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Enzyme-Catalyzed Organic Synthesis: NAD(P)H Cofactor Regeneration by Using Glucose 6-Phosphate and the Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*[1]

Chi-Huey Wong and George M. Whitesides*

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received December 11, 1980*

Abstract: Glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) and glucose 6-phosphate comprise a useful system for regeneration of reduced nicotinamide cofactors for use in enzyme-catalyzed organic synthesis. This enzyme is approximately equally active in reduction of NAD and NADP. It is commercially available, inexpensive, stable, and easily immobilized. Glucose 6-phosphate can be prepared in quantity by hexokinase-catalyzed phosphorylation of glucose by ATP (coupled with ATP regeneration) or by other methods. The operation of this regeneration system is illustrated by syntheses of enantiomerically enriched D-lactic acid (0.4 mol, enantiomeric excess 95%) and (*S*)-benzyl-α-d_1 alcohol (0.4 mol, enantiomeric excess 95%) and by a synthesis of *threo*-D_s(+)-isocitric acid (0.17 mol). Factors influencing the stability of NAD(P)(H) in solution have been explored.

Enzyme-catalyzed reactions that require reduced nicotinamide cofactors (NADH, NADPH) are not widely used in preparative chemistry.[2,3] Although many of these reactions are potentially valuable in synthesis, the cofactors required are expensive and significantly unstable in solution and can be used economically in stoichiometric reactions only on a small scale. A number of procedures for regenerating the reduced cofactors have been proposed and tested.[4] Of these, the use of formate and formate dehydrogenase (EC 1.2.1.2) to reduce NAD to NADH is, in principle, one of the most practical,[5,6] although the enzyme used in this procedure (from *Candida boidinii*) is still expensive when purchased commercially. This formate dehydrogenase does not reduce NADP,[7] although formate dehydrogenase from *Clostridium thermoacticum* does do so.

This paper describes a regeneration system based on dehydrogenation of glucose 6-phosphate (G-6-P) catalyzed by glucose-6-phosphate dehydrogenase (G-6-PDH, G-6-P, NAD(P) oxidoreductase, EC 1.1.1.49, from *Leuconostoc mesenteroides*). The G-6-P required can be prepared by several procedures. The most convenient for syntheses carried out on a ~1 mol scale is the hexokinase-catalyzed phosphorylation of glucose with ATP, with coupled acetate kinase catalyzed regeneration of ATP by using acetyl phosphate. The preparations of acetyl phosphate[8] and glucose 6-phosphate[9] have been described previously. For larger scale work, routes based on conversion of starch and phosphate to glucose 6-phosphate (using phosphorylase *a* and phosphoglucose mutase) may also prove useful. The advantages of the G-6-PHD from *Leuconostoc mesenteroides* as a catalyst for use in organic synthetic procedures are that it is almost equally effective in reducing NAD and NADP[10,11] and that it is stable,

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commercially available, and inexpensive. Thus, a single, practical, experimental protocol can be used interchangeably to regenerate both of the reduced nicotinamide cofactors. The latter feature has been noted by others, and this system has been used previously in small-scale experiments.[12] Until recently, however, the cost of G-6-P (~$1800 mol^{-1}) has been sufficiently high to discourage its use as a stoichiometric reagent in synthesis on the scale of moles. Here we establish that the G-6-P required can be prepared conveniently and inexpensively in large quantities by several methods. We illustrate the operation of this regeneration system by the preparation of three chiral materials—D-lactic acid, *threo*-D_s-(+)-isocitric acid, and (*S*)-benzyl-α-d_1 alcohol[13]—in 0.1–0.5 mol scale.

Results

Stability of Nicotinamide Cofactors in Solutions. NAD(P)H. In order to develop practical protocols for using and regenerating the nicotinamide cofactors, it is useful to know the factors which influence the lifetimes of these intrinsically unstable species in solution. Qualitatively, the stability of NAD(P)H is greater in basic than in acidic solutions, and the stability of NAD(P) is greater in acidic than in basic solutions.[14] Several of the reactions that transform NADH and NAD into enzymatically inactive substances have been studied: in particular, NADH undergoes acid-catalyzed hydration and, ultimately, transformation to a cyclic ether;[14] NAD reacts with hydroxide ion and with other nucleophiles.[15] NAD is also subject to enzyme-catalyzed addition of nucleophiles.[16] Analogous reactions undoubtedly occur with NADP(H) but have been studied in less detail. Of these reactions, the most important in determining the stability of NAD(P)H under the conditions encountered during enzyme-catalyzed organic synthesis is the Lewis acid catalyzed hydrolysis reaction. This process has been studied in some detail by Johnson and Tuazon, who have suggested protonation of C5 of the nicotinamide ring as rate limiting.[14] Our studies of this reaction paralleled those of these workers but have had a different and more practical emphasis. Where the two studies can be compared, agreement is satisfactory.

The pH of a solution used in an enzyme-catalyzed synthetic reaction is usually fixed (±0.5 pH units) by the stabilities and pH–rate profiles of the enzymes involved, and it is not practical to adjust the stability of the cofactors by changing the solution pH. Our interest in the subject of the solution stability of these substances was therefore centered on the influence of buffers and other solution components and especially on the influence of potential Lewis acid catalysts.

