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NADPH Regeneration by Glucose Dehydrogenase from *Gluconobacter scleroides* for *l*-Leucovorin Synthesis†

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A new process for (6*S*)-tetrahydrofolate production from dihydrofolate was designed that used dihydrofolate reductase and an NADPH regeneration system. Glucose dehydrogenase from *Gluconobacter scleroides* KY3613 was used for recycling of the cofactor. The reaction mixture contained 200 mM dihydrofolate, 220 mM glucose, 2 mM NADP, 14.4 U/ml dihydrofolate reductase, and 14.4 U/ml Glucose dehydrogenase, and the reaction was complete after incubation at pH 8.0, and 40°C for 2.5 hr. With (6*S*)-tetrahydrofolate as the starting material, *l*-leucovorin was synthesized *via* a methenyl derivative. The purity of the *l*-leucovorin was 100%, and its diastereomeric purity was >99.5% *d.e.* as the (6*S*)-form.

l-Leucovorin is widely used in rescue therapy for patients undergoing cancer chemotherapy with methotrexate.[2-5] On the other hand, the use of *l*-leucovorin with 5-fluorouracil may have better effects towards cancer than 5-fluorouracil alone.[6,7]

Dihydrofolate reductase (DHFR; EC 1.5.1.3) catalyzes the reduction of dihydrofolate (DHF) to give (6*S*)-tetrahydrofolate (THF), from which *l*-leucovorin is made by formylation.[1] NADPH is an essential cofactor for DHFR, so one problem in the use of this enzyme is the consumption of the expensive cofactor. There are various methods for the recycling of the cofactor.[8] Some enzyme reaction systems have been proposed for recycling of NADPH in THF production, with for example, isocitrate dehydrogenase,[9] glucose-6-phosphate dehydrogenase,[9] malic enzyme,[10] and 6-phosphogluconate dehydrogenase.[11] These methods have disadvantages: expensive substrate, low yield of the enzyme, or inefficient recycling.

With NADP as a coenzyme, glucose dehydrogenase (GUDH; EC 1.1.1.119) catalyzes the oxidation of D-glucose to D-glucono-δ-lactone, which spontaneously hydrolyzes to gluconic acid. The equilibrium of the reaction is in favor of NADPH formation; so this enzyme is much used as a NADPH recycling system.[12,13]

In this paper, production of *l*-leucovorin combined with a NADPH recycling system with GUDH is described.

Materials and Methods

Chemicals and enzymes. DHFR and DHF were obtained as described befor.[1] All other chemicals were ordinary commercial preparations.

Purification of GUDH. The buffer used for GUDH purification was 20 mM phosphate buffer, pH 7.0.

Gluconobacter scleroides KY3613 (formerly *Gluconobacter oxydans*[14]) was cultured at 30°C in a medium containing 2% glucose, 0.5% yeast extract, 0.3% peptone, 0.3% malt extract, and 2% $CaCO_3$, pH 6.0. Harvested cells (100 g) were suspended in 1 liter of buffer and homogenized with glass beads (diameter, 0.25—0.5 mm) in a homogenizer. The cell debris was removed by centrifugation. To the supernatant, 2% protamine sulfate was added to the final concentration of 0.1%, and nucleic acids were removed by centrifugation. After ammonium sulfate fractionation (35—55% saturation), the precipitate was collected by centrifugation, dissolved in the buffer, and dialyzed against the same buffer.

The sample was put on a DEAE-cellulose column (bed vol., 550 ml) equilibrated with the buffer. The column was washed with the same buffer, and proteins were eluted with a concentration gradient of NaCl (20—300 mM). The active fraction eluted with 150 mM NaCl was collected, precipitated with ammonium sulfate (60% saturation), and suspended in 20 ml of the buffer containing ammonium sulfate (60% saturation).

This sample was put on a Sepharose 4B column (bed vol., 100 ml) equilibrated with the buffer containing ammonium sulfate (60% saturation). The column was washed with the same buffer, and proteins were eluted by a concentration gradient of ammonium sulfate (60—10% saturation). The active fraction was collected, concentrated by precipitation with ammonium sulfate, and dialyzed against 30 mM phosphate buffer, pH 7.0. This sample was passed through a Sephadex G-100 column (bed vol., 400 ml). The active fraction was put on a hydroxyapatite column (bed vol., 70 ml) equilibrated with 30 mM phosphate buffer, pH 7.0. The column was washed with 60 mM buffer, and proteins were eluted with 80 mM buffer. Then the enzyme was lyophilized.

Preparation of l-leucovorin from DHF with DHFR coupled with a GUDH-NADP recycling system. To 2000 ml of an aqueous solution containing 200 mM DHF, 220 mM glucose, and 2.0 mM NADP adjusted to pH 8.0 were added 14.4 U/ml DHFR and 14.4 U/ml GUDH. The reaction was at 40°C with stirring and nitrogen gas bubbling. During the reaction, the pH was kept at 8.0 by the addition of 3 N NaOH. Two hours after the start of reaction, when all of the DHF had been converted to THF, 70 g of sodium ascorbate was added, and stirring was continued for 20 min more. After the pH was lowered to 3.5 by the addition of conc. HCl, the mixture was stirred for 1 hr with ice cooling. A precipitate of THF was collected by filtration and washed with water. To the precipitate, 1130 ml of formic acid was added, and the mixture was left at room temperature for 16 hr with nitrogen gas bubbling. The reaction mixture was concentrated under reduced pressure, 300 ml of 0.5 N HCl was added to the concentrate, and the resulting mixture was left overnight with stirring. A precipitate was collected by filtrating and dried, giving 163.8 g of the methenyl derivative of THF (5, 10-CH-THF).

