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# A Study on Physiological conditions for hydrogen evolution by *Rhodopseudomonass phaeroides* K-7

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# *Rhodopseudomonas sphaeroides* K-7에 의한 수소생성에 미치는 생리적 조건에 관한 연구

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## ABSTRACT

Physiological conditions for hydrogen evolution by *Rhodopseudomonas sphaeroides* K-7 are examined. Larger amount of molecular hydrogen was evolved at 30°C, pH 6.8~7.0 under anaerobically illuminated condition of about 12,000 lux by the organism. The heighest rates of hydrogen evolution were observed in the culture with the organic acids such as acetate, DL-lactate or DL-malate in media containing L-glutamate as a nitrogen source. Hydrogen was also evolved from glucose with the rate of 178.9 μl/hr/mg cells (dry weight). When glucose was adopted as a sole carbon source, however, consid erable time lag of about 20 hours was required for hydrogen evolution. The resting cells stored at0°C under argon maintained the rate of hydrogen evolution in nearly about 90% of initial one even after 40 days of storation.

## INTRODUCTION

Photosynthetic bacteria are known to evolve molecular hydrogen when grown photoheterotrophically and anaerobically. The light-dependent evolution of molecular hydrogen was first observed in *Rhodospirillum rubrum* by Gest and Kamen.[1] Thereafter, similar observations have been made with various photosynthetic bacteria.[2~6] Hydrogen evolution has been suspected to be mediated by nitrogenase on the basis of many indirect evidences.[4,6,7] But more detailed studies on natures of hydrogen evolution still remain in order to increase the rate of hydrogen evolution for utilization of the photosynthetic bacteria with practical purposes. In present work, we examine the physiological conditions in which molecular hydrogen could be evolved more effectively with purple nonsulfur photosynthetic bacterium, *Rhodopseudomonas sphaeroides* K-7 isolated by the authors.[8]

## MATERIALS AND METHODS

**Bacterial strain**

Among the several strains isolated and identified as previously reported,[8] *Rhodopseudomonas sphaeroides* K-7 was selected and used because of its excellent capacity of hydrogen evolution.

**Growth media and conditions**

The medium of Ormerod *et al.*[2] was used with slight modification. Yeast extract was omitted. Instead, thiamine (1 $\mu$g/m*l*) and *p*-aminobenzoic acid (0.2 $\mu$g/m*l*) were added as growth factors. To the above medium, DL-malate (30mM) was added as a electron donor (minimal malate medium). In addition, ammonium sulfate was replaced by L-glutamate (7mM) as a nitrogen source. In plate culture, 1.5% agar was added to the medium.

Cells were grown at 30°C under anaerobic condition illuminated laterally with two 200-W incandescent lamps (ca. 10,000~15,000 lux). Intensity of illumination was measured by Rodiometer/Photometer Model 550—1 (EG & G, Electro-Optics Div., Salem, Mass.)

**Preparation of resting cell suspensions**

Resting cells were prepared by the method of kelley *et al.*[7] Cultures were harvested at late exponential phase and centrifuged to precipitate cells. The pellets were washed twice with 10mM potassium phosphate buffer (pH 6.8) finally resuspending in the same buffer. The resting cell suspensions were stored at 0°C under argon until required.

**Measurement of hydrogen evolution**

Hydrogen evoution was carried out in serum bottles (25m*l*) containing 10m*l* mixtures of resting cell suspension ($A_{660}$, 0.7) and DL-malate (30 mM), L-glutamate(7mM) medium. After capping the reaction vessels with gas-tight rubber stoppers, the mixtures were gassed with argon for 3 min and incubated in a shaker bath at 30°C under illumination by two 200-W incandescent lamps. After a given time passed, samples (300$\mu$*l*) of gas phase were withdrawn with a gas syringe (Precision Sampling Co.).