We have determined rates of disappearance of NAD(P)H spectrophotometrically. These rates follow eq 1 and 2. Figure

$$-\mathrm{d}\ln[\mathrm{NADH}]/\mathrm{d}t = k_{\mathrm{NADH}}^{\mathrm{obsd}} = k_{\mathrm{H}}[\mathrm{H}^+] + k_{\mathrm{HA}}[\mathrm{HA}] + k_{\mathrm{H_2O}} \quad (1)$$

$$-\mathrm{d}\ln[\mathrm{NADPH}]/\mathrm{d}t = k_{\mathrm{NADPH}}^{\mathrm{obsd}} \quad (2)$$

$$k_{\mathrm{NADPH}}^{\mathrm{obsd}} - k_{\mathrm{NADH}}^{\mathrm{obsd}} = \frac{k_{\mathrm{I}}[\mathrm{H}^+]}{K_a + [\mathrm{H}^+]} = \frac{k_{\mathrm{I}}}{1 + 10^{\mathrm{pH}-\mathrm{p}K_a}} \quad (3)$$

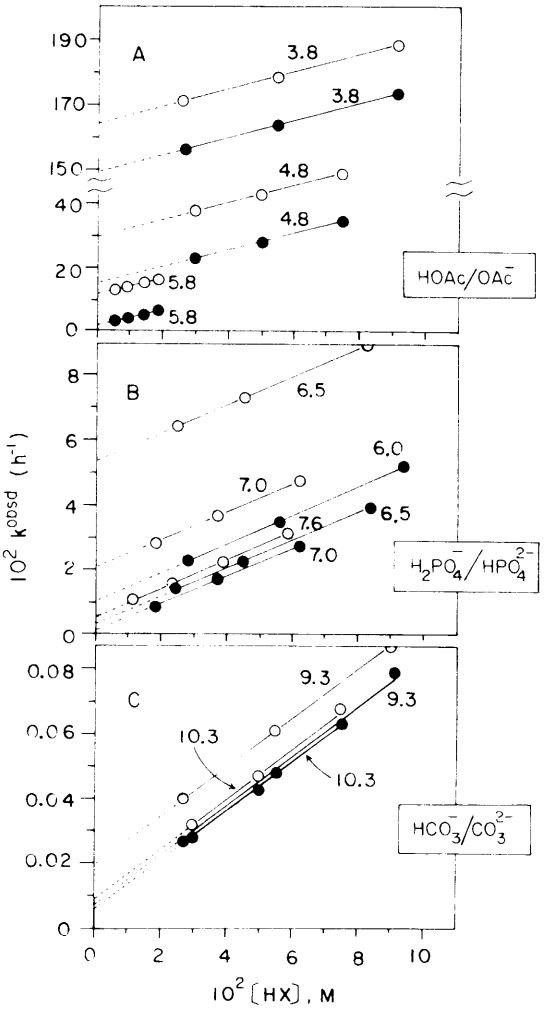

Figure 1. Catalysis of disappearance of NADH (●) and NADPH (○) by acids as a function of [HX]. Values of pH used are listed on the plots.

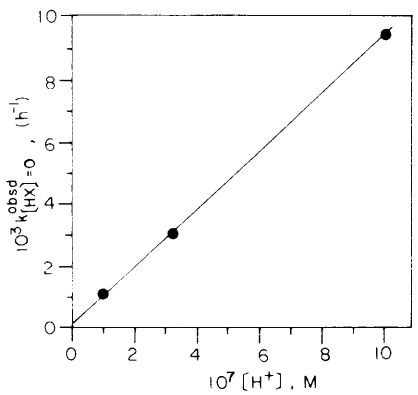

Figure 2. A plot of $k_{[\mathrm{HX}]=0}^{\mathrm{obsd}}$ (obtained by extrapolating the lines of Figure 1 to $[P_i] = 0$) vs. $[H^+]$ yields slope = 9.4×10^3 M^{-1} h^{-1} = k_{H^+} and intercept = 0.7×10^{-4} h^{-1} ($\rightarrow k_{H_2O} = 1.26 \times 10^{-6}$ M^{-1} h^{-1}).

1 summarizes representative data showing the dependence of k^{obsd} on typical buffers ($HOAc/OAc^-$, $H_2PO_4^-/HPO_4^{2-}$, and HCO_3^-/CO_3^{2-}) used to determine k_{HA}. Rate constants were determined as a function of the concentration of the acidic component of the buffer. In the case of phosphate buffer, the slope of a plot of $k_{\mathrm{NADH}}^{\mathrm{obsd}}$ vs. $[H_2PO_4^-]$ yielded $k_{H_2PO_4^-}$; the intercept at $[H_2PO_4^-] = 0$ yielded the quantity ($k_H[H^+] + k_{H_2O}$) (eq 1, Figure 1). Repeating this procedure at several values of pH and plotting the resulting values of $k_{\mathrm{NADH}}^{\mathrm{obsd}}([H_2PO_4^-] = 0)$ vs. $[H^+]$ gave a straight line with slope k_H and intercept k_{H_2O} at $[H^+] = 0$ (eq 1, Figure 2).

This procedure was repeated for a number of different buffers and Lewis acids, although not necessarily using as many points

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Table I. Rate Constants for General Acid-Catalyzed Decomposition of NAD(P)H (eq 1-3)[a]

HA[b]	pK_a	k_{HA}, M^{-1} h^{-1}	$\tau_{1/2}$,[c] h	
			NADH	NADPH
H_3O^+	−1.74	[9.4 × 10^3 = k_{H^+}] (7.3 × 10^3)[d]	690	32 ([HA] = 0)
CH_3COCO_2H	2.5	260	460	31
HCO_2H	3.8	(19)[d]	340	31
$CH_3CHOHCO_2H$	3.9	(1.9)[d]	320	31
CH_3CO_2H	4.8	2.5 (4.4)[d]	270	30
citric acid	5.4	1.2 (2.1)[d]	180	28
G-6-P ($ROPO_3H^-$)	6.1	0.97	59 (60)[e]	21 (21)[e]
6-PG ($ROPO_3H^{2-}$)	6.2	0.96	59	22
maleic acid	6.2	0.22 (0.25)[d]	172	28
PP_i ($H_2P_2O_7^{2-}$)	6.5	0.33 (0.54)[d]	78	24
ImH^+	7.0	0.087	130 (180)[e]	27 (29)[e]
$MeImH^+$	7.1	0.068	140 (220)[e]	27 (28)[e]
NaPi	7.2	0.41 (0.69)[d]	27 (29)[e]	13 (13)[e]
guanidinium P_i			(27)[e]	(15)[e]
Hepes	7.5	0.048	150 (170)[e]	28 (29)[e]
TRA	7.8	0.018	280 (330)[e]	30 (32)[e]
Tris	8.1	0.012	330 (340)[e]	31 (34)[e]
HCO_3^-	10.3	0.0081	520 (80)[e,f]	32 (28)[e]
H_2O	15.5	[1.26 × 10^{-6} = k_{H_2O}] (11.7 × 10^{-6})	690	32
P_i/$HOCH_2CH_2OH$			(30)[e]	(12)[e]
P_i/glycerol			(30)[e]	(14)[e]
P_i/sorbitol			(29)[e]	(14)[e]
P_i/PEG			(29)[e]	(12)[e]
P_i/Me_2SO			(38)[e]	(14)[e]
P_i/DMF			(38)[e]	(16)[e]
P_i/PA			(31)[e]	(14)[e]
P_i/(EtOH)			(29)[e]	(13)[e]

