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Regeneration of NAD(P)H Using Glucose 6-Sulfate and Glucose-6-phosphate Dehydrogenase

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Glucose 6-sulfate and glucose-6-phosphate dehydrogenase have been used for NAD(P)H cofactor regeneration in preparations of (*S*)-benzyl-α-d_1 alcohol and *threo*-D_s(+)-isocitrate (0.1-mol scale). The reduced nicotinamide cofactors are more stable in solutions of glucose 6-sulfate than in solutions of glucose 6-phosphate and their lifetimes in solution are correspondingly longer. The specific activities of the enzymes are, however, lower with glucose 6-sulfate than with glucose 6-phosphate. Glucose 6-sulfate appears to be a useful and practical reducing agent for NADP; glucose 6-phosphate is clearly superior for NAD. Comparisons of several methods for making glucose 6-phosphate indicate that phosphorylation of glucose with ATP (using ATP cofactor recycling and hexokinase as catalyst) is the most effective method for laboratory-scale syntheses.

The combination of glucose 6-phosphate (G-6-P) and G-6-P dehydrogenase (G-6-PDH from *Leuconostoc mesenteroides*) provides a useful method for large-scale regeneration of NAD(P)H.[2] This procedure has the ad-

Table I. Methods for the Preparation of Glucose 6-Phosphate[a]

	method	% yield[b]
1.	G + ATP $\xrightarrow{HK}$ G-6-P + ADP	100[c]
2.	G-1-P $\xrightarrow{PGM}$ G-6-P	95[c]
3.	FDP $\xrightarrow{H^+, PGI}$ G-6-P + Pi	50[c]
4.	$(G)_n$ + Pi $\xrightarrow{Pase, PGM}$ G-6-P	30[c]
5.	$(G)_2$ + Pi $\xrightarrow{CT}$ G-1-P + G	25[d]
6.	$(G)_2$ + Pi $\xrightarrow{CT, PGM}$ G-6-P + G	45[d]
7.	G + $POCl_3$ + lutidine → G-6-P	32[e]
8.	G + pyridine·P_2O_5 → G-6-P	10[f]

[a] Abbreviations: G-6-P, glucose 6-phosphate; G-1-P, glucose 1-phosphate; G, glucose; HK, hexokinase; PGM, phosphoglucomutase; FDP, fructose 1,6-diphosphate; PGI, phosphoglucoisomerase; $(G)_n$, starch; Pase, phosphorylase a; $(G)_2$, cellobiose; CT, resting cells of *C. thermocellum*. [b] Calculated as moles of G-6-P per mole of G or its derivative based on the concentration in the reaction mixture. [c] See ref 2. [d] The yield reached its maximum value after a 60-h incubation at 50 °C, and no further increase of G-1-P or G-6-P was observed after that time. [e] $POCl_3$ and lutidine were first allowed to react at 0 °C in triethyl phosphate, and glucose was then added to the reaction mixture. [f] Equivalent pyridine and P_2O_5 (0.5 M each) were mixed first in $CHCl_3$ followed by addition of glucose in DMF (0.5 M) at 25 °C.

vantage that it is applicable to the regeneration of both NADH and NADPH and that the enzyme is inexpensive, stable, and highly active. It has the disadvantage that it requires glucose 6-phosphate as a stoichiometric reagent and that phosphate ion and alkyl phosphates are active acid catalysts for the decomposition of the reduced nicotinamide cofactors NAD(P)H.

Several preparations of the G-6-P required in this procedure have been reported (entries 1–4, Table I).[2] Although these procedures are easily capable of generating kilogram quantities of G-6-P, none are ideally convenient. In this work, we have examined three other methods (entries 5–8, Table I). None have proved as satisfactory as hexokinase-catalyzed phosphorylation of glucose by ATP (entry 1). The chemical methods are unsatisfactory in that they give low yields and require extensive purification. The phosphorolysis of cellobiose to glucose 1-phosphate by cellobiose phosphorylase (EC 2.4.1.20, provided by resting cells of *Clostridium thermocellum*) is also less practical than hexokinase-catalyzed phosphorylation. The phosphorolysis is limited by the equilibrium constant of the reaction:[3]

$$K = \frac{[\text{glucose 1-phosphate}][\text{glucose}]}{[\text{cellobiose}][\text{phosphate}]} = 0.23 \quad (\text{pH } 7.0)$$

