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Hydrogen evolution of *Enterobacter aerogenes* depending on culture pH: mechanism of hydrogen evolution from NADH by means of membrane-bound hydrogenase

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The pH dependency of cell mass productivity, the hydrogen evolution rate and the yield of hydrogen from glucose was measured by controlling the pH of the culture automatically. The cell mass productivity of *Enterobacter aerogenes* increased in a linear fashion up to a pH value of approx. 7.0. In contrast, both the evolution rate and the yield of hydrogen showed convex relationships up to a pH value of 7.0, both having maximum values at a pH of approx. 5.8. The maximum evolution rate was approx. 11.3 mmol H_2 per g dry cell per h at 38°C. A hypothetical mechanism for hydrogen evolution was proposed by taking our results and other research work into consideration. The proposed mechanism of hydrogen evolution was that NADH was oxidized on the inside surface of the cell membrane and protons were reduced on the outside surface by means of membrane-bound hydrogenase. This mechanism explains in a thermodynamic context the relation between the activity of the hydrogen evolution and the pH of the culture.

Introduction

Biological hydrogen production has been investigated by many authors (for reviews, see Refs. 1 and 2). In general, there are two ways of producing hydrogen using living organisms: one is photo-hydrogen production by photosynthetic organisms and the other is fermentative hydrogen production by chemoheterotrophic bacteria. *Rhodobacter sphaeroides* 8703, a phototrophic bacterium, is capable of evolving hydrogen at a rate of 10.4 mmol/g dry cell per h by using lactate as an electron donor [3]. Clostridia and *Escherichia coli* are also widely studied for their use as fermentative hydrogen-producing organisms. *Clostridium butyricum*, for example, was reported to evolve hydrogen at a rate of 7.0 mmol/g dry cell per h under anaerobic cultivation [4]. We have also reported hydrogen production by using the *Enterobacter aerogenes* strain E.82005, which evolves hydrogen at a rate of 17 mmol/g dry cell per h [5]. These bacteria evolve hydrogen under a very high partial pressure of hydrogen, e.g., 0.3 atm. for *E. aerogenes* [6]. Why do these bacteria evolve hydrogen so vigorously and what is the mechanism by which such evolution takes place?

In 1965, Fredricks and Stadtman reported that hydrogen uptake occurred in a ferredoxin-dependent protein system with NAD^+ as an electron acceptor [7].

$$H_2 \xrightarrow[\text{hydrogenase}]{} \text{Ferredoxin} \xrightarrow[\text{Fd}:NAD^+\ \text{reductase}]{} NAD^+$$

In 1973, Jungermann et al. demonstrated the reverse electron flow from NADH to proton with the aid of acetyl-CoA and crude lysate from clostridia (*C. kluyveri, C. pasteurianum* and *C. butyricum*) [8–12]. Naturally, since the redox potential of NAD at pH 7 ($E_{m7} = -0.320$ V) is far more positive than those of hydrogen ($E_7 = -0.413$ V) and ferredoxin ($E_{m7} = -0.398$ V), the electron flow from H_2 to NAD^+ can be easily understood. However, in the past, it has been difficult to give a thermodynamic explanation of the reverse electron flow. For example, it is known that ferredoxin will be reduced by NADH if the concentration ratio of [NADH]/[NAD^+] is greater than 1000 in living cells or if the partial pressure of H_2 is kept under 10^{-3} atm. as in the rumen [1,13]. However, the concentration ratio of [NADH]/[NAD^+] was reported as approx. 0.3 in *C. kluyveri* cells [14] and the partial pressure of H_2 was very high in the case of these hydrogen-evolving bacteria [6,8]. Therefore, it is still questionable whether the elec-

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tron flow from NADH to H^+ actually proceeds in the living cells.

