

Efficient Production of Hydrogen from Glucose-6-Phosphate

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Abstract

The maximum molar yield of hydrogen that can be obtained from a renewable sugar such as glucose is 12 mol H₂ per mole glucose. In this work it has been shown that the enzymes of the oxidative cyclic pentose phosphate pathway coupled to hydrogenase, using NADP⁺ as the electron carrier, is capable of generating 11.6 mol H₂ per mole of glucose-6-phosphate (G6P). These results provide the first evidence to indicate that the maximum molar yield of hydrogen from a renewable sugar, such as glucose, is possible enzymatically, and, as such, could lead to an efficient process for biohydrogen production.

Introduction

The development of alternative sources of energy to oil, is essential as geologists have predicted that oil shortages will begin in about 10 to 20 years. Even with the discovery of new major oil fields such as those of the Caspian Basin, oil shortages are still inevitable within a generation (Kerr, 1998). Introduction of a hydrogen economy into society now seems even more important than a generation ago. Biohydrogen production *in vivo* and the enzymatic conversion of glucose to H₂ has been demonstrated as a method for H₂ production (Zaborsky, 1998, Woodward et al., 1996, Hershlag et al. 1998). Glucose, a sugar produced by photosynthesis, is renewable, unlike fossil fuels such as oil. Examples of other renewable sugars include xylose, lactose and sucrose (Goyal et al., 1991, Woodward, 1984) all of which have the potential to be converted into hydrogen gas utilizing two enzymes, glucose dehydrogenase (GDH) and hydrogenase from the extremophiles *Thermoplasma acidophilum* and *Pyrococcus furiosus* respectively (DeLong, 1998, Bonete et al., 1996, Smith et al., 1989, Woodward and Orr, 1998). In this enzymatic pathway for H₂ production, GDH catalyzes the oxidation of glucose to gluconic acid (via the lactone) using NADP⁺ as the electron acceptor.

NADPH simultaneously reduces hydrogenase and H₂ is evolved. The maximum stoichiometric yield of hydrogen possible from glucose by this procedure is only 1 mol per mole sugar which can be achieved (Woodward et al., 1996). However, this value represents only 8.3% of the theoretical yield. We postulated that the enzymatic conversion of glucose to H₂ might result in the maximum yield (~12 mol of H₂ per mole of glucose) if hydrogenase was included with the enzymes of the oxidative cyclic pentose phosphate pathway. This was based on results from the pioneering experiments of Horecker and Racker who showed, respectively, that the oxidation and decarboxylation of 6-phosphogluconate (6PG) resulted in the formation of pentose phosphate and demonstrated the cyclic nature of the complete oxidation of glucose-6-phosphate (G6P) to NADPH and CO₂ (Horecker and Smyrniotis, 1950, Couri and Racker, 1959).

Methods

Yeast enzymes of the pentose phosphate pathway were obtained from Sigma Chemical Company. In experiments measuring NADPH (NADH) or hydrogen production the reaction mixture contained 1 manufacturer's unit of G6P dehydrogenase, 6PG dehydrogenase, phosphoribose isomerase, Ru5P-epimerase, transaldolase, transketolase, aldolase, phosphoglucoisomerase, fructose-1,6-bisphosphatase and 50 units of triosephosphoisomerase. Other details are given below. Fructose 1,6 diphosphatase was dialyzed against buffer, pH 7.5 prior to use because the commercial preparation was shown to contain an inhibitor of the G6P/6PG dehydrogenase couple. Hydrogenase was purchased from the University of Georgia, Athens. Hydrogen evolution was measured using an in-line hydrogen sensor with a flow-detection system described in detail previously (Greenbaum, 1984). The reaction mixture for measuring Hydrogen production by enzymes of the oxidative branch of the pentose phosphate cycle contained 2 μmol of G6P, 1 unit each of G6P and 6PG dehydrogenases, 63 units of *Pyrococcus furiosus* hydrogenase, and 4 μmol of NADP⁺ in 2.0 mL 50 mM sodium phosphate buffer, pH 7.5, at 40°C. The reaction mixture for measuring the enzymatic conversion of G6P to H₂ by enzymes of the oxidative and regenerative branch of the pentose phosphate cycle contained 1 μmol of G6P, each of the enzymes of the pentose phosphate cycle (see ref. 6), 1 μmol of co-carboxylase, 1.0 μmol of NADP⁺, and 63 units of hydrogenase in 2.0 mL of 200 mM Hepes buffer, pH 7.5, containing 10 mM MgCl₂, at 30°C. The starting pH was measured at 7.4; and after 92 h of reaction, the pH was 7.2.