Although the reaction can be driven by adding phosphoglucomutase to convert glucose 1-phosphate to G-6-P (95% of an equilibrium mixture of G-1-P and G-6-P is G-6-P), phosphoglucomutase is not stable at the temperature (55 °C) used for the phosphorolysis.[4] Moreover, since high concentrations of phosphate are required, purification of the G-6-P again presents a technical problem. Thus, the best current procedure for the preparation of G-6-P seems to be hexokinase-catalyzed phosphorylation of glucose. Although this procedure is straightforward, it does require two enzymes (hexokinase and acetate kinase, for ATP recycling) and a separate preparation of acetyl phosphate. Moreover, glucose 6-phosphate is itself an effective acid catalyst for decomposition of NAD(P)H.[2] In an effort to avoid these problems, we have examined the utility of glucose 6-sulfate (G-6-S) as a reducing agent. This material

Table II. Calculated and Observed Half-lives for NAD(P)H (0.1 mM) in 0.2 M Glucose 6-Phosphate or Glucose 6-Sulfate Solution at 25 °C[a]

pH	[G-6-P$^-$], mM[b]	$\tau_{1/2}$ NADH, h G-6-P	$\tau_{1/2}$ NADH, h G-6-S	$\tau_{1/2}$ NADPH, h G-6-P	$\tau_{1/2}$ NADPH, h G-6-S
7.0	27.4	25	686	14	32
7.8	4.9	139	3164	81	182
		(128)[c]	(3100)[c]	(77)[c]	(170)[c]
8.0	3.1	218	4225	126	277

[a] The concentration of active NAD(P)H was determined enzymatically,[2] using G-6-P and G-6-PDH. [b] The concentration of glucose 6-phosphate monoanion (= [G-6-P]/(1 + 10^{pH-pK_a}) where [G-6-P] is the total concentration of G-6-P, and pK_a = 6.2 for G-6-P). [c] The numbers in parentheses were observed values. The first-order rate constant[2] (k_{obsd}) for NADH is $k_H[H^+] + k_{HA}[HA] + k_{H_2O}$; for NADPH it is $k_H[H^+] + k_{HA}[HA] + k_{H_2O} + k_I/(1 + 10^{pH-pK_a})$. In these expressions, the constants have the following values: $k_H = 9.4 \times 10^3$ M^{-1} h^{-1}, $k_{H_2O} = 0.7 \times 10^{-4}$ h^{-1}, $k_I = 0.15$ h^{-1}, k_{HA} for G-6-P = 0.97 M^{-1} h^{-1}, and pK_a = 6.2 for the 2′-phosphate of NADPH. We assume that [HA] = 0 for G-6-S at pH ≥ 7. k_I is the intramolecular rate constant for 2′-phosphate-catalyzed decomposition of NADPH.

Table III. Kinetic Parameters for Glucose-6-phosphate Dehydrogenases[a]

	yeast (*S. cerevisiae*) NAD	yeast (*S. cerevisiae*) NADP	*L. mesenteroides* NAD	*L. mesenteroides* NADP
G-6-P				
K_m(G-6-P), mM		0.051	0.053	0.053
K_m(NAD(P)), mM		0.0075	0.106	0.0057
sp act., U mg^{-1}	0	400	700	400
G-6-S				
K_m(G-6-S), mM		~30	~50	
K_m(NAD(P)), mM		0.002	0.106	0.0057
sp act., U mg^{-1}	0	60	2	10

[a] The numbers are cited from: H. R. Levy, *Adv. Enzymol.* **1979**, *48*, 141. Specific activities (at V_{max}) for G-6-S were determined in this laboratory. The activities were measured in a triethanolamine buffer (0.1 M, pH 7.8) containing 2 mM NAD or NADP, 0.1 mg/mL of enzyme, and varied concentrations of G-6-S. K_m for NAD(P) was determined at 0.8 M G-6-S. 1 U = 1 μmol of product produced per min. K_m = the concentration of indicated substrate at which the enzyme showed its half-maximum activity.