Since the work of Jungermann et al., several new facts have been reported, such as the pH gradient between the inside and outside of the cell [15,16], and the activity and conformation of membrane-bound hydrogenase [17–21]. In this paper, we report on the pH dependence of hydrogen evolution and the ATP productivity of living cells. Based on these new facts, we propose a mechanism for hydrogen evolution from a thermodynamic point of view, and also suggest a reason why bacteria evolve hydrogen.

Materials and Methods

Organism and culture conditions

E. aerogenes st. E.82005 was isolated from leaves of the *Mirabilis jalapa* (L.) plant as mentioned in a previous paper [6]. The species has been inoculated periodically in our laboratory. For this experiment, a pre-culture was made by transferring the bacterial colony grown on an agar plate and inoculating them into a pre-culture liquid. The culture was then incubated aerobically and stirred constantly for 20 h at 38° C. The culture medium consisted of 1.5% glucose, 0.5% peptone, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% citrate · $2H_2O$ and 0.02% $MgSO_4 \cdot 7H_2O$, all measured in weight percent.

10 ml of the incubated culture was inoculated onto the 250 ml of glucose peptone culture (GP culture). The GP culture contained 10 g of glucose and 50 g of peptone (Nissui Seiyaku Co. Ltd) in 1 l of ion-exchanged water. Incubation was conducted under anaerobic conditions at a temperature of 38° C and the liquid was stirred with a magnetic stirrer.

The pH was controlled with an automatic pH controller (Tokyo Rikakikai Co. Ltd., FC-1), which added 30% NaOH or 30% H_2SO_4 solution as required.

H_2 production rate and dry cell weight

The hydrogen evolution rate was measured by means of a liquid-gas exchange method using an inverted measuring cylinder. The gas was collected over a 30% NaOH solution. 1 ml of the gas exchanged was withdrawn from the cylinder periodically (30 min in the case of rapid evolution and 1 h during periods of slow evolution) and its components were analyzed by gas chromatography using activated carbon as packings for CO_2 and molecular sieves 5A as packings for H_2. However, because of the absorption of carbon dioxide by the NaOH solution, the carbon dioxide peak was not observed. Therefore, the gas evolution rate measured by the measuring cylinder could then be considered to be the hydrogen evolution rate.

Cells in a 30 ml culture liquid were separated in a centrifuge (10000 rpm for 10 min) and then washed by ion-exchanged water. After repeating the procedure twice, the cells were dried in a dryer and weighed.

Results

The pH dependency of hydrogen evolution was studied by varying the pH of the culture in a 300 ml fermenter. The mass of cells grown on 250 ml of GP culture was weighed at the end of the cultivation period, which was assumed to be the time when the evolution rate of hydrogen started to show a significant decrease [5].

Cell mass productivity

The maximum production of cell mass was at approx. pH 7.0. About 468 mg of the cells were obtained after 5 h of cultivation at pH 7.0. However, at pH 5.0, cell weight was only 168 mg after 6 h cultivation, and this decreased to 106 mg over a period of 23 h of cultivation. Over the same period, the amount of hydrogen increased from 43 to 254 ml, but it is worth noting that this extraordinary increase of hydrogen volume was observed only at pH 5.0. Cell mass productivity over the cultivation period is shown in Fig. 1. The productivity increased linearly from pH 5.0 to 7.0, and then decreased above pH 7.0. Therefore, this result indicates that the optimum pH for cell growth is around pH 7.0.

The yield of hydrogen and the evolution rate

The yields of hydrogen from glucose ($Y_{H_2/S}$) obtained from this study are shown in Fig. 1. The maximum yield was about 1.0 mol H_2/mol glucose in the pH range of 5.5–6.0. Above pH 6.0, the hydrogen yield decreased rapidly in contrast with the continuing upward trend shown by cell mass productivity.