Production of reductant and regeneration of G6P by the oxidative pentose phosphate cycle was measured in a reaction mixture containing 3.0 mL of 0.2 M Hepes buffer, pH 7.5 at 23°C, contained 0.05 μmol of G6P, each of the enzymes of the oxidative pentose phosphate cycle, 1.0 μmol of thiamine pyrophosphate (cocarboxylase), 1.0 μmol of NADP⁺, and 100 μg of the antibiotic spectomycin. An increase in absorbance at 340 nm due to the formation of NADPH was monitored over time. For measuring G6P formation from Ru5P, the reaction mixture (total volume, 3.19 ml) consisted of 3.0 ml of hexokinase assay reagent, containing G6P dehydrogenase (Sigma), each of the enzymes of the oxidative pentose phosphate cycle except G6P and 6PG dehydrogenases, 1.0 μmol of cocarboxylase, 1.5 μmol of Ru5P, and 100 μg of spectomycin. The increase in absorbance at 340 nm due to the formation of NADH by the action of G6P dehydrogenase in the hexokinase reagent was measured at 23°C.

The simultaneous production of H₂ and CO₂ by the oxidative branch of the oxidative pentose phosphate cycle was measured in a reaction mixture (2.0 ml) containing 1 μmol G6P, 2 μmol EDTA, 10 μmol MgCl₂, 1 unit each of G6P dehydrogenase and 6PG dehydrogenase, 4 μmol of NADP⁺, and 63 units of hydrogenase in 0.2 M HEPES, pH 7.5 in 2.0 mL 0.2M HEPES buffer, pH 7.5, at 30°C. For further details see Lee et al. (1996).

Results and Discussion

We conducted experiments to determine whether the oxidative pentose phosphate cycle could be utilized to improve the molar yield of H₂ from glucose by using G6P as the starting substrate. First, G6P dehydrogenase, 6PG dehydrogenase and hydrogenase were employed to convert G6P to ribulose-5-phosphate (Ru5P). Since, in these reactions, 2 mol of NADPH and 1 mol CO₂ are formed per mole G6P, it follows that if hydrogenase is present, 2 mol of hydrogen could also be generated since NADPH is the physiological electron donor to *P. furiosus* hydrogenase (Ma et al., 1994). This was indeed the case and the oxidative branch of the pentose phosphate pathway was capable of generating the maximum theoretical yield of hydrogen possible (i.e. 2.0 H₂ mol/mole G6P). The yield of CO₂ (0.85 mol/mole G6P) gives a ratio of H₂ to CO₂ of 2.3 and agrees quite well with the expected theoretical value (Fig. 1).

