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Regeneration of NADPH and Hydrogenation of Ketones to Alcohols with the Combination of Hydrogenase, Ferredoxin-NADP Reductase, and Alcohol Dehydrogenase

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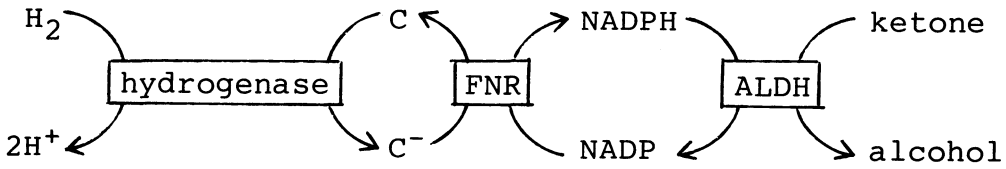
The regeneration of nicotinamide-adenine dinucleotide phosphate (reduced form, NADPH) by the reduction of NADP by hydrogen gas was carried out with the [hydrogenase-ferredoxin-NADP reductase (FNR)] system. Ketone hydrogenation was tried by the combination of the above system and alcohol dehydrogenase (ALDH), as shown in the following scheme:



C: electron carrier

Scheme 1.

In the [hydrogenase-FNR] system, NADP was easily reduced by hydrogen to form NADPH. By adding ALDH to the above system, the hydrogenation of ketone to alcohol was observed. The ketones used in this study were 2-butanone, 2-pentanone, and 2-hexanone. After 8 h, the turnover number of NADP was 13 for 2-butanone, 15 for 2-pentanone, and 10 for 2-hexanone, showing a recurrence of NADP by hydrogen gas.

Some photochemical,[1-3] electrochemical,[4-7] and enzymatic[7-9] systems for NAD(P)H regeneration have been reported. Among these systems, enzymatic systems are of great advantage to produce compounds with a high optical purity, and to the simplicity of the systems.

In this study the [hydrogenase-FNR] system for the regeneration of NADPH from NADP was established by using hydrogen gas as a reducing agent; ketone hydrogenation was tried by a combination of the system with ALDH as shown in Scheme 1.

Experimental

The bipyridinium compounds used in this experiment are listed in Fig. 1. Compounds **A**, **B**, and **C** were synthesized according to the literature.[10,11] The hydrogenase from *Desulfovibrio vulgaris* (Miyazaki type, IAM 12604) was purified according to Yagi's method.[12] The activity (1 unit) of hydrogenase used was to reduce 1 μmol of methyl viologen for 1 min in the system containing hydrogenase and methyl viologen (3.7×10^{-2} mol dm^{-3}) in 5.0 ml of a 0.02 mol dm^{-3} Tris-HCl buffer (pH 7.0) under 1 atmospheric pressure of hydrogen at 30 °C. FNR from spinach leaves was purified by Shin's method.[13] The concentration of FNR is not known but it had the ability to reduce 0.37 μmol of 2,6-dichlorophenol-indophenol (DPIP) in the following system: NADPH (1.2×10^{-4} mol dm^{-3})-DPIP (7.4×10^{-5} mol dm^{-3})-FNR (2.0%) in 5.0 ml of 0.05 mol dm^{-3} Tris-HCl buffer (pH 7.0) at 30 °C for 1 min. ALDH from *Thermoanaerobium brockii* was obtained from SIGMA Co.

The sample solution, which consisted of hydrogenase, bipyridinium salt, FNR, NADP, and ketone (if included) in a Tris-HCl buffer (pH 7.0), was deaerated by repeated freeze-pump-thaw cycles. The reaction was carried out at 30 °C by the introduction of hydrogen gas into the above system.

Ketones and alcohols were analyzed by gas chromatography with a 3-m long column of PEG 6000 at 80 °C by using nitrogen as a carrier gas. The electronic spectra were measured using a Shimadzu MPS 5000 spectrometer, and the concentration of NADPH was determined from the absorbance at 340 nm.

