CONF-9504160--2

ENZYMATIC PRODUCTION OF HYDROGEN FROM GLUCOSE[1]

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To be presented at the
Hydrogen Review Meeting
April 18-21, 1995
Coral Gables, Florida

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[1] Research sponsored by the Office of Utility Technologies, Advanced Utility Concepts Division, U.S. Department of Energy.

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Abstract

The objective of this research is to optimize conditions for the enzymatic production of hydrogen gas from biomass-derived glucose. This new project is funded at 0.5 PY level of effort for FY 1995. The rationale for the work is that cellulose is, potentially, a vast source of hydrogen and that enzymes offer a specific and efficient method for its extraction with minimal environmental impact. This work is related to the overall hydrogen program goal of technology development and validation. The approach is based on knowledge that glucose is oxidized by the *NADP*$^+$ requiring enzyme glucose dehydrogenase (GDH) and that the resulting *NADPH* can donate its electrons to hydrogenase (H_2ase) which catalyzes the evolution of H_2. Thus hydrogen production from glucose was achieved using calf liver GDH and *Pyrococcus furiosus* H_2ase yielding 17% of theoretical maximum expected. The cofactor *NADP*$^+$ for this reaction was regenerated and recycled. Current and future work includes understanding the rate limiting steps of this process and the stabilization/ immobilization of the enzymes for long term hydrogen production. Cooperative interactions with the Universities of Georgia and Bath for obtaining thermally stable enzymes are underway.

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Introduction

Cellulose represents a vast supply of reducing power and, although non-reducing, the individual glucose molecules of the cellulose polymer can be oxidized using the enzyme glucose dehydrogenase (GDH). GDH, first described in 1931, catalyzes the oxidation of glucose and hexose-6-phosphates using both nicotinamide adenine dinucleotide *(NAD^+)* and nicotinamide adenine dinucleotide phosphate *($NADP^+$)* as cofactors (Tranulis *et al.*, 1994). The reduction potential (E'_0) for the half-reaction for the reduction of *$NADP^+$* is -320 mV which is too high for the reaction of *NADPH* with most soluble hydrogenases for H_2 evolution (Greenbaum, 1980). However two hydrogenases have been shown to be capable of interacting with reduced nicotinamide nucleotides with concomitant hydrogen evolution. These are the soluble hydrogenases of *Alcaligenes eutrophus* (Egerer *et al.*, 1982) and *Pyrococcus furiosus* (Ma *et al.*, 1994). Since cellulose is an abundant primary and secondary resource that can be enzymatically degraded to glucose (Woodward, 1987) development of an enzymatic process for the generation of hydrogen from cellulose is feasible (Fig. 1).

It was the objective of this current work to demonstrate and optimize hydrogen production using this system (initially starting from glucose) and the results obtained so far are presented in this report.

Experimental Procedures

Materials

β-Glucose was purchased from Calbiochem. Glucose dehydrogenase (GDH) from calf liver and β-*$NADP^+$* were purchased from Sigma Chemical Company. Hydrogenase (H_2ase) from *Pyrococcus furiosus* was purchased from Dr. Michael Adams, University of Georgia.

Apparatus: Hydrogen Detection

A continuous flow system was constructed to measure the production of hydrogen as shown in Fig. 2. The system was continuously purged with helium and calibrated with an inline electrolysis cell and Faraday's law of electrochemical equivalence connected in tandem with the hydrogen detection system. The latter consisted of a combustible gas analyzer (Bio-Gas Detector Corporation, Okemos, MI) consisting of a Figaro semiconductor tin oxide gas sensor. A voltage increase occurs across the sensor when a combustible gas comes into contact with the sensor surface due to a decrease in the sensor resistance and this was measured by a Keithley Model 2000 multimeter. Current was measured with a Keithley Model 485 picoammeter. Data was analyzed using ASYST™ Technologies, Inc., 4.0 Analysis Software.

Enzyme Assay and Hydrogen Production

The activity of GDH was measured in a 1.0 ml reaction mixture containing 10 µmol glucose, 1 µmol *NADP*$^+$ and 0.75 mg GDH in 50 mM sodium phosphate buffer, pH 7.5 at room temperature (23°C). The production of *NADPH* was monitored at 340 nm in a Carey 219 spectrophotometer. One unit of activity is defined as that amount of enzyme which produces 1 µmol *NADPH* min^{-1} under the conditions of the assay. H_2ase was assayed in the flow system described above by measuring its *NADPH*-dependent H_2 evolution activity at room temperature (Ma *et al.*, 1994). The reaction mixture contained 0.4 mM *NADPH* and 0.1 ml H_2ase in 50 mM sodium phosphate buffer, pH 8.0. One unit of activity is defined as the production of 1 µmol H_2 min^{-1} under the assay conditions. A typical reaction mixture for the generation of hydrogen from glucose contained 10 µmol glucose, 1 µmol *NADP*$^+$, 1.0 mg GDH, 0.1 ml H_2ase all in 50 mM sodium phosphate buffer at a given pH. The reaction was run at room temperature.