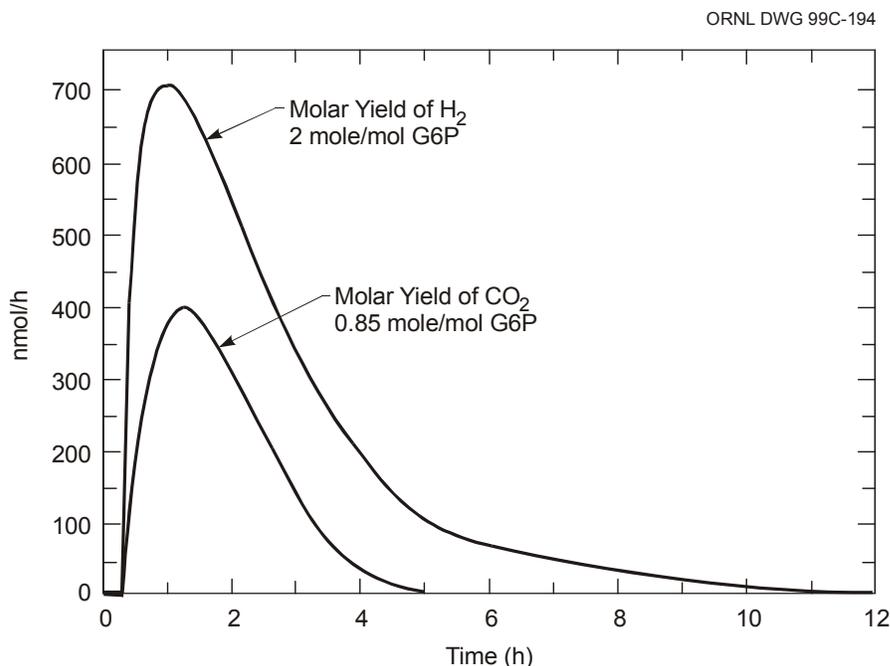


Fig. 1. Simultaneous production of H₂ and CO₂ by the oxidative branch of the oxidative pentose phosphate cycle.

A maximum rate of hydrogen evolution of ~700 nmol/h was observed and the reaction rate declined to zero after 5 h. No inhibition of G6P dehydrogenase by NADPH (Zubay, 1993) would be likely since it is oxidized by hydrogenase as soon as it is formed. The kinetics of these reaction remain to be optimized.

In an experiment to determine whether or not the non-oxidative branch of the pentose phosphate pathway was capable of regenerating G6P from Ru5P, enzymes of the pathway were incubated with 0.05 μmole G6P and 1.0 μmole NADP^+ and the formation of NADPH monitored. If the pathway is kinetically capable of cyclic behavior, a theoretical maximum of 0.6 μmole NADPH could be produced. The data of Fig. 2A show that after 80 min 0.16 μmole of NADPH was produced indicating 27% efficiency after this time. After 17 h of the reaction the 0.36 μmole of NADPH was produced (60% efficiency). This observed efficiency is expected if G6P is regenerated and indicates that greater than 3 cycles of the pathway have occurred after 17 h. The kinetics of this reaction are such that 0.1 μmole NADPH is formed rapidly (within 5 min) after the initiation of the reaction and can be explained by the rapid conversion of G6P to Ru5P with the concomitant generation of NADPH. Further production of NADPH is slow and limited by the enzyme-catalyzed reactions of the non-oxidative branch of the pathway. This would not be surprising since the pool of sugar phosphates is at equilibrium in cells containing transketolase and transaldolase (Glasser and Brown, 1955). The conversion of Ru5P to G6P suggested a similar kinetic pattern to that observed above in that there was an initial faster rate of G6P production in the first hour of the reaction. After 48 h, an initial 1.5 μmol Ru5P had led to the generation of 0.97 μmole G6P which represents ~65% of the maximum theoretical amount possible (Fig 2B). Other reasons for the slow kinetics of either NADPH or G6P production after the initial burst of activity could be product inhibition or enzyme inactivation over time. Inorganic phosphate is an inhibitor of G6PDH and triosephosphate isomerase (Zubay, 1993, Oesper and Meyerhof, 1950). NADPH is a potent inhibitor of yeast G6PDH with a K_i of 27 μM (Zubay, 1993). The concentration of NADPH reached 120 μM after 17 h of reaction and might suggest a reason for the slow production of NADPH (Fig. 2A). The G6PDH/6PGDH enzyme couple was also determined to be unstable when heated at 35.5°C losing all of its activity after 60 h.