Results and Discussion

Reduction of Bipyridinium Compounds by Hydrogen. When hydrogen gas was introduced into a system containing hydrogenase and methyl viologen, a

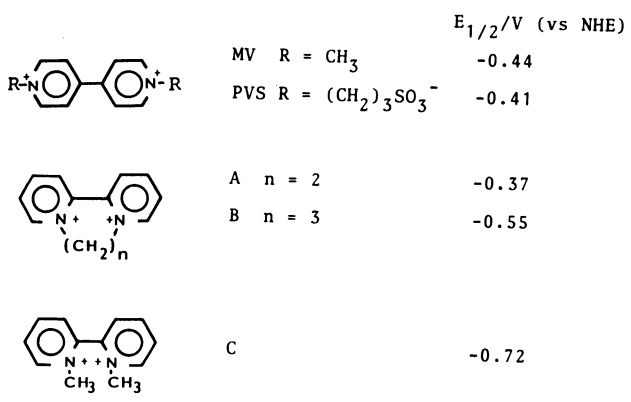


Fig. 1. Bipyridinium salts as electron carriers.

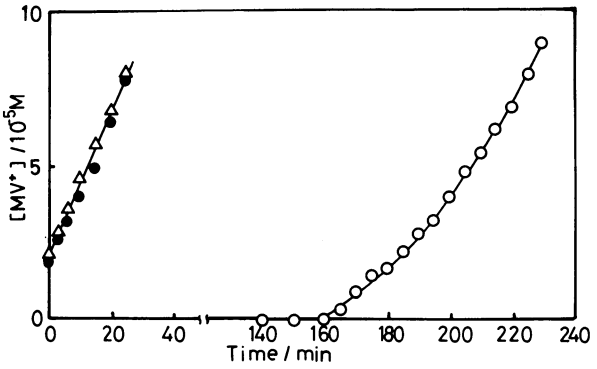


Fig. 2. Reduction of methyl viologen by hydrogen gas with hydrogenase. Reaction conditions: Methyl viologen, 3.7×10^{-2} mol dm^{-3}; hydrogenase, 4.9 units dm^{-3}; hydrogen, 0.97 atm; Tris-HCl buffer, 0.4 mol dm^{-3}, pH 7.0; reaction temp, 30°C. a (○): No pretreatment, b (△): pretreated with RSH (2.0×10^{-4} mol dm^{-3}) for 1 h, c (●): pretreated with $Na_2S_2O_4$ (5.0×10^{-4} mol dm^{-3}) for 1 h.

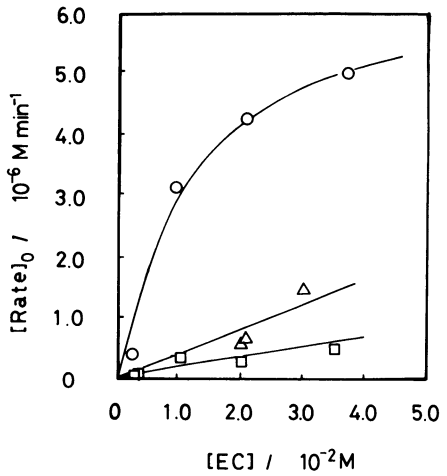


Fig. 3. Initial reduction rate vs. bipyridinium salt concentration. Reaction conditions: Hydrogenase pretreated with RSH, 4.9 units dm^{-3}, hydrogen, 0.97 atm; Tris-HCl buffer, 0.4 mol dm^{-3}, pH 7.0; reaction temp, 30°C. ○: Methyl viologen; △: A; □: PVS.

reduced type of methyl viologen was detected as shown in Fig. 2-(a). The induction period, ca. 150 min, was observed for the reduction. When the hydrogenase was pretreated with $Na_2S_2O_4$ or 2-mercaptoethanol, the induction period disappeared and a reduced form of methyl viologen was detected, as shown in Fig. 2-(b) and (c). As $Na_2S_2O_4$ and 2-mercaptoethanol are well-known reducing agents, the hydrogenase may be activated by reduction. The hydrogenase, sensitive to oxygen,[12,14] may be inactivated during the purification of the enzyme. The inactivated hydrogenase, however, may be re-activated by treatment with a reducing agent. Though hydrogen is also a reducing agent, it takes time to re-activate the hydrogenase. A long induction time appeared when hydrogen was used, as shown in Fig. 2-(a). In the following experiments, the hydrogenase pretreated by 2-mercaptoethanol was used.