Results and Discussion

The activity of calf liver GDH was calculated to be 2.0m units/mg at 23°C and its K_m and V_{max} determined to be 59 mM and 0.02 µmol min^{-1} with respect to glucose. The K_m with respect to *NADP*$^+$ was estimated to be 10 µM. The activity of H_2ase was calculated to be approximately 0.011 units ml^{-1} at 23°C. According to Ma *et al.*(1994) the K_m of H_2ase for *NADPH* is 200 µM. The enzymatic generation of hydrogen gas from glucose (and ultimately cellulosic materials) requires that the properties of GDH and H_2ase be compatible with respect to pH and temperature optimum/stability. Both enzymes are active between pH 7.0 and 9.0 with a respective optimum of pH 7.5 for GDH and pH 8.0 for H_2ase (Fig. 3). A comparison of the effect of temperature on the activity of GDH and H_2ase showed the difference between these two enzymes (Fig. 4) with respect to their temperature optimum. This is expected since *P. furiosus* is an hyperthermophile that grows optimally at 100°C (Ma *et al.*, 1993). It can be seen that there is little difference in the rate of *NADPH*-dependent hydrogen evolution between room temperature (23°C) and 60°C; however above 70°C the rate does increase over two-fold in agreement with the data of Ma *et al.* (1994) that showed less than a two-fold rise in activity from 60-80°C using *NADPH* (1.5 mM) as the electron donor. In our experiments a concentration of 0.2 mM *NADPH* was used.

The stability of *NADPH* at pH 8.0 was determined by measuring the yields of hydrogen from 0.2 mM *NADPH* at several temperatures. The data in Fig. 5 indicate that the yields obtained were close to theoretical maximum at both 60°C and 80°C. The major difference in these two sets of data is that it took over 12 h to reach theoretical maximum at 60°C unlike 2.5 h at 80°C. This would be explained by the difference in H_2ase activity between the two temperatures but also suggests that *NADPH* is stable under these experimental conditions. Clearly compatibility in the thermal stability of GDH and H_2ase is necessary for long-term hydrogen production at the fastest rates. Calf liver GDH is stable at 40°C for at least 5 h but above this temperature the rate of *NADPH* production becomes non-linear after 1 h suggesting instability above 40°C (Fig. 6).

Hydrogen was generated from the reaction mixture containing glucose, GDH, H_2ase and *NADP*$^+$ (Fig. 7). As far as is known, this is the first demonstration of the enzymatic conversion of glucose to hydrogen by such an *in vitro* system. The two experimental runs shown, were conducted at pH

7.0 and pH 8.0. At pH 8.0 the rate of H_2 evolution rose to a maximum after 6 h and then declined to zero after 20 h. The percentage of the theoretical yield of H_2 was 17%. At pH 7.0 the time to reach the maximum rate of H_2 evolution was 20 h at which time the yield of H_2 was 16% of theoretical maximum. At this point the experiment was stopped (by the addition of hydrogen peroxide; 0.025% final concentration in the reaction mixture) and so the maximum yield was not determined. It was also evident from these data that the co-factor *$NADP^+$* was regenerated and reused during the course of the reaction. Recently, we have shown that at pH 7.0, yields of H_2 at 30 h can be obtained close to theoretical maximum. Furthermore injection of another 10 µmol of glucose yields a further 10 µmol H_2 after 10 h. These data (not shown) are particularly exciting because they demonstrate: 1. the theoretical maximum yield of H_2 is obtainable, 2. the system had long term stability, at least at 23°C and, 3. the co-factor was recycled 20 times.

Future work will focus on obtaining maximum yields of H_2 in shorter reaction times. This will be achieved by increasing the rate of glucose oxidation and hydrogen evolution by increasing the reaction temperature. A thermally-stable glucose dehydrogenase from *Thermoplasma acidophilum* has been expressed in *Escherichia coli* from which it can be extracted and purified (M. Danson, University of Bath, personal communication). A sample of this GDH will be obtained from Dr. Danson's laboratory and will be used in our system. Ultimately, GDH and H_2 ase will be immobilized in order to recover them from the reaction mixture and for their subsequent reuse. It should also be pointed out that the other product of this reaction, gluconic acid, is a valuable commodity used as a sequestering agent and in the dairy and brewing industries (Szmant, 1986). Such a reaction may also have advantages for the manufacture of gluconic acid over fermentation methods currently used (Godfrey and Reichelt, 1983).