[a] T = 25 °C for all measurements. Rate constants (eq 1) refer to the disappearance of the NAD(P)H chromophore (0.1 mM) at 340 nm. Organic cosolvents were present at concentrations of 20% v/v (except for sorbitol and polyethylene glycol, which were 20% w/w). [b] Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonate; TRA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane; EG, ethylene glycol; G-6-P, glucose 6-phosphate; 6-PG, 6-phosphogluconate; Im, imidazole; MeIm, methylimidazole; PEG, poly(ethylene glycol) (M_r 7500); PA, 1-propanol. [c] The estimated half-life for disappearance of NAD(P)H in a solution containing 0.1 M [HA] + [A^-], pH 7.0, T = 25 °C. [d] Values of rate constants in parentheses are from Johnson and Tuazon (ref 14). [e] Measured values of $\tau_{1/2}$. [f] The difference between observed and calculated values of τ in this instance is probably due to contributions from catalysis by H_2CO_3.

as for $H_2PO_4^-$. For each acid, measurements were carried out over a range in pH of approximately 2 units, bracketing its pK_a. Table I gives values for k_{HA} obtained for these buffers and acids and for solutions containing other components. The measured rate constants k_{HA} were used to calculate the half-life ($\tau_{1/2}$) for disappearance of NAD(P)H in a solution containing the acid HA (pH 7, [HA] + [A^-] = 0.1 M, 25 °C); for convenience, these values are also listed in Table I. In the instances in which the measurements of k_{HA} reproduce those reported by Johnson and Tauzon (shown in brackets in Table I), agreement is adequate. Figure 3 shows the Brønsted plot derived from these data (α = 0.54; lit.[14] α = 0.47) and the relation between the lifetime of NAD(P)H in solution and the pK_a of the buffer acid. These plots provide a check on the consistency of the data and offer a way of estimating the efficiency of other solution or buffer components in catalyzing the decomposition of NAD(P)H. In particular, the most effective catalyts for this decomposition will be those for which the pK_a of the acid is approximately numerically equal to the pH of the solution (for $H_2PO_4^-$, pH 7). This condition reflects a compromise between acid strength (stronger acids are intrinsically more effective catalysts) and degree of ionization (stronger acids have a greater tendency to exist as the catalytically inactive conjugate anions at a particular pH).

The practical consequence of this analysis is the conclusion that acids having pK_a values of ~7 should provide the most rapid catalysis of the decomposition of NADH. The most important of these acid catalysts for the regeneration system discussed here are the phosphate derivatives ($HOPO_3H^-$, $ROPO_3H^-$). The catalytic efficiency of these species is a disadvantage for the system, because G-6-P, 6-PG, and P_i (present as an impurity in the G-6-P) are all necessarily present. All accelerate the decomposition of NAD(P)H.

We briefly examined the ability of organic cosolvents to stabilize NAD(P)H against phosphate-catalyzed decomposition and found only small effects (Table I). This observation is interesting in light of the discovery that attachment of organic polymer chains to the adenine moiety of NADH provides significantly increased stability. This stabilization apparently is not due to a general solvent effect, since addition of high concentrations of organic cosolvents does not have the same effect.

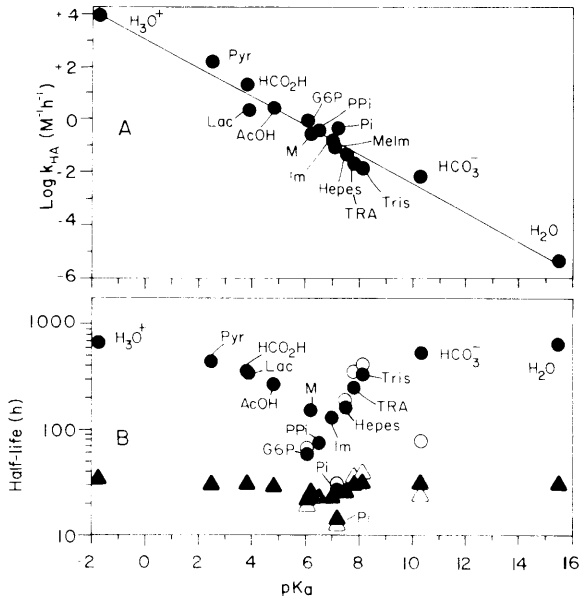

Figure 3. (A) Brønsted plot for acid-catalyzed decomposition of NAD(P)H. Least-squares analysis of the points generated the solid line, described by the equation log k_{HA} = −5.44pK_a + 4.09. (B) Plots of calculated half-life vs. pK_a for decomposition of NADH (●) and NADPH (▲) in 0.1 M buffer, pH 7.0, 25 °C. The observed values are indicated by (○) for NADH and (△) for NADPH. Abbreviations: Pyr, pyruvic acid; Lac, lactic acid; M, maleic acid; MeIm, 1-methylimidazole; Im, imidazole; TRA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonate.

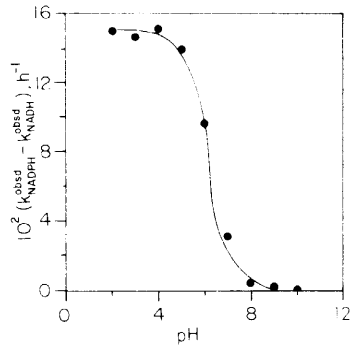

Figure 4. Difference between the observed rate constants for decomposition of NADH (0.1 mM) and NADPH (0.1 mM) as a function of pH (25 °C, 50 mM buffer; pH 3.8–5.8, sodium acetate; pH 6.5–8.8, TRA; pH >9, sodium bicarbonate). The solid line was calculated, by using eq 3 with $pK_a^{NADPH} = 6.2$, $k_I = 0.15\ h^{-1}$.