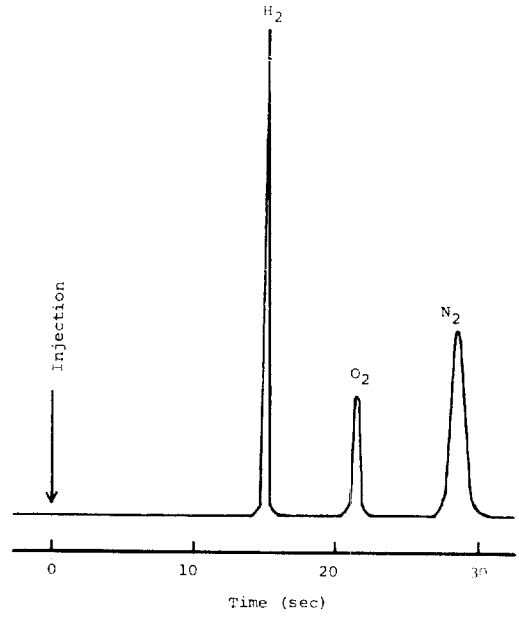


**Fig. 1.** Gas chromatographic pattern of $H_2$, $O_2$ and $N_2$.

It was analyzed for hydrogen with a Varian 3,700 gas chromatograph with a thermal conductivity detector which was set at 120°C. The stainless-steel colum (2m×1/8 inch) was packed with molecular sieve 5A (45 to 60 mesh) and was maintained at 80°C. Argon was used as a carrier gas (flow rate, 40m*l*/min). The results were quantitated by relating the peak height to a calibration curve made with pure hydrogen obtained from hydrogen generator (General Electric Co.): Retention time was identified by standard gases (Fig. 1). Other variations in physiological conditions for hydrogen evolution are detailed in connection with the experimental results.

**Cell mass determinations**

Bacterial concentrations were measured either by determining culture absorbancy at 660 nm or dry weight of centrifuged cells(washed

once with distilled water and dried in preweighed aluminum cups at 105°C for 6 hrs).

## RESULTS AND DISCUSSION

### Effects of temperature and pH on hydrogen evolution

Fig. 2 shows effects of temperature on cell growth and hydrogen evolution. At optimum temperature, 30°C, for cell growth, hydrogen was also evolved more largely. At 40°C, cell growth occurred in the nearly same extent as at 20°C, but hydrogen evolution decreased largely. This indicates that hydrogen evolving system in *R. sphaeroides* K-7 may be rather heat-labile. In Fig. 3, cell growth and hydrogen evolution at different pH are shown. Optimum pH for hydrogen evolution lies around 6.8 where optimum growth was also obtained. In two other sides of pH 6.8, both cell growth and hydrogen evolution decreased markedly. After measurement for hydrogen evolution, final pHs were checked. These values were somewhat different from initial ones presumably due to accumulation of metabolites.

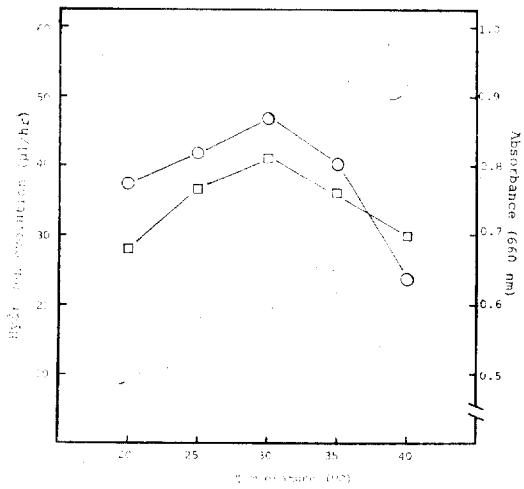


**Fig. 2.** Effect of temperature on cell growth and hydrogen evolution. ○: Hydrogen evolution, □ : Difference of cultural absorbance between initial and final one.

### Effect of illumination intensity on hydrogen evolution

In Fig. 4, effect of illumination intensity on cell growth and hydrogen evolution is shown. The optimum range for hydrogen evolution lies around 12,000 lux. Above 12,000 lux, cell

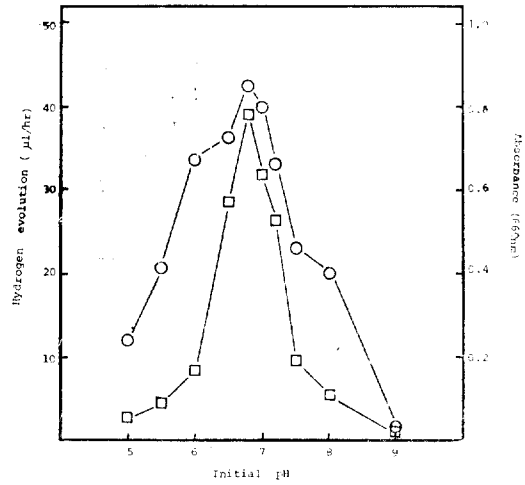


**Fig. 3.** Effect of pH on cell growth and hydrogen evolution. ○ : Hydrogen evolution, □ : Difference of cultural absorbance between initial and final one.