Acknowledgements

We would like to thank Steve Blankinship for his continuing assistance with the experimental apparatus for this project, Eli Greenbaum, Barbara Evans and Lynette Stephan for useful technical discussions. We are also grateful to the Office of Utility Technologies, Advanced Utility Concepts Division, U.S. Department of Energy for financial support.

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Legend to Figures

Figure 1- Reaction pathway for the conversion of cellulose (glucose) to hydrogen.

Figure 2- Schematic illustration of the reaction system and detection apparatus used in the enzymatic conversion of glucose to hydrogen. The data for hydrogenase was taken from Ma et al., 1994.

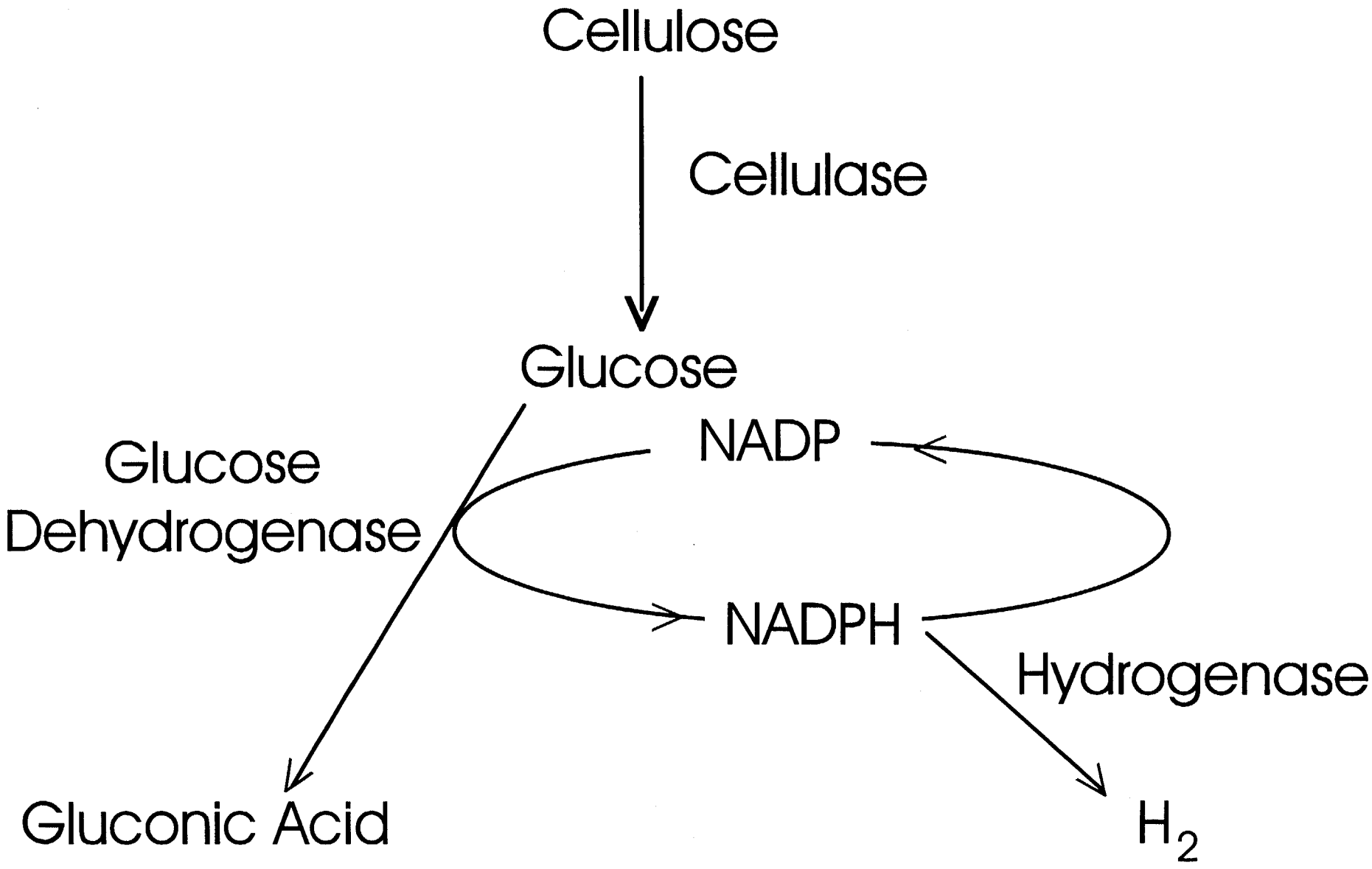
Figure 3- Activity of glucose dehydrogenase and hydrogenase as a function of pH.