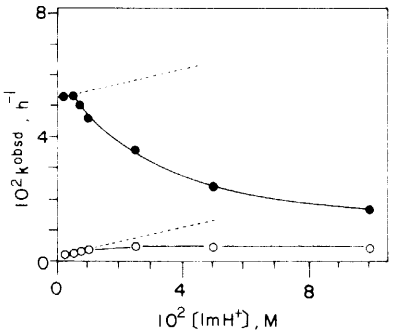

Figure 5. Plots of the rates of decomposition of NADH (○) and NADPH (●) against the concentration of imidazolium ion (ImH^+) at pH 7.0, 25 °C. The dotted lines were those anticipated if the magnitude of k^{obsd} was a linear function of [ImH^+].

The decomposition of NADPH follows a different pattern from that for NADH. Qualitatively, the factors leading to decomposition of NADH also contribute to the decomposition of NADPH, but there is in addition a large contribution to the rate that appears to reflect *intramolecular* acid catalysis by the 2′-phosphate group of NADPH. The decomposition of NADPH is first order in NADPH (eq 2). The difference between the rate constants for decomposition of NADPH and NADH is given by eq 3, where $K_a = 6.2$ is the dissociation constant of the 2′-phosphate, and $k_I = 0.15\ h^{-1}$ is a constant representing intramolecular catalysis. Figure 4 shows the data from which k_I was derived. At high pH, where the 2′-phosphate is entirely ionized, NADH and NADPH decompose at the same rate; at low pH ([buffer] ≃ 0.1 M) NADPH decomposes approximately 3 times more rapidly than NADH. The intramolecular interaction of the 2′-phosphate with the dihydronicotinamide ring proposed here is consistant with previous NMR studies.[17]

Some buffers showed deviations from the Brønsted plot of Figure 3 that were sufficiently large to be of practical interest in efforts to maximize the lifetime of NADPH in solution: in particular, inorganic phosphate is more effective as a catalyst for the decomposition of NAD(P)H than expected from its pK_a value, and imidazole, Hepes, TRA, and Tris buffers were less effective. Moreover, as observed previously,[14] the rates of decomposition of NADH in these organic buffers were not linear in the buffer acid concentration but instead fell off markedly at high concentrations of buffer (Figure 5). This observation may be of some practical value in prolonging the lifetime of NADPH. The origin of this effect is not certain: it may reflect interactions of the organic buffer with the stacked conformation of NAD(P)H[14] or aggregation of the buffer with a resulting decrease in the rate of proton transfer to NAD(P)H, or some other effect. In any event, the k_{HA} values in Table I were estimated from the linear portions

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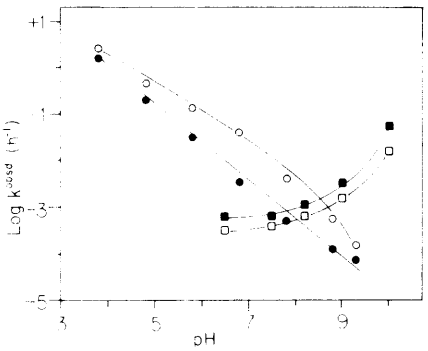

Figure 6. Rate constants for decomposition of NAD (■), NADP (□), NADH (●), and NADPH (○) (0.1 mM each) at 25 °C in 50 mM buffer; pH 3.8–5.8, acetate; pH 6.5–8.8, TRA; pH >9, bicarbonate.

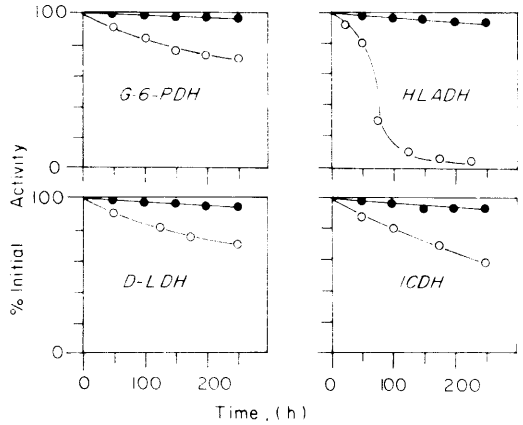

Figure 7. Stabilities of immobilized (●) and soluble (○) enzymes. G-6-PDH: pH 7.6, 5 mM Mg^{2+}, 0.1 M TRA; immobilized, 4 U mL^{-1} gel, soluble, 45 U mL^{-1} solution. HLADH: pH 7.0, 0.1 M phosphate; immobilized, 2 U mL^{-1} gel, soluble, 9 U mL^{-1} solution. D-LDH: pH 7.6, 0.1 M TRA, 5 mM Mg^{2+}; immobilized, 4 U mL^{-1} gel, soluble, 34 U mL^{-1} solution. ICDH: pH 7.6, 0.1 M TRA, 5 mM Mg^{2+}; immobilized, 0.5 mL^{-1} gel, soluble, 2.2 U mL^{-1} solution. Enzyme solutions were kept in 5-mL vials at room temperature under air. The observed losses in activity represent both thermal denaturation and oxidative inactivation. In synthetic experiments, the oxidative inactivation was avoided by keeping the reactions under argon.

of plots such as those shown in Figure 5.