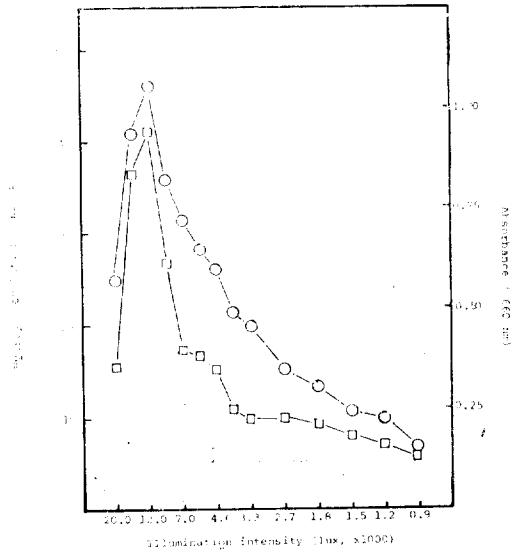


**Fig. 4.** Effect of illumination intensity on cell growth and hydrogen evolution. ○ : Hydrogen evolution, □ : Difference of cultural absorbance between initial and final one.

**Table 1.** Hydrogen evolution[a] with various amino acids.

| Amino acid | Rate of hydrogen evolution (μl/hr/mg cells(dry weight)) | Amino acid | Rate of hydrogen evolution (μl/hr/mg cells(dry weight)) |
|---|---|---|---|
| Glutamate | 58.5 | Glycine | 24.4 |
| Aspartate | 35.0 | Proline | 23.9 |
| Asparagine | 34.6 | Lysine | 21.5 |
| Tryptophan | 31.8 | Arginine | 21.3 |
| Methionine | 30.3 | Glutamine | 20.4 |
| Histidine | 30.0 | Serine | 20.2 |
| Valine | 28.3 | Tyrosine | 19.3 |
| Phenylalanine | 28.2 | Threonine | 17.1 |
| Alanine | 27.1 | Cysteine | 10.9 |
| Isoleucine | 27.0 | None[b] | 34.2 |
| Leucine | 24.8 | | |

a Incubation was carried out for 15hrs in mixtures containing DL-malate (30mM) and either the indicated amino acids (7mM) of L-forms.
b Mixture containing only DL-malate (30mM) was used.

**Table 2.** Hydrogen evolution[a] with various electron donors.

| Substrate | Rate of hydrogen evolution (μl/hr/mg cells(dry weight)) |
|---|---|
| Glucose | 178.9[b] |
| Acetate | 131.1 |
| DL-Lactate | 95.1 |
| DL-Malate | 79.6 |
| Succinate | 77.8 |
| Formate | 76.9 |
| Sucrose | 71.1 |
| Fumarate | 70.0 |
| Gluconate | 69.5 |
| Maleate | 62.9 |
| Tartarate | 0 |
| Propionate | 0 |
| Control[c] | 0 |

a Incubation was carried out for 45hrs in mixtures containing either the indicated electron donors (30mM) and L-glutamate (7mM).
b The rate was calculated from data obtained after lag period (ca. 20hrs).
c Mixture containing only L-glutamate(7mM) was used.

growth and hydrogen evolution decreased as in the range of low illumination intensity. From the result, it is evident that hydrogen-evolving system in this bacterium is dependent on light.

**Hydrogen evolution with various amino acids**

Twenty kinds of amino acids were examined as nitrogen sources in special regard to rate of hydrogen evolution with DL-malate as a primary carbon source (Table 1). Hydrogen was evolved from each of all amino acids with different rates. The rate of hydrogen evolution was best with L-glutamate. If no amino acids were added in the mixture, hydrogen was evolvveed with the rate of 34.2 μl/hr/mg cells (dry weight). This low rate in compared with that from L-glutamate was due to absence of nitrogen sources to support cell growth. Hence only hydrogen-evolving systems already present in resting cells might be largely responsible for hydrogen evolution. In addition, this rate was higher than the rates from amino acids other than glutamate, aspartate and asparagine although all twenty amino acids supported cell growth. When DL-malate was omitted from the mixture (Table 2), hydrogen was not evolved. From the results, it is evident that hydrogen was evolved through metabolism of DL-malate, and its evolution might be inhibi-

ted by the metabolites of amino acids rather than by amino acids themselves.

