributed to inhibition of the reducing systems of the cell, *viz.* the dehydrogenases. The following figures show that hydrogenase is also inhibited by urethane:

Conc. of urethane (%)	Reduction time (mins.)	% inhibition
0	4¼	0
0·025	7¼	41
0·25	8¾	51·5
2·5	11¾	64

In its reactions to cyanide and urethane hydrogenase behaves as a typical dehydrogenase.

Reduction of other hydrogen acceptors.

One would expect that any substance that can be reduced by a given organism with the aid of reducing substances will also be reduced by it with molecular hydrogen provided that the organism possesses hydrogenase. This has been tested by means of the Barcroft differential manometer in the cases of *Bact. coli* and strain 111.

Reduction of molecular oxygen. For this purpose the right-hand cup of the apparatus was separately evacuated and filled with a gas mixture containing 20 % oxygen and 80 % hydrogen. The left-hand cup remained filled with air. Each cup contained 3 cc. of bacterial suspension in phosphate buffer. A typical result is shown in Fig. 4, curve 1. It is seen that in this case the activity of the enzyme falls off rapidly; we first suspected the formation of hydrogen peroxide but failed to demonstrate it by the peroxidase and guaiacum test. It seems however that mere shaking with air inactivates the enzyme; this we demonstrated by removing the contents of the left-hand Barcroft cup and placing it in a vacuum tube with methylene blue and adding hydrogen in the usual way. The reduction time was > 1 hour, while that of the untreated control was 7 mins.

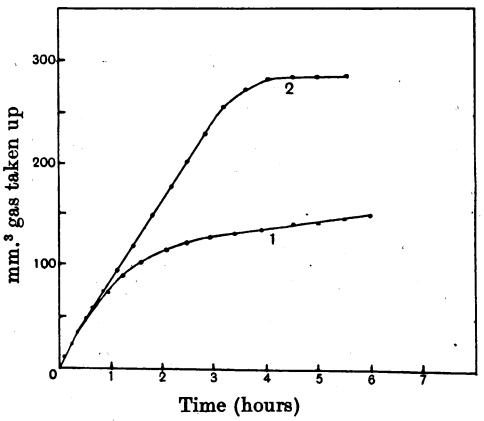

Fig. 4. 1. H_2+O_2. 2. Nitrate in H_2.

Reduction of nitrate. For these experiments both cups of the Barcroft apparatus were completely evacuated and filled with hydrogen. Potassium nitrate (*M*/1000–*M*/300) was present only in cup 2. The uptake of hydrogen proceeded linearly, but never attained completion; Fig. 4, curve 2, gives a typical result, the hydrogen here taken up corresponding to 53 % reduction of the nitrate to nitrite. Estimation of the nitrite at the end of the experiment, however, showed that the nitrate was completely reduced. A further addition of nitrate (in a companion experiment) showed that reduction again proceeded to the same extent, though slightly more slowly, and the same was true of a third and fourth addition (Fig. 5). The explanation of this phenomenon was obtained by carrying out parallel experiments in vacuum tubes where no

hydrogen was present. Here it was found that the nitrate was also reduced by the donators of the cell alone; hence in the Barcroft cup we have the result of a competition for the nitrate between the donators of the cell and the hydrogen.

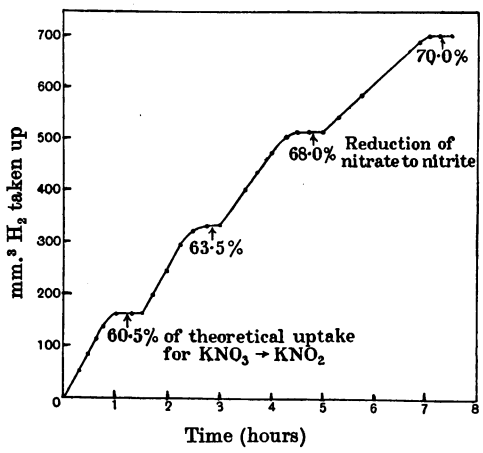

Fig. 5.

Exp.		Nitrate used (*M*)	Nitrite found (*M*)	H_2 uptake (% of theory for $NO_3' \rightarrow NO_2'$)
1	In hydrogen	0·0040	0·0044	41·0
2	,,	,,	0·0043	33·0
3	,,	0·0080	0·0070	39·5
4	,,	,,	0·0072	42·5
5	*In vacuo*	0·0040	0·0042	—
6	,,	,,	0·0037	—
7	,,	,,	0·0040	—

We attempted to obtain a reduction of nitrate by hydrogen and hydrogenase dissociated from reductions by cell substances; for this purpose we incubated the cell suspension with a 0·3 % solution of potassium nitrate for varying periods; the resulting nitrite was then removed by centrifuging and the suspension was tested as before with fresh nitrate in the Barcroft apparatus. This was however unsuccessful as the preliminary treatment so damaged the hydrogenase that the rate was greatly reduced and the hydrogen uptake was even less complete than when the untreated suspension was used.