Stability of Nicotinamide Cofactors: NAD(P). The oxidized nicotinamide cofactors are more stable than the reduced ones in the absence of strong nucleophiles (SO_3^{2-}, CN^-)[15] and/or reducing agents for pH <8–9 (Figure 6). Decompositions (measured by enzymatic determination of NAD(P)) follow first-order kinetics (eq 4), with rate constants that are insensitive to buffer compo-

$$-\mathrm{d}\ \ln\ [\mathrm{NAD(P)}]/\mathrm{d}t = k_{\mathrm{NAD(P)}}{}^{obsd} \qquad (4)$$

sition. The rates of decomposition are effectively independent of pH over the range 4–9. Similar results have been obtained by others.[18]

Enzyme Immobilization and Enzyme Stability. Enzymes were immobilized by using procedures described elsewhere[19] by copolymerization into gels formed from poly(acrylamide-*co*-*N*-(acryloxy)succinimide) (PAN-1000 or PAN-500) and triethylenetetraamine (TET). Immobilization yields were satisfactory (see Table III). The stabilities of the immobilized enzymes were higher than those of the same enzymes in solution (Figure 7). With the exception of G-6-PHD from *L. mesenteroides*,[20] all of the enzymes used in this work (HLADH, LDH, ICDH,

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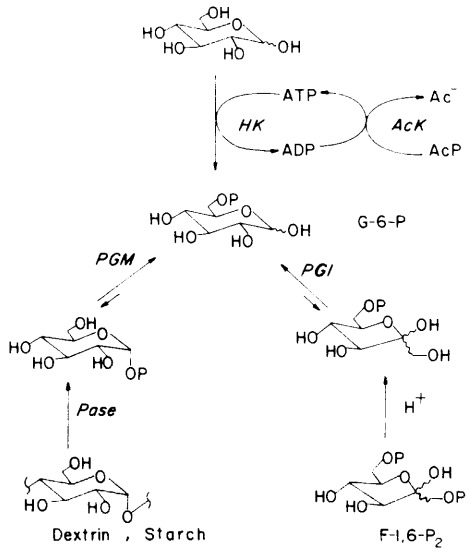

Figure 8. Syntheses of glucose 6-phosphate (G-6-Pe. Abbreviations: HK, hexokinase; AcK, acetate kinase; PGI, glucose phosphate isomerase; Pase, phosphorylase *a*; PGM, phosphoglucomutase.

Table II. Conversion to G-6-P with Phosphorylase and Phosphoglucomutase in the Presence of 0.2 M P_i, pH 7.2, 25 °C

starch[a]	rel re-activity[b]	G-6-P pro-duced,[c] mM
oyster glycogen	1.00	
dextrin	0.25	36
potato starch, soluble	0.18	26
potato starch	0.12	
corn starch	0.12	

[a] Substrate 2% (w/w) in 0.2 M P_i (pH 7.2). [b] Results were obtained by using the *soluble* component of the mixture. [c] The concentration of G-6-P was determined enzymatically after reaction of the 2% solution/suspension of substrate in 0.2 M phosphate (1 L) for 5 days with immobilized Pase (124 U) and PGM (330 U). No additional G-6-P production was observed after 5 days. The recovered enzyme activities were Pase, 71%; PGM, 82%.

G-6-PDH, Pase, PGM, PGI, AcK) contain thiol groups close to or in the active site and are sensitive to autoxidation: reactions were therefore conducted under argon.

Glucose 6-Phosphate. Three routes to G-6-P were explored (Figure 8). The selective phosphorylation of glucose with hexokinase (HK, EC 2.7.1.1) and ATP, with coupled regeneration of ATP from ADP by using acetate kinase (AcK, EC 2.7.2.1) and acetyl phosphate, provides one practical route.[9] The enzymes are commercially available and inexpensive, and acetyl phosphate is easily prepared.[8] The glucose 6-phosphate can be isolated and stored (as the barium salt), or used without isolation.

The enzymatic conversion of an appropriate starch to glucose 1-phosphate (G-1-P) by phosphorylase (Pase, EC 2.4.1.1.)-catalyzed reaction with phosphate and subsequent conversion of G-1-P to G-6-P with phosphoglucomutase (PGM, EC 2.7.5.1) provide a second procedure. We have briefly screened possible polysaccharide starting materials for this reaction (Table II). The forms of starch that seem to offer the best combination of cost, commercial availability, and reactivity are preheated (100 °C, 1 min) soluble starch and dextrin. (Glycogen is the best substrate but is too expensive to be useful in most synthetic applications.) The equilibrium constant for formation of G-6-P from G-1-P is $K = 17$ (pH 6.5–7.2, 30 °C);[21] thus, ~95% of the equilibrium mixture of G-6-P and G-1-P can be utilized by the G-6-PDH. Because the phosphorolysis of starch requires a high concentration of phosphate in solution ($[P_i] \geq 0.2$–0.4 M), it is necessary to isolate the G-6-P produced in order to avoid unacceptably rapid phosphate-catalyzed hydration of NAD(P)H. The presence of inorganic phosphate and unreacted starch make the isolation less convenient than that used in isolating G-6-P prepared by hexokinase-catalyzed phosphorylation of glucose.

The third route to G-6-P examined begins with the conversion of fructose 1,6-diphosphate (F-1,6-P_2) to fructose 6-phosphate (F-6-P)[22] with mild acid catalysis. F-6-P is converted to G-6-P by using phosphoglucomutase (PGM): The equilibrium mixture contains 70% G-6-P.[22] Isolation of G-6-P from the mixture is not necessary for the purpose of NAD(P)H regeneration. The F-6-P solution is used directly in the presence of PGI, G-6-PDH, and the synthetic enzyme (see Experimental Section). This route is not a practical route for large-scale preparations, since F-1,6-P_2 is moderately expensive (~$80/kg). It may, however, be useful in laboratory work, since F-1,6-P_2 is commercially available and no ATP regeneration system is required.

Two other routes leading to G-6-P were also explored briefly. In one, direct reaction of glucose with an anhydrous polyphosphoric acid[24] gave a mixture of glucose phosphates (including ~14% phosphorylation at the 6 position). Since G-6-P is resistant to acid hydrolysis (reflux in 8.7 N HBr for 16 h results in only 10% hydrolysis), other esters can be destroyed with little loss of G-6-P. In practice, separation of G-6-P from phosphate and from other impurities in these mixtures proved too inconvenient for this route to be practical. In the second, hydrolysis of starch to glucose-1-P has been reported to occur in high yields by using an acidic ion-exchange resin (KUZ.EDE10P).[25] We were not able to obtain the specific resin used in this work, and our efforts with an available resin (Dowex 50) led to low conversions (~10%).