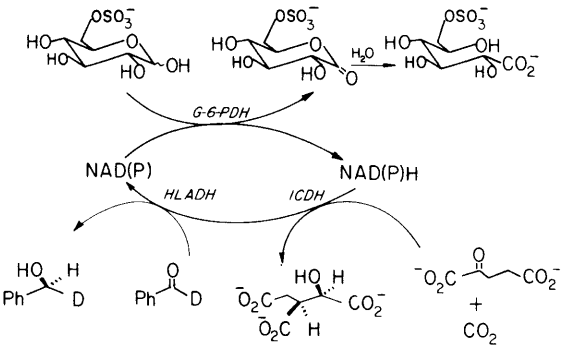
(1) Supported by NIH (GM 26543, to G.M.W.) and the Department of Energy (EG-77-5-0204198; XR-9-8109-1, to C.L.C.).

(2) Wong, C. H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890.

(3) Cohn, M. *Enzymes*, **1961**, *5*, 192.

(4) The PAN-immobilized PGM was not stable at 50 °C (half-life = 6 h), and the reaction catalyzed by the resting cells of *C. thermocellum* was very slow at lower temperatures (for example, the rate of G-1-P production at 37 °C was ~10% of that at 50 °C). Other thermostable enzymes such as glucose isomerase (= xylose isomerase, EC 5.3.1.5) might, in principle, be used to convert glucose in the equilibrium mixture to fructose, which might then be stabilized as a germanate complex (optimal pH ~6; the optimal pH for fructose borate complex is 9; Barker, S. A.; Bowers, H. A.; Somers, P. J. *Enzyme Eng.* **1980**, *5*, 329). Since glucose isomerase is not available and germanate might influence the activity of the enzymes, we did not test this system.

Scheme I. Regeneration of NAD(P)H Using G-6-S/G-6-PDH[a]



[a] Abbreviations: G-6-PDH, glucose-6-phosphate dehydrogenase; HLADH, horse liver alcohol dehydrogenase; ICDH, isocitrate dehydrogenase.

has been reported to be a substrate for the glucose 6-phosphate dehydrogenase from *L. mesenteroides*.[5] It is not an acid catalyst for the decomposition of NAD(P)H (Table II). It is easily prepared[6] by reaction of glucose and pyridine–sulfur trioxide[7] in DMF in 58% yield. After removal of DMF and pyridine, the resulting G-6-S solution is used directly for cofactor regeneration without further purification.

The G-6-PDH from yeast is specific for NADP, but its specific activity in catalysis of the reduction of NADP by G-6-S is sufficiently high to be useful (Table III). The G-6-PDH from *L. mesenteroides* accepts both NAD and NADP, but specific activities with both cofactors are low. Nonetheless, it is possible to carry out syntheses on the scale of 100 mmol with NAD-requiring enzymes by using G-6-S as a reducing agent.

Scheme I illustrates syntheses carried out to demonstrate the practicality of these regeneration systems. Each was carried out on 100-mmol scale. The (*S*)-benzyl-α-d_1 alcohol preparation utilized NAD; the *threo*-D_s(+)-isocitrate synthesis required NADP. The turnover numbers (TN = moles of product produced per mole of cofactor present during the reaction) and residual activities of the cofactors remaining at the conclusion of these reactions were TN_{NAD} = 1000 (90%) and TN_{NADP} = 1500 (84%). These values indicate good lifetimes for the cofactors. The enzymes used also proved stable and the yields of reaction products were high (see the Experimental Section).

Discussion

The combination of G-6-PDH from yeast and glucose 6-sulfate provides a useful method for regenerating NADPH from NADP. Although the specific activity of the system comprising G-6-S/G-6-PDH (yeast) is less than that of G-6-P/G-6-PDH (*L. mesenteroides*), it is comparable with other regeneration systems such as formate/formate dehydrogenase,[8] dihydrogen/hydrogenase,[9] and combined electrochemical/enzymatic systems.[10,11] It has two advantages over the G-6-P/G-6-PDH system. Glucose 6-sulfate is more easily prepared than glucose 6-phosphate. Because glucose 6-sulfate is inactive as an acid catalyst for the decomposition of NAD(P)H, the lifetimes of these cofactors are higher in solutions containing G-6-S than in those containing G-6-P. For these reasons, and because this regeneration system has high specific activity relative to others,[9,11] we believe it is one of the best available for NADPH in laboratory-scale preparations (several moles of product). For larger scale manipulation, however, the 6-sulfogluconate formed may complicate product isolation. Under such circumstances, we believe that methods using H_2[9] or electrochemistry[11] are more practical.