Reduction of fumarate. The reduction of fumarate by hydrogen was shown in the same way as that of nitrate, *M*/30 to *M*/120 fumarate being used in place of nitrate. Hydrogen was taken up linearly but the uptake ceased when only about 30 to 60 % of that required for the complete reduction to succinate had disappeared. This incomplete uptake is probably due to the same cause as in the case of nitrate, *i.e.* to a part of it being reduced by the hydrogen donators of the cell; possibly also the anaerobic decomposition of fumarate occurring alongside the reduction may be a contributory cause.

The reduction of bacterial substance. It is well known that bacteria, in common with other living tissues, effect two kinds of oxidation, an endogenous

oxygen uptake in which the cell substance alone is concerned [Callow, 1924] and a much more rapid exogenous uptake when an oxidisable substance is added [Cook and Stephenson, 1928]. We therefore sought to discover whether, in addition to the exogenous reductions already demonstrated as the result of the action of hydrogenase, an endogenous reduction of cell material could also occur. For this purpose 3 cc. of a suspension of *Bact. coli* in buffer at p_H 6·5 was placed in the right-hand Barcroft cup and 3 cc. of buffer alone in the left-hand cup; both cups were evacuated and filled with hydrogen; no hydrogen uptake was observed. We therefore conclude that the cell has no store of hydrogen acceptors, and therefore no endogenous reduction occurs comparable with cell respiration.

The relation of hydrogenase to dehydrogenases and its function in cell metabolism.

From its general character it seems justifiable to regard hydrogenase as an enzyme closely related to the dehydrogenase class. It also seems likely that the enzyme which catalyses the change of molecular to activated hydrogen catalyses also the reverse reaction, *viz.* the production of molecular hydrogen from activated hydrogen atoms, that is, the reversible reaction, $H_2 \rightleftharpoons 2H$, should be catalysed in both directions. Previous studies by one of us [Stickland, 1929] have made it clear that the production of molecular hydrogen from formate is the work of two enzymes; it was there shown (1) that non-gas-producing organisms (*e.g. Bact. typhosum*) may possess an active formic dehydrogenase, (2) that in the case of *Bact. coli*, which possesses the double mechanism, treatment with trypsin augments the dehydrogenase and destroys the mechanism producing gaseous hydrogen. We therefore tentatively suggest that the production of molecular hydrogen from formate is the work of the two enzymes formic dehydrogenase and hydrogenase. Such a hypothesis would of course be invalidated if an organism could be found able to produce hydrogen from formate yet not possessing hydrogenase. So far, among the organisms investigated, we have not come across a definite exception of this sort; the following list illustrates this. In the case of *Bact. lactis aerogenes* the presence of

Organism	Ability to produce hydrogen from formate	Presence of hydrogenase
Bact. coli (Escherich)	+	+
Bact. coli (Houston 1)	+	+
Bact. coli (Houston 2)	+	+
Bact. coli (Houston 3)	+	+
Bact. acidi lactici	+*	+
Bact. lactis aerogenes	+	?
Bact. alcaligenes	−	−
Ps. pyocyanea	−*	−
Chromobact. prodigiosum	−†	−
Bac. megatherium	−*	−
Bac. subtilis	−*	−

* Pakes and Jollyman [1901].

† Pakes and Jollyman obtained a positive result with *Chromobact. prodigiosum*, but our strain, tested by ourselves, gave completely negative results.

hydrogenase is not thoroughly established, the reduction of methylene blue being slow and sometimes absent; we propose to investigate this point further.

According to the view suggested above, we should expect organisms possessing hydrogenase to liberate molecular hydrogen from other donators; two conditions at least would however have to be satisfied before this could occur. First, no hydrogen acceptors must be present since these would presumably seize the hydrogen before it was liberated; second, the r_H of the system must be such that the hydrogen would be liberated at some measurable pressure, *i.e.* $r_H \not> 3$, say. For instance, in the case of

$$\text{succinate} \rightleftharpoons \text{fumarate} + 2H,$$

the theoretical pressure of hydrogen in equilibrium with the system is of the order of 10^{-8} atmospheres.

Summary.

1. An enzyme has been found in a number of bacterial species which activates molecular hydrogen; for this the name "hydrogenase" is suggested.

2. By means of this enzyme hydrogen reduces molecular oxygen, methylene blue, nitrate and fumarate.

3. The properties of this enzyme are described, and its relation to other bacterial enzymes and also its function in the cell are discussed.

We wish to record our thanks to Dr Malcolm Dixon for very useful criticism in connection with this work, and to Sir Frederick Hopkins for his kind interest and advice.

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