Of the several procedures explored for the preparation of G-6-P, that based on *HK*-catalyzed phosphorylation of glucose by acetyl phosphate seems the most convenient, primarily because isolation of the product is simple. For most procedures, isolation is unnecessary, although it may be useful to reduce the volume of the solution by rotary evaporation ($T \simeq 35$ °C). In certain circumstances, however, isolation may be required. The desirability of isolation depends on the enzyme, on the method of preparation of the G-6-P, and on the lifetime required for NAD(P)H (i.e., on the quantity of phosphate which can be tolerated). Acetate ion is present in G-6-P prepared by hexokinase-catalyzed phosphorylation. Acetate is an inhibitor of horse liver alcohol dehydrogenase (HLADH, $K_i = 9.5$ mM)[26] and must be removed in reactions involving this enzyme. This separation can be effected either by isolation of Ba(G-6-P) (see below) or by adjusting the G-6-P solution to pH ~2 with H_2SO_4 and concentrating at reduced pressure to volatilize acetic acid. HCl cannot be used in this operation or other operations that would leave significant concentrations of chloride ion present in solutions to be used with HLADH, since Cl^- is also an inhibitor of this enzyme ($K_i = 100$ mM for NAD and 30 mM for ketone substrates).[27]

Isolation of G-6-P as a solid is best accomplished by preparing the barium salt. The convenience of this procedure depends upon the amount of phosphate present in the solution but is generally straightforward. Although isolations are described in the Experimental Section, several explanatory comments are valuable. Isolations are typically accomplished by addition of barium chloride to precipitate excess phosphate as barium phosphate, separation, and addition of more barium chloride to precipitate the barium salt of G-6-P. An alternative procedure for removing inorganic phosphate involves its precipitation as magnesium ammonium phosphate. This procedure is also followed by isolation

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Table III. Enzymatic Reactions Used to Prepare D-Lactic Acid, *Threo*-D_s(+)-Isocitric Acid, and (*S*)-Benzyl-α-d_1 Alcohol

	D-lactic acid	isocitric acid	(*S*)-benzyl-α-d_1 alcohol
reactor characteristics			
solution volume (L)[b]	5	2	3
pH, temp (°C)	7.8, 25	7.5, 25	7.5, 25
reaction time (days)	4	4	4
G-6-PDH			
immobilization yield[c] (%)	30	30	30
immobilized units[d]	400	100	400
gel volume (mL)[e]	2.0	1.5	2.0
recovery[f] (%)	92	82	90
TN[g]	2.6×10^7	1.3×10^7	2.5×10^7
synthetic enzyme	DLDH	ICDH	HLADH
immobilization yield[c] (%)	40	56	60
immobilized units[d]	300	100	400
gel volume (mL)[e]	2.0	25	13
recovery[f] (%)	94	86	86
TN[g]	4.3×10^7	8.0×10^5	1.0×10^6
G-6-P (mol)	0.60	0.12	0.50
NAD(P) (mol)	5×10^{-4}	2×10^{-4}	4.8×10^{-4}
TN[g]	1200	1020	1000
remaining[f] (%)	85	50	76
isolated products	$Zn(lactate)_2$	$Ba_3(isocitrate)_2$	PhCHDOH
g, purity[h] (%)	53, 95	78, 88	44, 80
mol	0.42	0.17	0.38
yield (%) (based on)	70 (pyruvate)	58 (G-6-P)	80 (PhCDO)
ee (%)	95		95
6-phosphogluconate[i]	$Ba_3(6\text{-PG})_2$	$Mn_3(6\text{-PG})_2$	$Ba_3(6\text{-PG})_2$
g, purity (%)	250, 92	96, 84	215, 90
mol	0.48	0.23	0.41
yield from G-6-P (%)	80	75	82

[a] Reactions were run with positive pH control under argon. Details are given in the experimental section. [b] These volumes are those of the starting solutions. Volumes at the conclusion of the reaction were ~2% larger due to addition of reagents and acid or base to maintain pH. [c] Immobilization yield = 100 (observed activity in the enzyme-containing gel)/starting activity. [d] Unit = μmol min^{-1}. [e] Approximate volume of swollen enzyme-containing gel used in the reactor. [f] "Recovery" represents the percentage of the enzymatic activity that survives the reaction. "Remaining" is the percentage of the NAD(P)(H) present in biochemically active form at the conclusion of the reaction. [g] TN (turnover number) = moles of product generated per mole of enzyme (cofactor) originally present. The turnover numbers are not corrected for residual enzyme (cofactor) activity remaining at the conclusion of the reaction. [h] The quantity of product isolated is given by "g". The percentage of this material that could be assigned the indicated composition is given by "purity". Thus, the lactic acid preparation yielded 53 g of solid. Enzymatic assays indicated that 50.4 g (95%) of this could be assigned the structure Zn(D-lactate)$_2$, and 0.8 g (1.5%) Zn(L-lactate)$_2$. [i] The quantity of solid isolated in grams is given by "g"; the fraction of this material that assays as the indicated product is given by "purity"; the calculated number of moles of this product produced (based on the assumption of 100% purity) is given by "mol".

of G-6-P as the barium salt. Barium salts can inactivate enzymes and precipitate with some anions and are toxic. They must be removed before use of the G-6-P. Removal of barium is accomplished by dissolving the Ba(G-6-P) in 0.1–0.2 M H_2SO_4 solution, separating the insoluble precipitate of barium sulfate, and neutralizing the resulting solution with sodium hydroxide.

The stability of G-6-P, both under conditions experienced during operation of an enzymatic reactor and during storage, is pertinent to the use of this regeneration system. Figure 9 summarizes semiquantitative kinetic data describing to the decomposition of G-6-P at 25 °C: these decompositions seemed to follow first-order kinetics. Figure 9 gives both half-lives for decompositions and first-order rate constants. On the scale of times required for completion of the synthetic reactions (~5 days), the decomposition of G-6-P presents no problem. For long-term storage in solution, decomposition is a modest problem. By addition of ethylene glycol or other organic components to these solutions (20% v/v), decomposition can be slowed significantly: these components are often (but not always) innocuous toward the enzymes used.[28] Sorbitol, for reasons that are unclear, appears to destabilize G-6-P. Storage at 0 °C also greatly increases stability (at 0 °C, the half-life for decomposition of 1 mM G-6-P in 0.1 M phosphate buffer (pH 7) is 400 days).