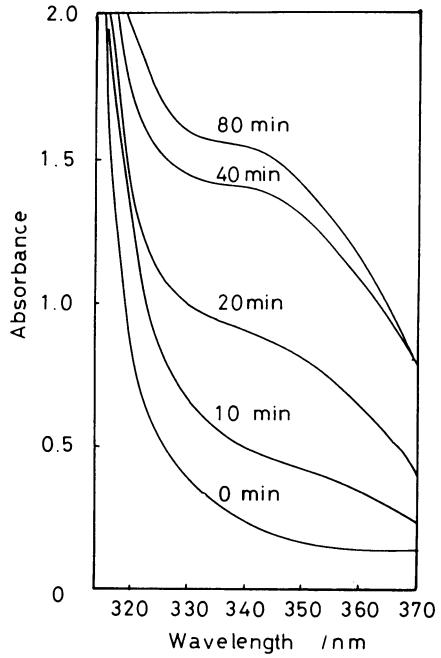


Fig. 4. Typical spectrum change during the reaction. Reaction conditions: Methyl viologen, 3.7×10^{-2} mol dm^{-3}; hydrogenase pretreated with RSH, 20.2 units dm^{-3}, hydrogen, 0.97 atm; FNR, 1.0 ml; NADP, 9.7×10^{-4} mol dm^{-3}; Tris-HCl buffer, 0.4 mol dm^{-3}, pH 7.0; reaction temp, 30°C.

When 3-sulfonatopropyl viologen (PVS) or compound A was used in place of methyl viologen, these compounds were also reduced by hydrogen with hydrogenase. The dependence of the initial reduction rate on the concentration of these compounds is shown in Fig. 3. The order of the reduction rate does not coincide with that of the redox potential (see Fig. 1) of these compounds, suggesting that methyl viologen is the most effective substrate of the hydrogenase among these compounds.

Reduction of NADP by Hydrogen. When hydrogen gas was introduced to a system containing hydrogenase, FNR, and NADPH, the spectrum of the solution changed with reaction time (Fig. 4). The characteristic absorption band of the reduced form (NADPH) of NADP was observed at 340 nm.

As shown in Fig. 5, the NADPH concentration increased linearly with the reaction time at low NADP conversion; then, the NADPH formation rate decreased. The rate decrease was caused by a decrease in the concentration of the starting material, NADP. When 4.5×10^{-3}, 9.7×10^{-4}, and 4.9×10^{-4} mol dm^{-3} of NADP were used, the conversion of NADP after 1 h was 26, 67, and 70%, respectively. In the case of the system shown in Fig. 5-(a), NADPH formed with the reaction time and no deactivation of the enzymes was observed within the reaction time.

The time dependence of the amount of NADPH

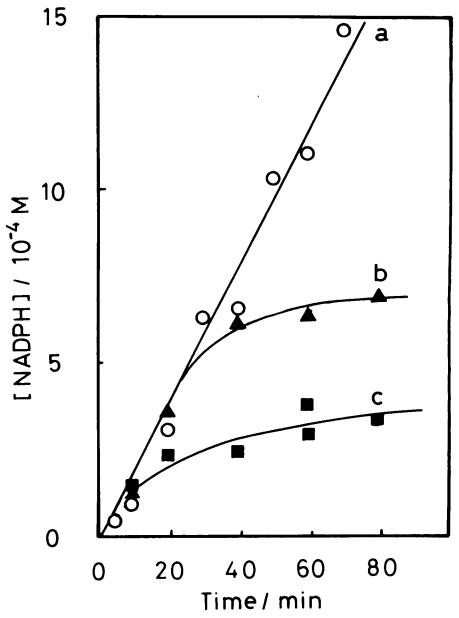


Fig. 5. Time dependence of NADPH formation. Reaction conditions: Methyl viologen, 3.7×10^{-2} mol dm^{-3}; hydrogenase pretreated with RSH, 20.2 units dm^{-3}, hydrogen, 0.97 atm; FNR, 1.0 ml; NADP, 4.5×10^{-3} mol dm^{-3} (○), 9.7×10^{-4} mol dm^{-3} (▲), 4.9×10^{-4} mol dm^{-3} (■); Tris-HCl buffer, 0.4 mol dm^{-3}, pH 7.0; reaction temp, 30°C.