Experimental Section

Enzymes and routine biochemicals were obtained from Sigma. Chlorosulfonic acid, lutidine, and $POCl_3$ were obtained from Aldrich. Other chemicals and solvents were reagent grade. Welding-grade argon was used as an inert atmosphere without purification.

UV spectra were obtained with a Perkin-Elmer 552 spectrophotometer, equipped with a constant temperature cell. NMR spectra were measured at 250 MHz (Bruker 270). Procedures for immobilizations and assays of enzymes have been described.[2,12]

Preparation of *C. thermocellum* Used in the Resting Cell System. *C. thermocellum* was obtained from the American Type Culture Collection (ATCC 27405). The composition of the medium for growth was as follows: cellobiose, 10 g L^{-1}; yeast extract (Difco Lab), 10 g L^{-1}; KH_2PO_4, 1.5 g L^{-1}; K_2HPO_4, 2.9 g L^{-1}; $(NH_4)_2SO_4$, 1.3 g L^{-1}; $MgCl_2$, 0.75 g L^{-1}; $CaCl_2$, 0.1 g L^{-1}; $FeSO_4$ (1.25% solution), 0.1 mL L^{-1}; Resazurin (0.2% solution), 1.0 mL L^{-1}; sodium thioglycollate, 0.5 g L^{-1}; sodium bicarbonate, 10 g L^{-1}. The culture vessel was a specially modified 500-mL Erlenmeyer flask for anaerobiosis[13] and contained 300 mL of medium. The procedures for medium preparation, sterilization, and inoculation were as follows: three portions of 100 mL of concentrated solution in distilled water of (1) the cellobiose, (2) chloride salts, and (3) the remaining medium components were steam sterilized in separate flasks for 15 min at 15 psi (121 °C). (This procedure was followed to avoid caramelization of the cellobiose and precipitation of the salts). After being autoclaved, the solutions were cooled to room temperature. The cellobiose and chloride salt solutions were added to the anaerobic flask containing the rest of the medium. Flasks were inoculated with 10 mL of *C. thermocellum* growing in the same medium. Prior to and during inoculation, the flasks were bubbled with sterile carbon dioxide to effect and maintain anaerobiosis and to complete the bicarbonate buffer system in the medium.

After 24 h of anaerobic incubation at 60 °C, the cells were harvested by centrifugation (10 000 rpm for 30 min in a Sorvall refrigerated centrifuge, SA-600 rotor), washed once in citrate–phosphate buffer, pH 5.6 (0.1 M citric acid–0.2 M disodium phosphate–distilled water, 2:3:5), resuspended in the same citrate–phosphate buffer containing cellobiose (10 g L^{-1}) and sodium thioglycollate (1.0 g L^{-1}). (Cells suspended in citrate–phosphate buffer alone were used as controls.) The cell/substrate suspensions were transferred from centrifuge tubes to Hungate tubes (Bellco Glass Inc., Vineland, NJ), 5-mL working volume. After harvesting, centrifugation, and resuspension in the substrate solution, the cells were effectively concentrated sixfold (4–5 g L^{-1}) from their original broth volume (0.7–0.8 g L^{-1}).

***C. thermocellum* Catalyzed Phosphorylation of Cellobiose.** A 5-mL solution containing cellobiose (60 mM), citrate–phosphate buffer (60 mM), and thioglycolate (8 mM), pH 6.0, was deoxygenated with argon. The resting cells (20 mg) were added, and the mixture was incubated at 50 °C anaerobically (deoxygenated with Ar). After 1 h, the immobilized phosphoglucomutase (20 U, 0.5 mL of gel) was added and the mixture kept at the same temperature anaerobically.[13] Aliquots were withdrawn periodically, and G-6-P or G-1-P was determined.[14]

Glucose 6-Sulfate (G-6-S). The procedure used was a modification of that described by Guiseley and Ruoff.[6] Pyri-

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(14) Bergmeyer, H. U. "Methods of Enzymatic Analysis"; Academic Press: New York, 1974. G-6-S was determined in the same way as G-6-P.