The methenyl derivative of THF (2.43 g) was added to 58 ml of hot water, and the mixture was refluxed for 5.5 hr in an oil bath of 120°C while the pH was kept in the range of 6.5 to 6.9 by the addition of 0.5 N NaOH. At the end of the reaction, 1.2 g of anhydrous calcium chloride and 11 ml of ethanol were added, and stirring was done for 1 hr. A precipitate was filtered off. To the filtrate, 400 ml of ethanol was added dropwise with cooling over 1 hr. A precipitate was collected by filtration and dried, giving 2.21 g of *l*-leucovorin.

† Asymmetric Synthesis of *l*-Leucovorin. Part 2. See ref. 1.

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Abbreviations: DHF, dihydrofolate; DHFR, dihydrofolate reductase; GUDH, glucose dehydrogenase; THF, (6*S*)-tetrahydrofolate.

Analysis. Assay of the DHF concentration and DHFR activity was as described in a previous paper.[1] Glucose was analyzed by the use of a glucose analyzer (GL-101; Mitsubishi Kasei Corporation). GUDH activity was measured by reaction at 30°C of a mixture containing 0.1 mM phosphate buffer (pH 7.0), 170 mM glucose, and 435 μM NADP in 3 ml. The reduction of NADP was observed spectrophotometrically at 340 nm. One unit of GUDH activity was defined as the amount that reduced 1 μmol of NADP per minute. The activity of NADPH oxidation was measured with D-glucono-δ-lactone and NADPH instead of glucose and NADP.

The optical purity of *l*-leucovorin was evaluated by chiral HPLC with a silica column treated with bovine serum albumin (Resolvosil).[15]

Results

Properties of GUDH

Table I lists some properties of GUDH. This enzyme reacted with NADP, but not with NAD. So it seemed to be D-glucose : $NADP^+$ 1-oxidoreductase (EC 1.1.1.119). The initial velocity of NADPH oxidation was 8% of NADP reduction.

Optimal conditions for NADPH recycling system with GUDH

Effect of pH. The optimal pH of GUDH was found at pH 8.0—9.0 (Table I); for DHFR, it is at pH 5.0.[1] Because the cycling system involves two enzymes, the optimal pH was between pH 5.0 and 9.0. The effect of pH in the NADPH recycling system is shown in Fig. 1. The optimal pH was 8.0. At this pH, the activities of GUDH and DHFR were almost the same (data not shown).

Coenzyme concentration. The reaction efficiency was examined with different ratios of enzyme concentrations. Based on the results, we decided to use the same enzyme concentration, 14.4 U/ml, for both enzymes throughout the experiments (data not shown).

The reaction time had decreased with the increase in coenzyme concentrations (Fig. 2). When only 0.2 mM coenzyme was used, the recycling of the coenzyme was faster than NADPH consumption. In these conditions, the turnover number was about 850 for 3 hr.

Effect of temperature. The highest reaction rate was at about 37 to 45°C (Fig. 3).

Optimal conditions for NADPH recycling. For industrial applications, the concentration of DHF should be high and the concentrations of the enzymes and coenzyme should be low. However, solutions of high concentrations of DHF are viscous. Considering the viscosity, 200 mM DHF was best. DHF and THF were not stable, so the reaction time should preferably be less than 3 hr under anaerobic conditions. Suggested production conditions for THF are given in Table II.

Synthesis of l-leucovorin

Figure 4 shows a scheme for *l*-leucovorin production with DHFR. The THF decomposed in a few hours under aerobic conditions, so the reaction mixture must be kept anaerobic. At the reaction step from methenyl derivative of THF (5,10-CH-THF) to *l*-leucovorin (5-CHO-THF), the conditions for reflux are important. At a low temperature or at a high pH such as pH 7.8, the product of the reaction was 10-CHO-THF.

The yield of the methenyl derivative of THF based on

Table I. Properties of GUDH from *Gluconobacter scleroides*

Mol. wt.	89,000 (2 subunits)
pH	8.0—9.0
pH stability	6.0—9.0 (100%)
K_m (glucose)	6.3 mM
(NADP)	70 μM
Thermostability (15 min)	50°C (100%)
Substrate specificity	D-Glucose, 100%
	D-Mannose, 94%
	D-Galactose, 3%
	2-Deoxy-D-glucose, 120%

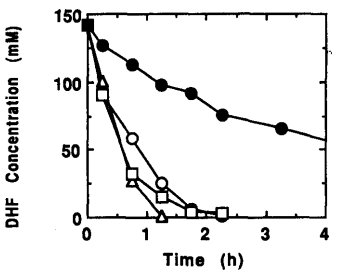

Fig. 1. Effect of pH on NADPH Regeneration.