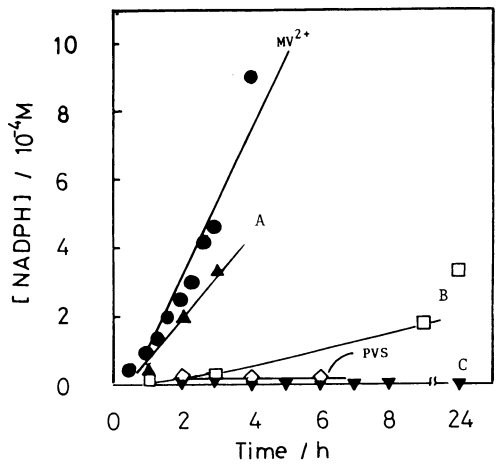


Fig. 6. Time dependence of NADPH formation. Reaction conditions: Bipyridinium salt 1.9×10^{-3} mol dm^{-3}; hydrogenase pretreated with RSH, 7.4 units dm^{-3}, hydrogen, 0.97 atm; FNR, 1.0 ml; NADP, 3.3×10^{-2} mol dm^{-3}; Tris-HCl buffer, 0.4 mol dm^{-3}, pH 7.0; reaction temp, 30°C. ●: Methyl viologen, ▲: A, □: B, ▼: C, ◇: PVS.

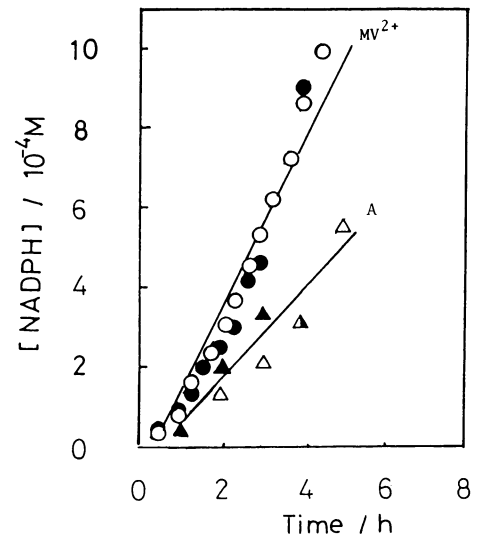


Fig. 7. Time dependence of NADPH formation. Reaction conditions: Same as the caption of Fig. 6 except for the amount of FNR. FNR: 1 ml (●,▲), 2 ml (○,△), 3 ml (▲).

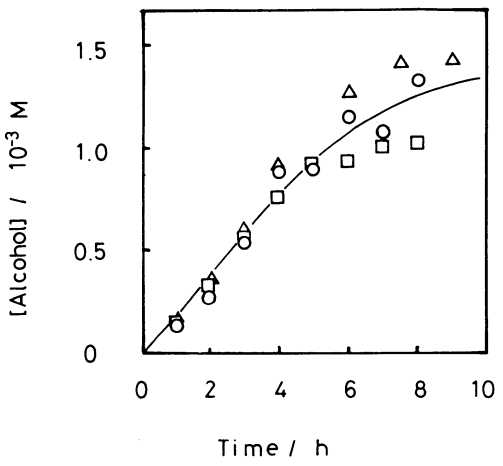
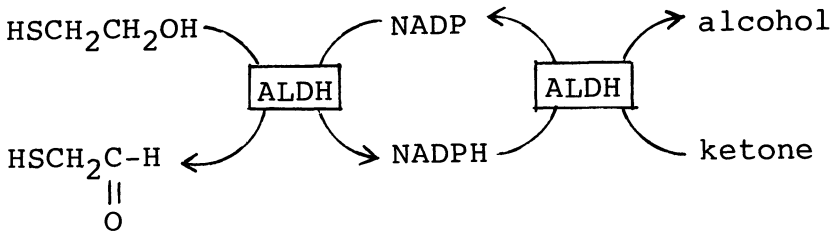


Fig. 8. Time dependence of alcohol formation. Reaction conditions: Methyl viologen, 3.7×10^{-2} mol dm^{-3}; hydrogenase pretreated with RSH, 5.25 units dm^{-3}, hydrogen, 0.97 atm; FNR, 2.0 ml; NADP, 1.0×10^{-4} mol dm^{-3}; NADP, 1.0×10^{-4} mol dm^{-3}; ALDH, 500 units dm^{-3}; Tris-HCl buffer, 0.4 mol dm^{-3}, pH 7.0; reaction temp, 30°C. ○: 2-Butanone (3.15×10^{-2} mol dm^{-3}), △: 2-pentanone (1.24×10^{-2} mol dm^{-3}), □: 2-hexanone (3.6×10^{-3} mol dm^{-3}).