**Hydrogen evolution with various organic substrates**

It has been known that purple nonsulfur photosynthetic bacteria require various organic acids to supply additional energy needed for hydrogen evolution though a significant portion comes from light through photophosphorylation.[3,4] We examined the efficiency of various organic acids and sugars on hydrogen evolution as electron donors with L-glutamate as the source of nitrogen (Table 2.). Among them, higher rates of hydrogen evolution were observed with the organic acids such as acetate, DL-lactate or DL-malate. The rate from DL-malate, 79.6 $\mu l$/hr/mg cells (dry weight), was different from that shown in Table 1. This was due to the difference in incubation time during which hydrogen evolution increased in fact not linearly as shown in Fig. 5. So the rate of hydrogen evolution should be considered with the incubation times described. Glucose had also excellent effect on the rate of hydrogen evolution with value of 178.9 $\mu l$/hr/mg cells (dry weight). With glucose, however, *R. sphaeroides* K-7 evolved hydrogen only after a considerable lag, about 20 hours (Fig. 5). During that time, glucose might be metabolized into various organic acids in cells. In addition, hydrogen could be evolved from sucrose and gluconate, while no hydrogen was evolved from tartarate and propionate. When intermediates of TCA cycle were used, the rates of hydrogen evolution were 70~80 $\mu l$/hr/mg cells (dry weight). Thus the rates of hydrogen evolution vary to a certain extent with different carbon sources, presumably because of differences in their reduction states and patterns of metabolism. From the result it is also found that *R. sphaeroides* K-7 has the capacity of utilizing various kindes of reduced carbon compounds as electron donors.

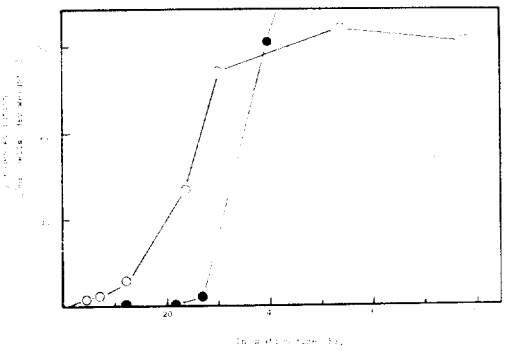

**Fig. 5.** Hydrogen evolution in mixtures containing L-glutamate (7mM) supplemented with DL-malate (30mM : ○) or glucose (30mM : ●)

**Table 3.** Hydrogen evolution in resting cells stored at 0°C under argon for 40 days.

| Duration (days) | Rate of hydrogen evolution[a] ($\mu l$/hr/mg cells (dry weight) | % |
|---|---|---|
| 1 | 34.7 | 100 |
| 13 | 35.6 | 102.6 |
| 26 | 33.4 | 96.3 |
| 40 | 31.3 | 90.2 |

a Incubation was carried out for 15hrs in mixture containing only DL-malate (30mM).

**Stable maintenance of resting cells**

Resting cells were prepared and stored at 0°C under argon until required. Cells were taken at appropriate time intervals and examined the capacity of evolving molecular hydrogen. As shown in Table 3, about 90% of the capacity was stably maintained after 40days of storation. The slight decrease in rate of hydrogen evolution was due to death of small amount of cells during the period.

## 摘 要

*R. sphaeroides* K-7에 의한 수소 생성에 미치는 생리적 조건을 조사한 결과 수소생성은 혐기성조건하에서 30°C, pH 6.8~7.0, 조명도 약 12,000 lux를 유지하였을 때 활발히 이루어지는 것으로 나타났다. 아미노산으로서는 글루타민산, 유기산으로는 초산, 젖산, 혹은 사과산을 이용했을 때 높은 수 소생성이 나타났으며 포도당과 같은 당류에서도 수소생성이 이루어졌다. 휴지기 세포는 약 40일 보관후에도 90% 정도의 수소생성능을 유지하고 있었다.

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