Synthetic Applications of the NAD(P)H Regeneration System. We have tested the practicality of the NAD(P)H regeneration system based on the G-6-PDH from *Leuconostic mesenteroides* for synthetic applications by applying it in enzyme-catalyzed preparations of three materials: D-lactic acid, *threo*-D_s(+)-isocritic acid, and (*S*)-benzyl-α-d_1 alcohol (Figure 10). D-Lactic acid is

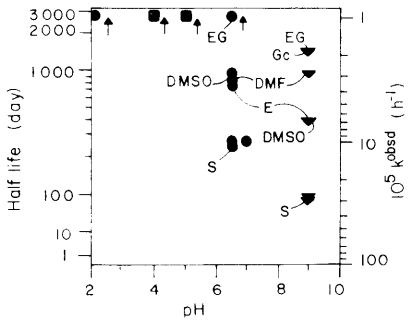

Figure 9. Estimated half-lives and observed first-order rate constants (k^{obsd}) for decomposition of glucose 6-phosphate. Buffers: (●) 0.1 M phosphate, (■) 0.1 M acetate, (▼) 0.1 M glycine. Organic cosolvents (20% v/v) are as follows: EG, ethylene glycol; Gc, glycerol; E, ethanol; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; S, sorbitol. Points with arrows represent lower limits for half-lives (or upper limits for k^{obsd}): no decomposition was detected in these solutions over the course of 1 month.

a useful synthon for the preparation of chiral substances and complements. L-Lactic acid is already readily available by fermentation. Isocitric acid contains an array of chiral functionality and may also be useful as a starting material in synthesis. Chiral benzyl-d_1 alcohol is used in research biochemistry. Table III summarized representative reactions; details are given in the Experimental Section. A few general comments on these reactions follow.

The enzymes in these preparations were immobilized separately and the enzyme-containing gels mixed only in the reactor. This procedure gives slightly lower overall reaction rates than one in which the enzymes are coimmobilized in the same gel.[29] It is,

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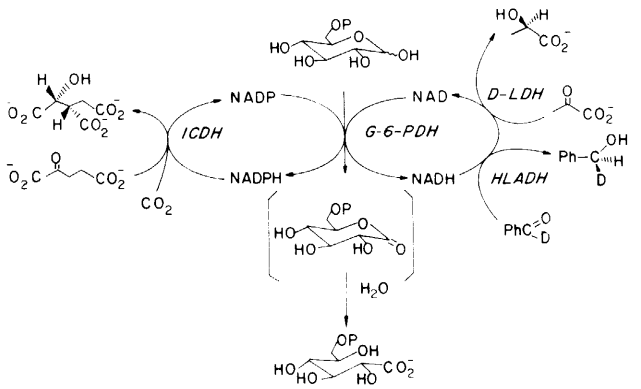

Figure 10. Syntheses based on NAD(P)H regeneration. Abbreviations: G-6-PDH, glucose-6-phosphate dehydrogenase; D-LDH, D-lactic dehydrogenase; HLADH, horse liver alcohol dehydrogenase; ICDH, isocitrate dehydrogenase.

however, more convenient and permits independent control of the amount of each enzyme present in the reaction. We have not explicitly determined the rate-limiting step in these reactions. The assayed activities of immobilized G-6-PHD and the coupled synthetic enzyme were approximately the same. We can only estimate the expressed activity—that is, the activity under the conditions of the synthetic reaction, rather than under the V_{max} conditions used in the assays—but we note that the quantities of products isolated over the indicated intervals in which the reactors were operating suggested average enzymatic activities ~30% those measured under assay conditions.

The solutions used in these reactions are relatively dilute, and the volumes required to generate a given quantity of product are accordingly somewhat larger than those usually used in conventional organic synthesis. The use of dilute solutions is dictated by product inhibition: thus, K_i = 0.13 M for lactate in the L-LDH-catalyzed reduction of pyruvate[30] (K_M = 0.019 *M* for D-lactate in the D-LDH catalyzed reduction of pyruvate)[31] and K_i = 0.060 M for isocitrate in the ICDH-catalyzed conversion of a α-ketoglutarate to isocitrate.[32]

The workup of the reactions consists of stopping the stirring, allowing the gel particles to settle (typically 1–2 h), and decanting the product-containing solution. The gel can usually be used again immediately. The acidic products in this work were usually isolated by conversion to metal salts and precipitation with organic solvent. The product purities were typically 70–90%; higher purities required crystallization (zinc lactate, $Ba_3(6\text{-PG})_2$) or distillation (benzyl alcohol). The initial concentration of NAD or NADP in these reactors was ~0.1 mM. This value is fixed for NAD by the requirement that it be above K_M for G-6-PDH (see Table IV). A lower concentration of NADP could, in principle, have been used. No effort was made to recover the nicotinamide cofactors at the conclusion of the reactions, although their residual activity was determined by enzymatic assays.

Discussion

This method for regeneration of reduced nicotinamide cofactors has both advantages and disadvantages relative to other methods. Its advantages are six: First, it can reduce both NAD and NADP. It is thus almost uniquely suitable for the few problems that might involve both types of cofactor simultaneously.[11] More importantly, it provides a single convenient procedure for either cofactor separately. Second, both G-6-P and 6-GP are innocuous to most enzymes, unlike the reactive aldehydes or ketones produced by methods based on alcohol dehydrogenases. Third, the equilibrium constants for reduced nicotinamide formation are high, because the reaction is rendered essentially irreversible by hydrolysis of the initially formed 6-phosphogluconolactone to 6-phosphogluconate. Fourth, glucose-6-phosphate dehydrogenase is commercially available, inexpensive, and easy to manipulate and immobilize. Fifth, because G-6-PDH from *L. mesenteroides* has no essential thiol groups, this enzyme is relatively stable under aerobic conditions and can be used for regeneration of reduced nicotinamide cofactors in oxygen-containing environments. Sixth, the values of K_M for the oxidized nicotinamide cofactors for G-6-PDH is not usually high, and the regeneration system based on G-6-PDH thus requires only the concentration of these cofactors that would be required for efficient operation of the synthetic enzymes (Table IV).