dine–sulfur trioxide[7] (135 g, 0.85 mol) in DMF (700 mL) was added to a solution of glucose (156 g, 0.87 mol) dissolved in DMF (1 L) over 3 h at 25 °C. The mixture was stirred for another hour and concentrated in vacuo (0.1 mmHg) at 35–40 °C to remove DMF and pyridine. The oily residue was dissolved in water (1 L), adjusted to pH 7.5 (2 N KOH), and concentrated again to remove pyridine. The process was repeated until neutrality was permanent (2–3 times). The aqueous solution (400 mL) contained 0.5 mol of G-6-S[14] (58% yield) and was used directly for the enzymatic syntheses.

Glucose 6-Phosphate. Some methods for the preparation of G-6-P are summarized in Table I. The procedure for $POCl_3$–lutidine was as follows. Triethyl phosphate (250 mL) and freshly distilled $POCl_3$ (18 mL, 200 mmol) were mixed at 0 °C. To this solution was added slowly 2,6-lutidine (20 mL, 200 mmol) with stirring over 15 min at 0 °C followed by addition of glucose (36 g, 200 mmol). The reaction mixture was stirred at 25 °C for 5 h. Aliquots were taken, diluted 10 times with water, and heated on a steam bath for 20 min. G-6-P was determined enzymatically.[14] This assay showed that 32% of the glucose had been converted to G-6-P. If chloroform (80 mL) was used instead of triethyl phosphate, and glucose dissolved in DMF (36 g in 200 mL) was added slowly (over 1 h) to the $POCl_3$–lutidine solution, a 28% reaction yield was observed in 5 h.

Synthesis of (*S*)-Benzyl-α-d_1 Alcohol. To a 1-L solution containing G-6-S (0.2 mol), $MgSO_4$ (3 mmol), NAD (0.16 mmol), β-mercaptoethanol (2 mmol), and 20 U each of immobilized G-6-PDH from *L. mesenteroides* (based on NAD and G-6-S as substrates, 10 mL of gel) and horse liver alcohol dehydrogenase (HLADH, based on benzaldehyde and NADH as substrates, 0.5 mL of gel)[2] was added slowly benzaldehyde-α-d_1 (17.1 g, 0.16 mmol)[2] over 3–4 days. The solution was kept under argon at pH 7.6.[2] After 8 days, the reaction was complete and (*S*)-benzyl-α-d_1 alcohol was isolated as described previously:[2] 15 g, 130 mmol, 81% yield, 95% ee. The turnover numbers (TN) and residual activities were as follows: NAD, 1000, 90%; G-6-PDH, 1.6×10^6, 80%; HLADH, 1×10^7, 78%. The TN for NAD is based on the quantity of NAD added at the beginning of the reaction, not the quantity lost during the reaction.

Synthesis of *threo*-D_s(+)-Isocitrate. A 1-L solution containing G-6-S (0.2 mol), α-ketoglutarate (0.15 mol), $NaHCO_3$ (0.2 mol), $MgCl_2$ (5 mmol), $MnCl_2$ (1 mmol), NADP (0.1 mmol), β-mercaptoethanol (2 mmol), and 80 U each of immobilized G-6-PDH from yeast (based on NADP and G-6-S as substrates, 3 mL of gel) and isocitrate dehydrogenase (ICDH, 12 mL of gel) was kept under CO_2 at pH 7.6 with stirring for 5 days, and isocitrate was isolated as its barium salt as described previously.[2] The solid (38 g) contained 94% of *threo*-D_s(+)-isocitrate (91.5 mmol), corresponding to 61% yield. The TN and residual activities were as follows: NADP, 1500, 84%; G-6-PDH, 5×10^6, 81%; ICDH, 1×10^6, 84%.

Registry No. G-6-S, 79084-12-1; G-6-P, 79101-58-9; NAD, 53-84-9; NADP, 53-59-8; NADH, 58-68-4; NADPH, 53-57-6; (*S*)-benzyl-α-d_1 alcohol, 3481-15-0; benzaldehyde-α-d_1, 3592-47-0; barium *threo*-D_s-(+)-isocitrate, 79120-64-2; α-ketoglutarate, 64-15-3.