Fig. 2 shows the evolution rate of hydrogen measured in terms of mmol/g dry cell per h (cf. Ref. 5). The optimum pH for the evolution rate was found to be around 5.8. The evolution rate of hydrogen at pH 5.8 was approx. 11.3 mmol/g dry cell per h. The optimum

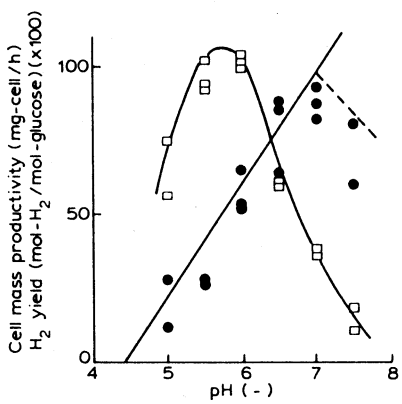

Fig. 1. Effect of cultural pH on cell mass productivity (●) and H_2 yield (□).

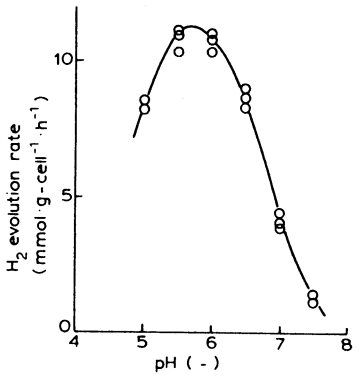

Fig. 2. Effect of cultural pH on H_2 evolution rate.

pH for the evolution rate was the same as the optimum pH for the yield of hydrogen, which we noted in Fig. 1. The evolution rate was seen to decrease rapidly above pH 6.0. At pH 7.0, where growth conditions were most suitable for the bacteria, the rate was approx. 4.0 mmol/g dry cell per h. At pH 7.5, the rate was only 1.3 mmol/g dry cell per h. On the other hand, at pH 5.0, where growth conditions were very unfavorable, the rate was relatively high at 8.5 mmol/g dry cell per h but still below the optimum rate. This difference in behavior between the suitable pH for growth and hydrogen evolution may provide an important clue when considering the mechanism of hydrogen evolution by the fermentation process.

Discussion

At this stage, we would like to discuss the mechanism of bacterial hydrogen evolution from a thermodynamic standpoint by combining our own experimental results with other work in this field.

Redox potential of hydrogen related to the pH of the culture

The hydrogen evolution of *E. aerogenes* was significantly affected by the pH of the culture. Estimating the rate of hydrogen evolution over unit dry cell weight, the optimum pH for hydrogen evolution was approx. pH 5.8 as seen in Fig. 2. The rate decreased rapidly as the pH of the culture increased, e.g., the rate of pH 7.0 was only 35% of the maximum rate. Thus, the pH value of the culture seems a very important factor for hydrogen evolution.

The redox potential of hydrogen is:

$$E = E_0 + \frac{RT}{2F}\ln\frac{[H^+]^2}{p_{H_2}} = -\frac{2.303RT}{F}pH - \frac{RT}{2F}\ln p_{H_2} \qquad (1)$$

where the square brackets denote activity and the parentheses denote partial pressure. Since the E_0 of hydrogen is 0 V and $2.303RT/F$ has a value of about 0.0592 V at 25 °C, the redox potential of hydrogen is calculated as −0.355 V at pH 6.0 and $p_{H_2} = 1$ atm. *E. aerogenes* evolved CO_2 concurrently with H_2 at a ratio of about 2 : 1 [6,22], and the total gas evolved amounted to several times the available gas space in the fermenter. Thus, as a result, the partial pressure of H_2 climbed to approx. 0.3 atm. The potential of hydrogen, E, at pH 6.0 and at a pressure of 0.3 atm. was therefore calculated to be −0.340 V, which may reflect the potential of hydrogen near the cells. The correlations between redox potential and the pH values are shown in Fig. 3.