Figure 4- Activity of glucose dehydrogenase and hydrogenase as a function of temperature.

Figure 5- NADPH-dependent evolution of hydrogen by hydrogenase as a function of temperature

Figure 6- Stability of glucose dehydrogenase as a function of temperature.

Figure 7- Enzymatic conversion of glucose to hydrogen gas.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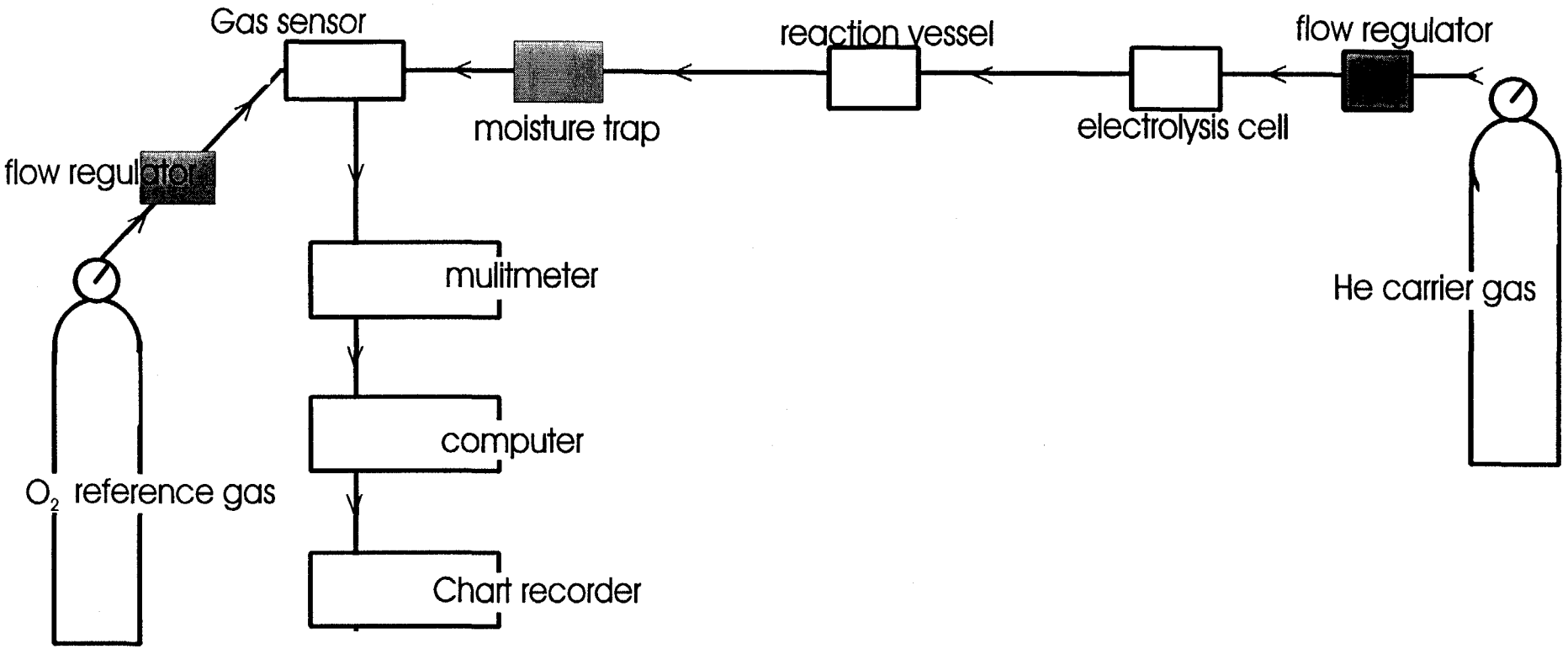
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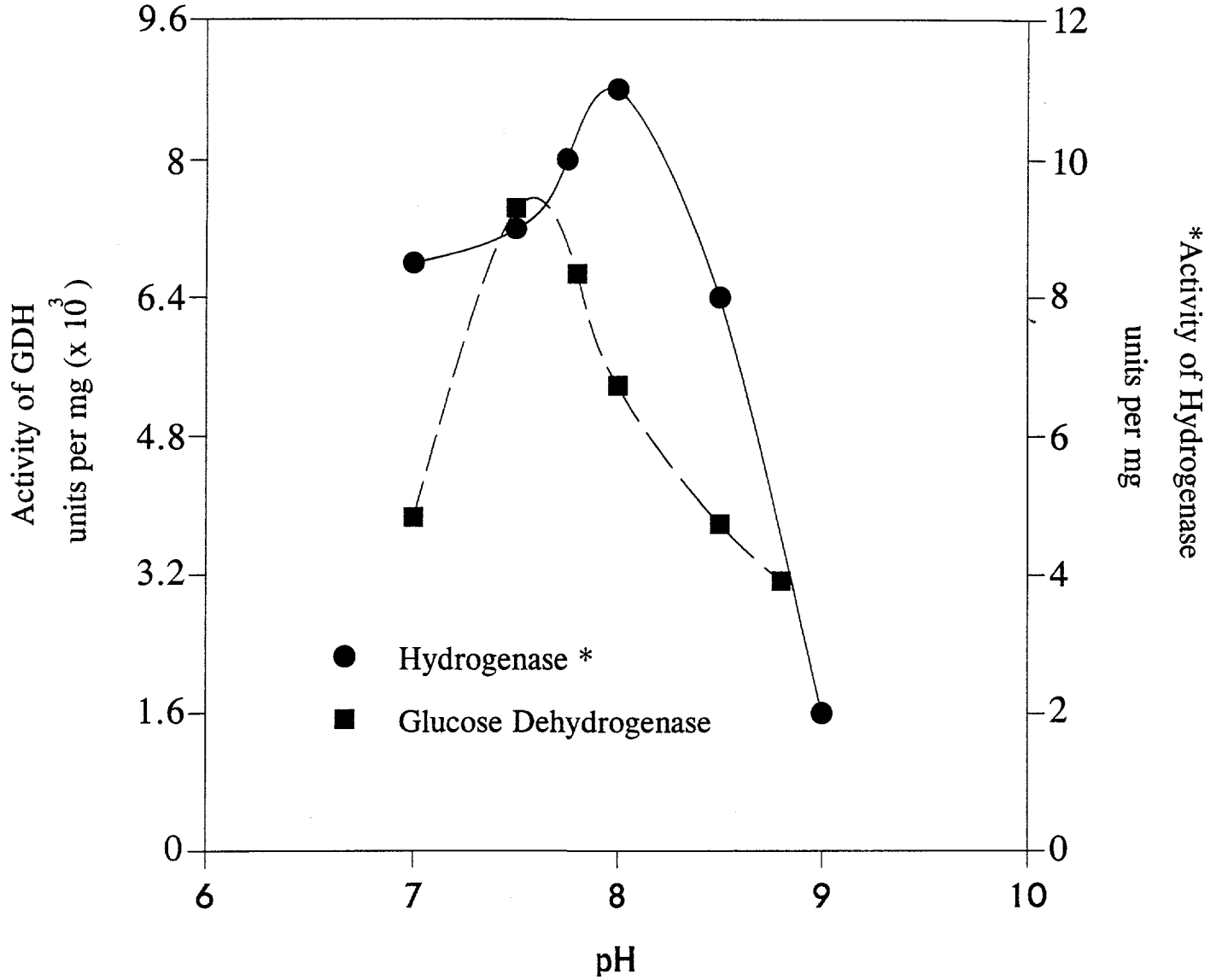