Hydrogen was evolved upon the addition of NADP^+ when the enzymes of the pentose phosphate pathway were mixed together with hydrogenase and 1.0 μmole G6P. The rate of hydrogen evolution reached a maximum of 425 nmol/h (Fig. 3, Curve A). These data are significant because 11.6 μmol hydrogen per mole of G6P were evolved after 92 h and are compared to the maximum yield of 2.0 mol H_2 from the oxidative branch of the cycle (Fig. 3, Curve B). This represented 97% of the maximum yield of hydrogen possible. Such high stoichiometric yields of hydrogen have never been obtained from glucose fermentation by anaerobic bacteria because most of the reducing potential produced by such cells is needed for ATP synthesis with subsequent growth and maintenance of the organism (Thauer et al., 1977). Typically, the molar yield of hydrogen from glucose is 1–4 mol H_2 per mole of glucose by anaerobic fermentation (Solomon, 1995). However, yields of 8.0 and 9.8 mol H_2 per mole of glucose have been reported by a combination of *Clamydomonas* and *Rhodospseudomonas* and by *Oscillatoria* sp. respectively (Miura, 1995). If glucose is the starting substrate, the requirement of ATP to generate G6P must be taken into consideration when calculating the energy efficiency of the process. The high molar yields of hydrogen obtained using enzymes of the pentose phosphate pathway coupled via NADP^+ with hydrogenase gives a thermodynamic efficiency of 98%. This value is much higher than the 25–50% energy efficiency common to the majority of anaerobic bacteria that ferment glucose (Thauer et al., 1977). Two other observations are noteworthy. Firstly, no G6P was detected in the reaction mixture at the completion of the experiment. Secondly, the cofactor NADP^+ was recycled 11.6 times during the experiment. This is important because co-factor requiring reactions are

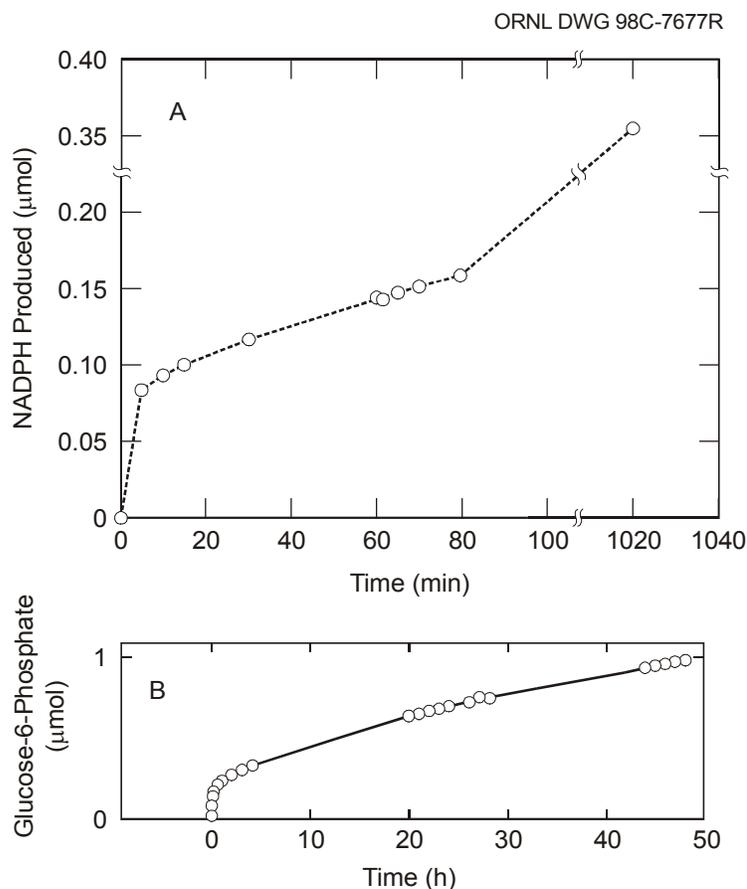


Fig. 2. Production of reductant (A) and regeneration of G6P (B) by the oxidative pentose phosphate cycle.

generally considered to be uneconomical if utilized on an industrial scale. The cost of NADP^+ could be reduced drastically if it was recycled. An example whereby the co-factor NAD^+/NADH is recycled on a practical scale is the commercial production of *L-tert-leucine*. In this case, polymer enlarged NADH was recycled 125,000 times reducing the co-factor costs by several orders of magnitude. It should also be noted that cofactors in bulk quantities are commercially available e.g. ATP \$0.33/g, NADP^+ \$2.06/g (Kragl, 1996). The production of G6P enzymatically, therefore, even without the regeneration of ATP from ADP and P_i should be commercially feasible.