This hydrogen evolution in *E. aerogenes* could be assumed to occur in the oxidizing reaction of excess NADH below pH 6.3 by stoichiometric consideration, since the bacteria change their metabolic pathway around the pH from the mixed acid production to the 2,3-butanediol production [22–24].

$$NADH + H^+ \rightarrow NAD^+ + H_2 \qquad (2)$$

The above reaction was catalyzed in the saccharolytic clostridia by enzymes such as NADH : ferredoxin oxidoreductase, ferredoxin and hydrogenase [1,13]. These enzymes have been isolated from many microbes (see Refs. 2,17,18,25–31). For example, Vetter and Knappe (in 1971) [32] and Bernhard and Gottschalk (in 1978) [33] isolated the ferredoxin and the hydrogenase of *E. coli*, respectively. Although it seems that very little work has been done on the enzymes of *E. aerogenes*, the taxonomical similarity between *E. coli* and *E. aerogenes* is about 75% [34], and so *E. aerogenes* may have the same or very similar enzymes (e.g., ferredoxin and hydrogenase) as *E. coli*.

Redox potential of NAD related to the internal pH of living cells

In 1976, Padan et al. [15] and Ramos et al. [16] reported independently the very interesting fact that the internal pH of *E. coli* cells was approx. 8.0 while the external pH ranged widely from 5.5 to 9.0. The neutral

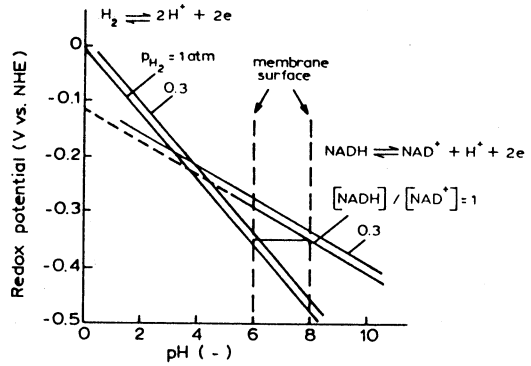

Fig. 3. Relation between redox potentials (25 °C) and pH. Bold broken lines indicate presumed pHs at the inside and outside of the cell.

pH had been generally considered to be favorable to the biochemical reactions, whereas in certain bacteria, the reactions inside the cell were proceeding under slightly basic conditions. This fact made us evaluate the redox potential of NAD at pH 8.0.

The redox potential of NAD is described as:

$$E = E_0 + \frac{RT}{2F}\ln\frac{[NAD^+][H^+]}{[NADH]} \quad (3)$$

$$= E_0 - \frac{2.303RT}{2F}pH + \frac{RT}{2F}\ln\frac{[NAD^+]}{[NADH]} \quad (4)$$

where E_0 is the midpoint potential ([NADH] = [NAD$^+$]) at pH 0 and 25 °C. Since the midpoint potential at pH 7 (E_{m7}) is −0.320 V [1,35], E_0 is postulated as −0.113 V. This correlation is also shown in Fig. 3. When the internal pH of the cell was 8.0, the E_{m8} of NAD was calculated as −0.349 V, which is significantly higher than the E_8 of hydrogen (−0.474 V) and is slightly lower than the potential of hydrogen under special conditions such as pH 6.0 and 0.3 atm H_2 (−0.340 V).

Correlation between the optimum pH for H_2 evolution rate and the activity of hydrogenase-bound cell membrane

In 1979, Adams et al. reported the activity of the membrane-bound hydrogenase of *E. coli* cells [17]. This hydrogenase, which was a dimer of identical subunits with a molecular weight of 113000, showed different optimum pHs for H_2 evolution and uptake. The optimum pH was 6.5 for hydrogen evolution and 8.5 for hydrogen uptake.

These results are very important because they imply that the optimum pHs of hydrogenase coincide very well with the optimum pH of the culture for H_2 evolution and with the internal pH of the cells, respectively. Since the hydrogenase is bound to the plasma membrane and consists of a dimer (recent research made it more clear that the hydrogenase is quaternary, consisting of two subunits with different molecular weights [19]), it seems reasonable to consider that the hydrogenase has an active site on each subunit and that the two active sites may be on different sides of the membrane, i.e., the one interacting with NADH on the cytoplasmic side and the other interacting with proton on the periplasmic side, as shown in Fig. 4.