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Fig. 3

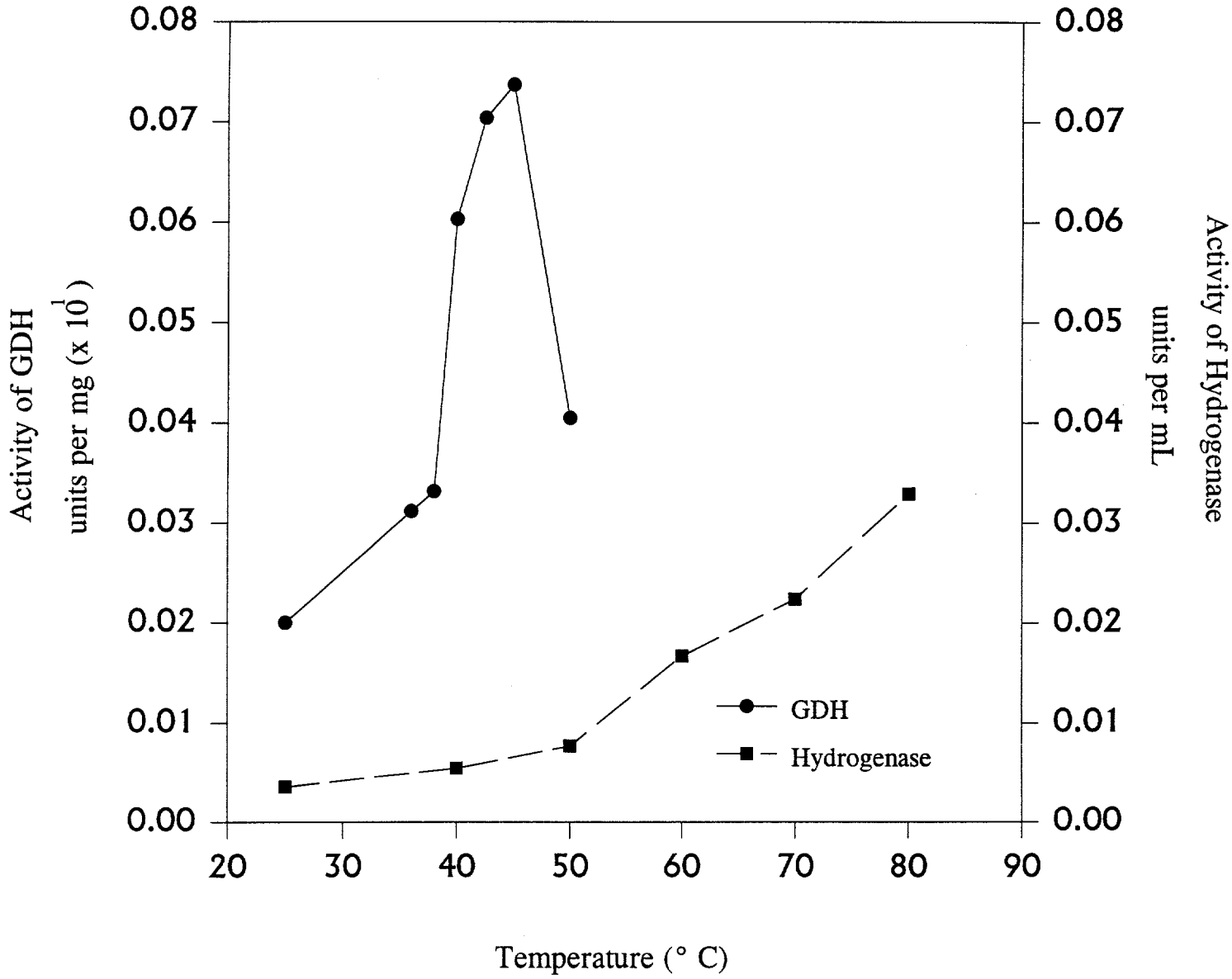

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Fig. 4