A process for the efficient production of hydrogen using enzymes of the pentose phosphate pathway coupled to hydrogenase could become practically feasible if they were isolated from thermophilic microorganisms (extremophiles). This could result in higher rates of hydrogen evolution and faster yields because *Pyrococcus furiosus*, the source of hydrogenase used in these studies, is a hyperthermophile whose hydrogenase activity is optimal at 85°C (Bryant and Adams, 1989). Pentose phosphate enzymes from yeast used in this study are rapidly inactivated above 45°C , hence the necessity for maintaining a reaction temperature of 30°C at which the activity of hydrogenase is an order of magnitude lower than that at 80°C . In this regard, the complete genome sequence has been determined for *Aquifex aeolicus*, one of the most thermophilic bacteria known and genes encoding most of the enzymes of the pentose phosphate pathway have been identified. The cloning

and expression of these genes from *A. aeolicus* could provide enzymes that are active and stable at 60°C and above. Also, plasmids containing genomic *Methanococcus jannaschii* DNA fragments encoding transaldolase, glucose-6-phosphate isomerase and triose phosphate isomerase have been cloned into expression vectors and expressed in active form. Obtaining thermally stable forms of the enzymes of the pentose phosphate pathway active at higher than ambient temperatures in large quantities therefore, is technically feasible. The possibility of producing hydrogen utilizing these enzymes in immobilized form also exists including a polymer enlarged NADP⁺ co-factor. Although this process also generates CO₂ as a by product (6 mol/mole G6P), it should not be considered as contributing to global warming but rather as green technology that recycles the CO₂ that was reduced to sugar by the process of photosynthesis. This is in contrast to fossil fuel utilization for the production of fuels and chemicals that simply adds CO₂ to the atmosphere.

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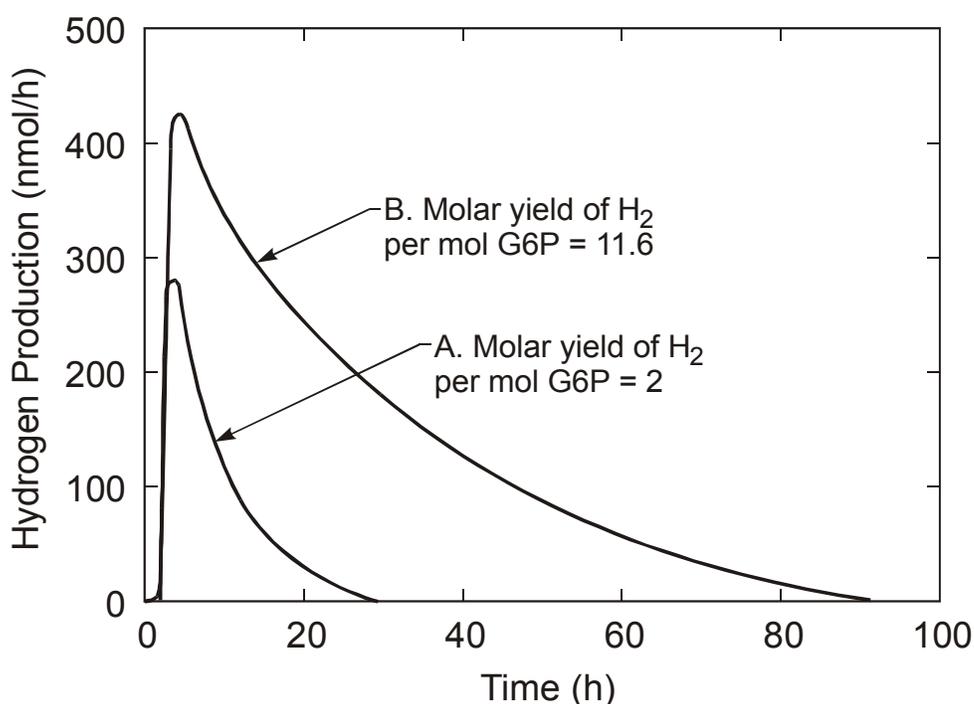


Fig. 3. Hydrogen production by enzymes of the oxidative pentose phosphate cycle.

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Legends to Figures

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