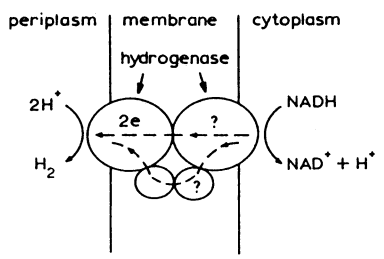

Fig. 4. Schematic diagram of the reduction-oxidation reaction of H^+ and NADH. Dotted lines show possibilities of the electron flow.

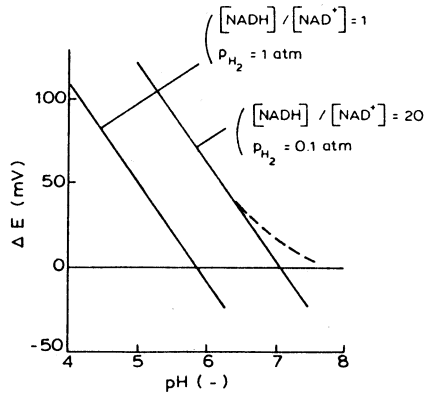

Fig. 5. Potential difference between NAD and hydrogen correlated to cultural pH. Redox potentials were calculated for NAD at pH 8.0 and for hydrogen as a function of cultural pH. Dotted line shows the potential difference calculated under conditions such that the pH of the periplasmic space is lower than the pH of the culture.

Taking the above assumptions into consideration, we are now at a stage to integrate all our results in order to explain the activity of hydrogen evolution.

Activity of hydrogen evolution correlated with potential difference and cell mass productivity

Subtracting Eqn. 4 from Eqn. 1, the potential difference between the outside and within the cells is described as a function of the cultural pH:

$$\Delta E = -59.2\{pH - 5.9\} \quad (5)$$

for $P_{H_2} = 1$ atm, [NADH]/[NAD$^+$] = 1, where the unit of ΔE is estimated in mV and the internal pH is kept at 8.0. If the conditions are $p_{H_2} = 0.1$ atm. around the cells and [NADH]/[NAD$^+$] = 20 in the cells, the potential difference is:

$$\Delta E = -59.2\{pH - 7.1\}$$

$$\text{for } p_{H_2} = 0.1 \text{ atm}, [NADH]/[NAD^+] = 20 \quad (6)$$

Eqns. 5 and 6 are illustrated in Fig. 5 with bold lines.

The potential difference becomes large as the cultural pH becomes small and the larger the potential difference, the easier the hydrogen evolution. However, the activity of hydrogen evolution decreased in Fig. 2 for the cultural pH values smaller than 5.5. This result is easily explained by taking into account the ATP productivity of the cells.

As seen in Fig. 1, the productivity of cell mass increased linearly up to pH 7.0. Considering that the growth yield for ATP (Y_{ATP}) is 10.0 g dry cell per 1 mol ATP [36], the ATP productivity (P_{ATP}) of the cell is