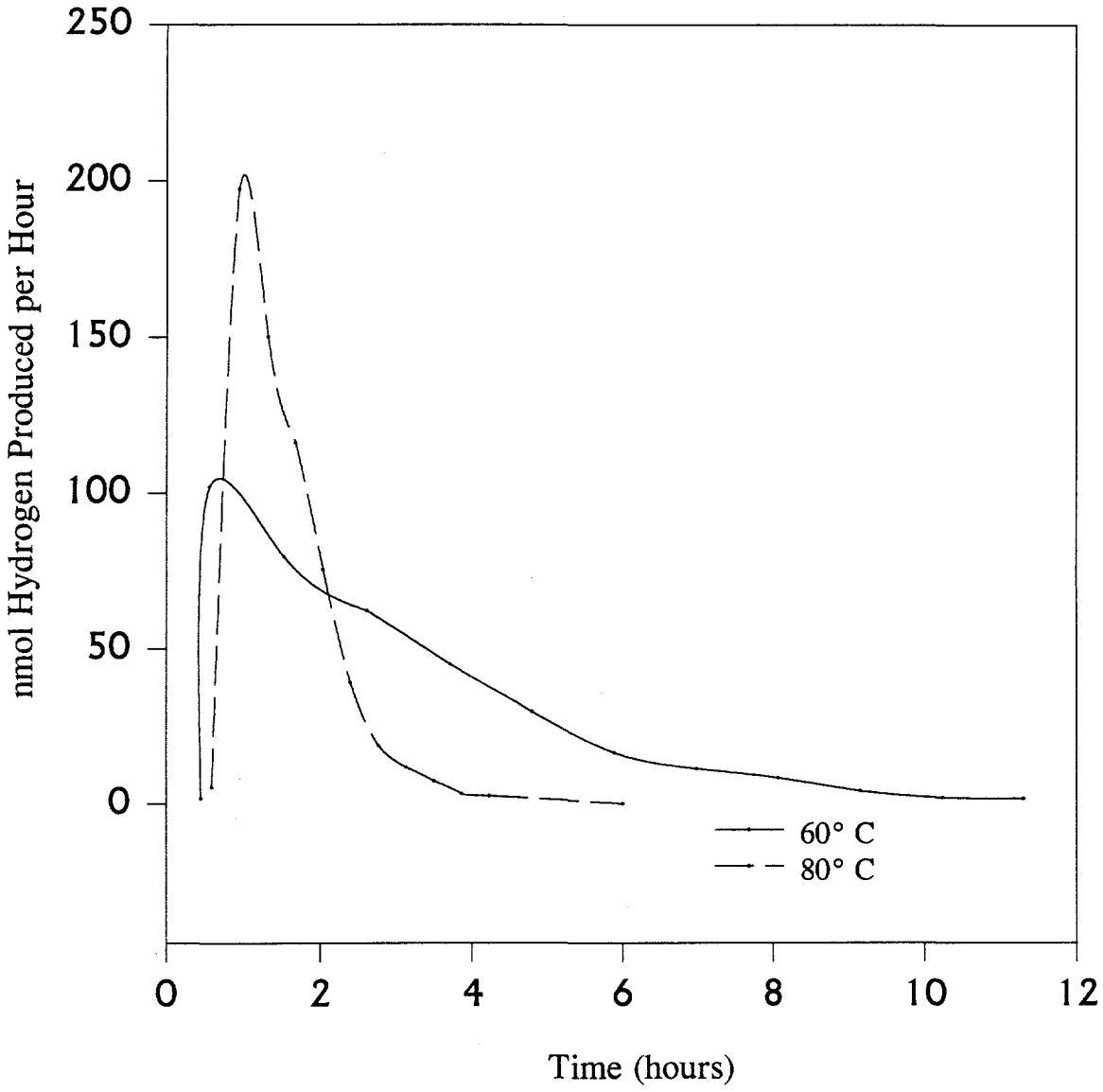

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Fig 5

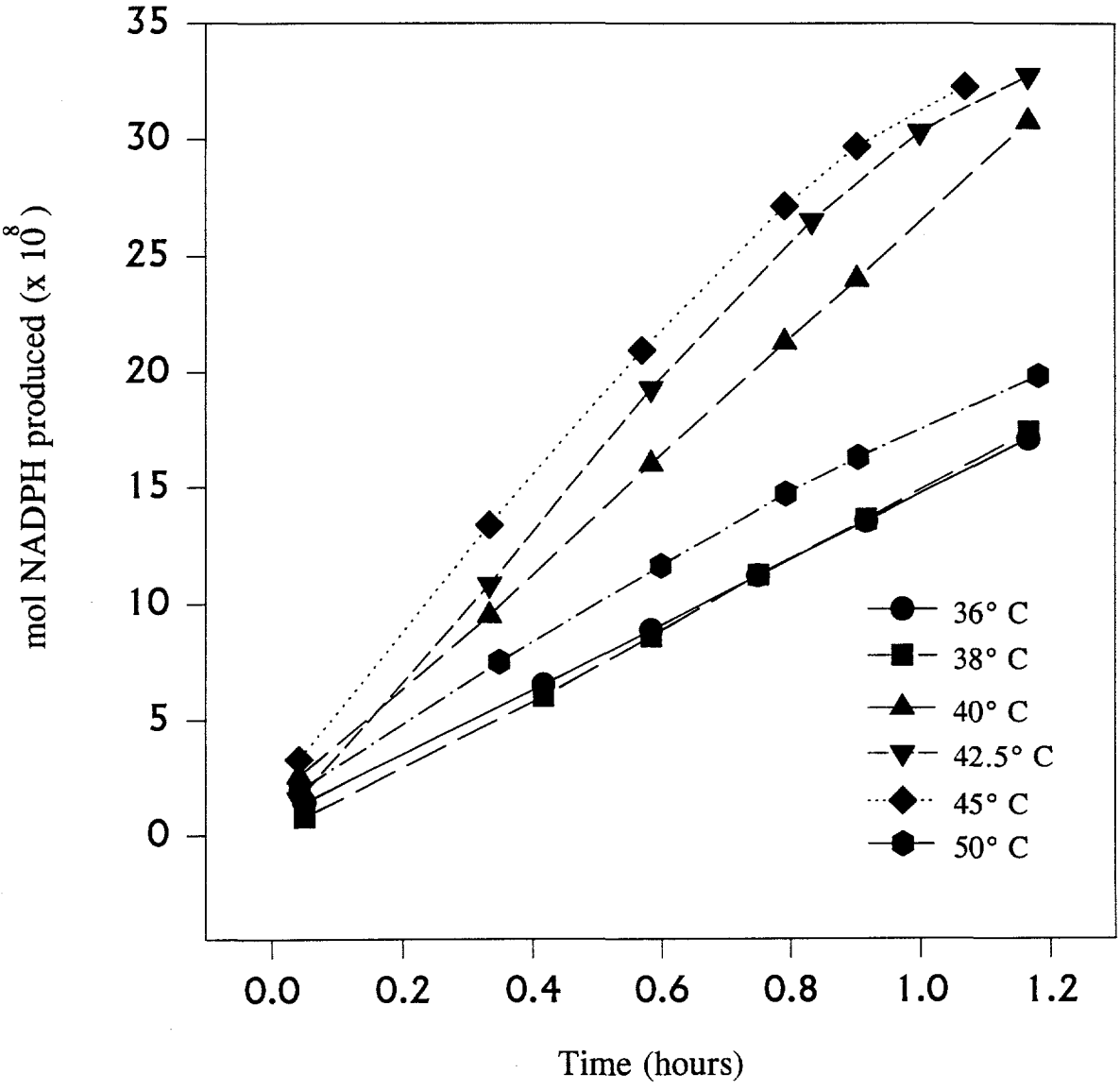