formation is shown in Fig. 6, when various bipyridinium compounds were used as electron carriers. Among these compounds, methyl viologen, compounds **A** and **B**, were effective for NADPH formation. PVS and compound **C** were inactive. As PVS was easily reduced by hydrogen with hydrogenase and its reduced form has a sufficient redox potential (see Fig. 1) to reduce NADP, it is apparent that PVS cannot be a substrate of FNR.

When other concentrations of methyl viologen and compound **A** were used, no change in the reduction rate of NADP was observed (Fig. 7). From the above results, it can be seen that the rate determining step of NADPH formation by hydrogen is not the reduction step of NADP with FNR, but the reduction step of a bipyridinium compound by hydrogen with hydrogenase.

Reduction of Ketone by Hydrogen. When NADP is reduced in a [hydrogenase-FNR] system, the



Scheme 2.

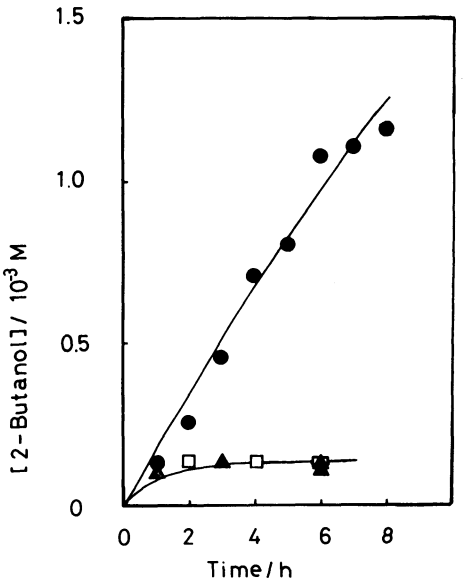


Fig. 9. Effect of RSH for alcohol formation. The system (shown by ●) contains methyl viologen (3.7×10^{-2} mol dm^{-3}), hydrogenase (5.25 units dm^{-3}), RSH (1.0×10^{-2} mol dm^{-3}), hydrogen (0.97 atm), FNR (2.0 ml), NADP (1.0×10^{-4} mol dm^{-3}), ALDH (500 units dm^{-3}), 2-butanone (0.18 mol dm^{-3}), Tris-HCl buffer (0.4 mol dm^{-3}, pH 7.0); reaction temp, 30°C. The systems (shown by ▲,□) contain RSH (1.0×10^{-2} mol dm^{-3}), hydrogen (0.97 atm), FNR (2.0 ml), NADP (3.0×10^{-3} mol dm^{-3}, (▲), 1.6×10^{-3} mol dm^{-3} (□)); ALDH (500 units dm^{-3}), 2-butanone (0.18 mol dm^{-3}), Tris-HCl buffer (0.4 mol dm^{-3}, pH 7.0); reaction temp, 30°C.

hydrogenation of ketones to alcohols could be accomplished by adding ALDH to the above system. The hydrogenation of some ketones were tried. The results are shown in Fig. 8. As ketones, 2-butanone, 2-pentanone, and 2-hexanone were used. All the ketones were hydrogenated and the corresponding alcohols were formed. The turnover numbers of NADP against alcohols after an 8-h reaction were 13, 15 and 10 for 2-butanone, 2-pentanone, and 2-hexanone, respectively. The results show that NADP recycled catalytically in this system, and that ketone hydrogenation by hydrogen gas was accomplished.

Since 2-mercaptoethanol was used in order to avoid the induction period of the hydrogenase activation (Fig. 2) in the system, there is a possibility of ketone hydrogenation through the following reaction: Thus, the hydrogenation of ketone was tried using a system containing 2-mercaptoethanol, ALDH, NADP, and 2-butanone. The results are shown in Fig. 9-(a). The above reaction proceeded and 2-butanol formation was observed. The amount of 2-butanol, however, was much less compared with that of the 2-butanone formed by a reduction with hydrogen gas as (Fig. 9-(b)). Ketone hydrogenation by 2-mercaptoethanol is neglected in this system.

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