The reaction mixture consisted of 142 mM DHF, 156 mM glucose, 2.8 mM NADP, 17 U/ml DHFR, and 17 U/ml GUDH. The reaction was at 30°C. ○, pH 7; △, pH 8; □, pH 9; ●, pH 10.

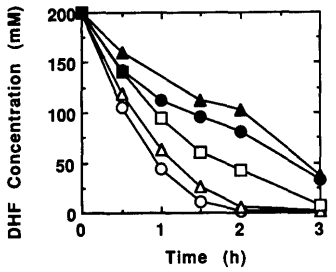

Fig. 2. Effect of NADP Concentration on NADPH Regeneration.

The reaction mixture consisted of 200 mM DHF, 220 mM glucose, 14.4 U/ml DHFR, and 14.4 U/ml GUDH. The reaction was at pH 8.0, and 30°C. NADP concentrations: ▲, 0.2 mM; ●, 0.5 mM; □, 1.0 mM; △ 2.0 mM; ○, 4.0 mM.

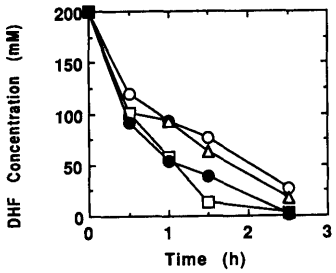

Fig. 3. Effect of Temperature on NADPH Regeneration.

The reaction mixture and conditions were as in the legend to figure 2, except for temperature: ○, 25°C; △, 30°C; □, 37°C; ●, 45°C.

Table II. Optimal Conditions for Cofactor Recycling during Reduction of DHF

DHF	200 mM
Glucose	220 mM
NADP	2 mM
DHFR	14.4 U/ml
GUDH	14.4 U/ml
pH	8.0
Temperature	40°C

The reaction is complete in 2.5 hr under anaerobic conditions.

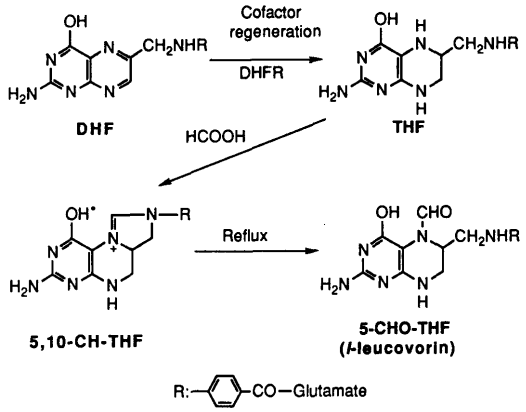

Fig. 4. Scheme for Production of *l*-Leucovorin.

DHF was 95%, and the yield of *l*-leucovorin based on the methenyl derivative was 91%.

The methenyl derivative was purified by treatment with $MgCl_2$ and $Ca(OH)_2$,[16] and then Florisil column chromatography.[16,17] *l*-Leucovorin was obtained as the Ca salt after crystallization caused by the addition of $CaCl_2$.

The final product was identified as *l*-leucovorin by NMR and TLC (data not shown). The purity of *l*-leucovorin, when measured by HPLC, was 100% as a substance, and its diastereomeric purity was >99.5%*d.e.* as the (6*S*)-form. Our sample had a specific rotation of $[\alpha]_D = -13.3°$ (*c* 1.0, H_2O).

l-Leucovorin stored in 0.1 M Tris–buffer, pH 7.0, did not decomposed within 10 days.

Discussion

As a rule, buffers are useful to keep the pH suitable for enzyme reactions, but in THF synthesis, any buffer could not be used, for two reasons. First, the activities of DHFR and GUDH were inhibited by phosphate, glucose, and NaCl, so in *l*-leucovorin synthesis, the additions should be avoided. These two enzymes were not inhibited by DHF, at concentrations of up to 200 mM (data not shown). Second, with a buffer, the recovery of the methenyl derivative of THF was poor. Phosphate buffer may interfere with the precipitation of the methenyl derivative.

In this report, GUDH was used as the enzyme for NADPH regeneration, but other enzymes may be used. Glutamate dehydrogenase (EC 1.4.1.4) from yeast was examined for its capacity to regenerate the cofactor, but DHF reduction was not unsatisfactory. For some dehydrogenases, reaction conditions can not be adjusted to give a satisfactory NADPH/NADP ratio, so dehydrogenases, that cause a practically irreversible NADP conversion, sould be used for *l*-leucovorin synthesis. The hydrolysis of the product D-glucono-δ-lactone to glucuronic acid by GUDH moves the reaction towards total conversion of NADPH to NADP.

When the recovery of the enzymes and cofactor is most important, immobilized enzyme(s) or cofactor (or both) might be useful.

The specific optical rotation of *l*-leucovorin was $[\alpha]_D = -13.3°$ (*c* 1.0, H_2O), unlike the reported value of $[\alpha]_D = +2.12°$ (*c* 1.32, H_2O).[9] The discrepancy may arise from different degrees of purity.

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