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Fig. 6

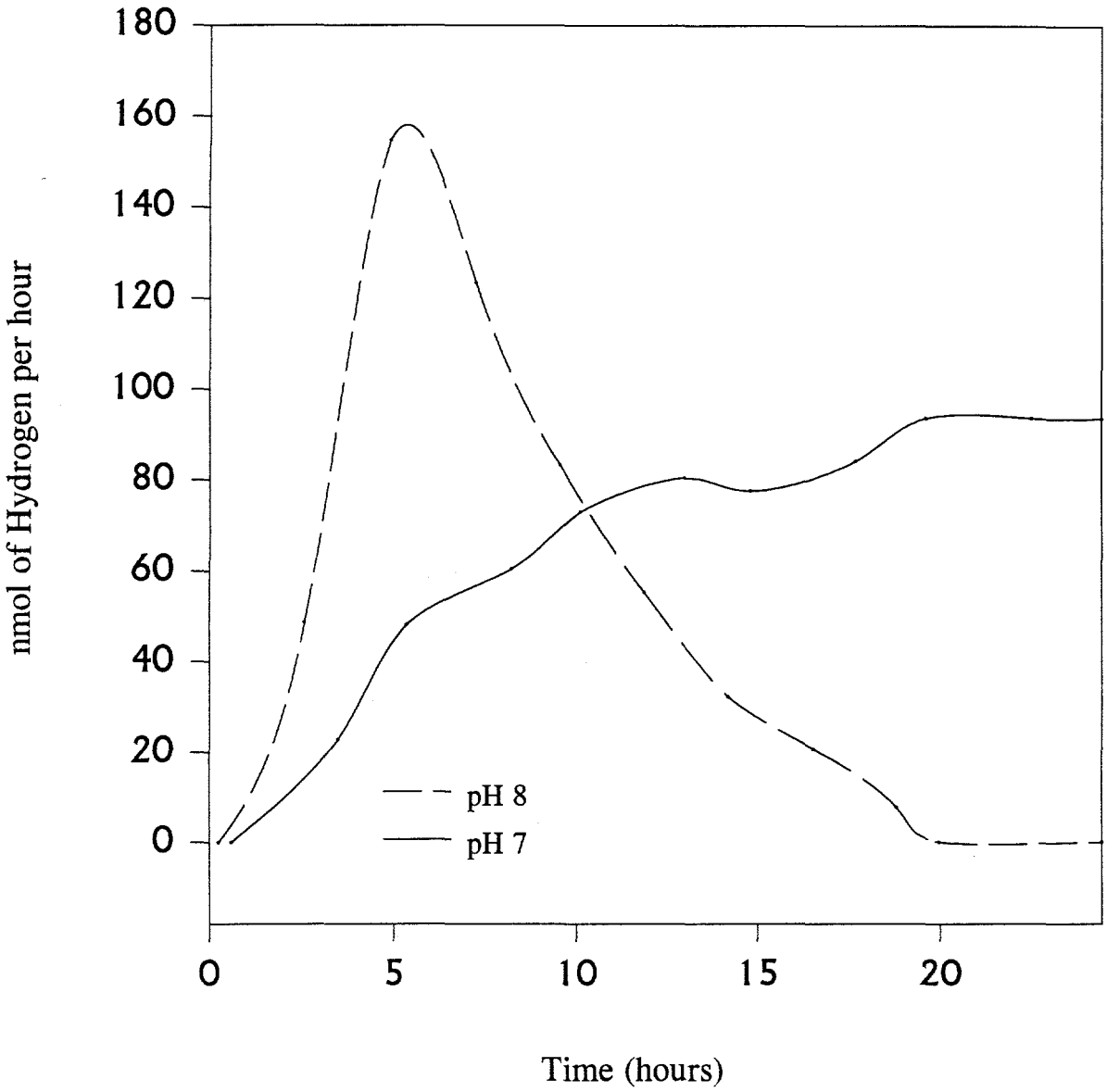

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Fig. 7