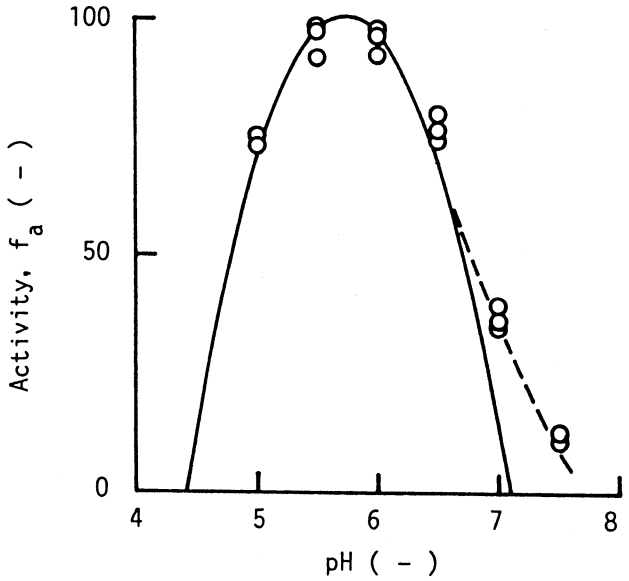

Fig. 6. Activity of hydrogen evolution predicted by Eqn. 9. Dotted line was calculated by using the dotted lines in Figs. 1 and 5. ○, experimental results.

described from the productivity of cell mass (bold straight line in Fig. 1) as a function of the cultural pH:

$$P_{ATP} = 3.8\{pH - 4.4\} \quad \text{for } pH < 7.0 \tag{7}$$

Enteric bacteria produce ATP from glucose mainly through the Embden-Meyerhof pathway under anaerobic conditions. In this pathway, NAD^+ is reduced to NADH in proportion to the production of ATP. Therefore, the activity of the hydrogen-evolving reaction will be described as:

$$f_a \propto (\Delta E)(P_{ATP}) \tag{8}$$

Substituting Eqns. 6 and 7, the above equation is described as

$$f_a = -52\{pH - 5.75\}^2 + 100 \quad \text{for } 4.4 < pH < 7.0 \tag{9}$$

This equations is illustrated in Fig. 6 with a bold line, where the symbol (○) marks the values calculated from the evolution rate of hydrogen in Fig. 2.

The curve shows a good agreement with the experimental results for pH values smaller than approx. 6.5, though the relationship is not so apparent at pH values greater than approx. 6.5. However, since bacteria pump out protons for energy production by the proton pumping theory of Mitchell and the pH difference between the inside and outside of the cell was reported to amount approx. 2 at cultural pH 6.0, proton concentration in the periplasmic space should be higher than that in the culture for the pH range of approx. 6.5–8.0. If the pH of the periplasmic space is 6.82 and 7.00 at cultural pH 7.0 and 7.5, respectively, the potential difference ΔE will still remain positive as shown in Fig. 5 with the dotted line, and consequently the activity of the hydrogen-evolving reaction will be positive and agree with the experimental results, as shown in Fig. 6 with the dotted line. Therefore, the above assumption that protons are reduced to molecular hydrogen on the outside of the cell may be an important explanation of our experimental work.

The reason why bacteria evolve hydrogen

The activity of hydrogen evolution of *E. aerogenes* has been explained in this paper by considering the potential difference and the ATP productivity. Why then do these bacteria waste protons and not use them for their own energy production? The reason for this can be explained by the growth conditions of the bacteria.

As seen in Fig. 1, a cultural pH value should be around 7.0 for rapid growth of the bacteria. However, owing to bacterial acid production, the pH of the culture soon decreases and becomes unsuitable for growth (see Ref. 5). Since, the bacteria cannot live at pH values smaller than approx. 4.4, they are obliged for their survival to stop acid production and/or to carry out a certain biochemical reaction which reduces the proton concentration on the outside of the cell in proportion to the pH of the culture. Taking the above into consideration, it will be agreeable that the bacteria evolved hydrogen vigorously around pH 5.8, and wasted protons which were pumped out from the inside of the cell in the process.

There are still many questions to be solved, such as why NADH does not react with hydrogenase in vitro. Also, how do we take the membrane potential into consideration when evaluating the potential difference. Furthermore, we know little about the actual ratio of $NADH/NAD^+$ in the cell or the partial pressure of hydrogen in the periplasmic space. However, the activity of hydrogen evolution and the reason for such evolution in *E. aerogenes* has been explained in this paper from a thermodynamical viewpoint by the suggestion that protons are reduced to hydrogen on the outside of the cell membrane.

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