Table IV. Values of K_M (mM) for NAD(P)(H) for Selected Enzymes

enzyme	NAD	NADH	NADP	NADPH	ref
G-6-PDH (*L. mesenteroides*)	0.11		0.0057		10
formate dehydrogenase (*C. boidinii*)	0.09				33
alcohol dehydrogenase (yeast)	0.074	0.011			34
HLADH	0.017	0.027			34
D-LDH	1.2[a]	0.071			35
L-LDH	0.253	0.011			35
ICDH			0.0001	0.001	32
glutamate dehydrogenase	0.7	0.024	0.047	0.025	36
lipoamide dehydrogenase	0.14	5.0			37

[a] Determined in this laboratory by D. Abril.

This regeneration procedure also has several disadvantages. First, although glucose 6-phosphate is easily prepared in quantity from inexpensive starting materials, it is intrinsically a more complex substance than the formate or simple alcohols used with other regeneration methods. We note that this disadvantage is not severe in our laboratory, since the 6-PG produced is itself a valuable intermediate for the production of pentose derivatives (ribose 5-phosphate, ribulose 5-phosphate, ribulose 1,5-diphosphate, NAD).[38,39] The ability of a particular laboratory to use these substances depends, however, on the research being conducted. Second, both G-6-P and 6-PG are general acid catalysts for the hydration of the reduced nicotinamide cofactors. At the concentrations of G-6-P used in this work, the lifetime of NADH is shortened by alkyl hydrogen phosphate catalyzed decomposition to ~15% of that in a phosphate-free solution, while that of NADPH is shortened to approximately 70% that in phosphate-free solution. In the synthesis reported here, this decrease has not been particularly important. The cofactor turnover numbers (1000–1200) reported in Table III were calculated conservatively: that is, they are based only on the quantity of NAD(P) originally added to the solution and contain no correction for the quantity of cofactor remaining at the end of the reaction. In a reaction designed to optimize cofactor utilization, either the cofactors would be recovered or the reaction would be run in a way that resulted in essentially complete consumption of cofactor. Under these circumstances, one can estimate from the data in Table III that these turnover numbers would range from 8000 (for D-lactic acid) to 2000 (for isocitric acid). Such turnover numbers are high enough for most syntheses of complex molecules. For slower reactions (in which decomposition of the NAD(P)H

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is more rapid relative to production of product) or for reactions in which maximum turnover numbers for the NAD(P) is important for economic or other reasons, the G-6-P- and 6-PG-catalyzed hydrations may become influential in determining the choice of a regeneration system. Third, the 6-PG produced as the product of oxidation of G-6-P must be separated from the product of the reduction. Although the separations reported here are straightforward, they are more complex than those required with formate/formate dehydrogenase or ethanol/alcohol dehydrogenase.

These problems notwithstanding, our practical experience with this and other NAD(P)H regeneration systems suggests that it is one of the most convenient (perhaps *the* most convenient) systems available for use in laboratory-scale synthesis (that is, syntheses on scales up to several moles). The low cost and ease of manipulation of G-6-PDH compensate for the inconvenience of preparing G-6-P. Formate/formate dehydrogenase is a very attractive and useful alternative for NADH regeneration, but the commercial enzyme is presently 35 times more expensive than G-6-PDH (for equivalent activities), more sensitive to autoxidation than G-6-PDH, and limited to the reduction of NAD to NADH. Ethanol/alcohol dehydrogenase is not a particularly attractive system: both acetaldehyde and ethanol deactivate enzymes rapidly, and the equilibrium constants for reduction of carbonyl groups by regeneration procedures based on this system are not high.

Chemical methods[3,4] for NAD(P)H regeneration lack the specificity required to achieve high turnover numbers. If reduction of NAD(P) to NAD(P)H is 95% selective for hydride donation to the 4 position of the nicotinamide ring, the residual activity of the cofactor after 64 turnovers would be $(0.95)^{64} \approx 4\%$. We require 10^3 turnovers in most synthetic applications. Thus, presently available procedures involving direct chemical or electrochemical reduction of NAD(P) to NAD(P)H are insufficiently selective to be practical.

Experimental Section

General. UV spectra were taken with a Perkin-Elmer 552 spectrophotomer, equipped with a constant-temperature cell. NMR spectra were measured at 250 MHz for determinations of enantiomeric excesses (Bruker 270). Water was doubly distilled in glass. The pH of reaction mixtures was controlled with a Weston Model 7561 pH controller coupled to a Monostat cassette peristaltic pump.

Materials. Enzymes were obtained from Sigma. NAD, NADP, dithiothretol (DTT), 1,3-dithiopropanol, dextrin and starches, and routine biochemicals were from Sigma. PAN was prepared as described.[19] Argon was used as an inert atmosphere; welding-grade material was used without purification.

Assays. Assays for the following group of enzymes and biochemicals were taken directly from Bergmeyer:[40] lactate dehydrogenase (LDH) and lactate (pp 480, 1464), G-6-PDH and G-6-P (pp 458, 1238), isocitrate dehydrogenase (ICDH) and isocitrate (pp 624, 1570), glutamate dehydrogenase (GluDH) and 2-ketoglutarate (pp 650, 1577), horse liver alcohol dehydrogenase (HLADH) (p 429), acetate kinase (AcK) and acetyl phosphate (pp 425, 1538), phosphorylase *a* (Pase) (p 505), phosphoglucomutase (PGM) (pp 798), phosphoglucoisomerase (PGI) (pp 501, 113), hexokinase (HK) (p 473), fructose 6-phosphate (F-6-P, p 1238), fructose 1,6-diphosphate (F-1,6-P_2, p 1314), 6-phosphogluconate (6-PG, p 1248). NAD(P)H was determined by using GluDH (p 2054); NAD(P) was determined by using G-6-PDH from *L. mesenteroides* with the same procedure used for NADP (p 2050).

Assays for the activities of immobilized enzymes were essentially the same as described.[19] Aliquots (20–100 μL) of immobilized enzyme suspended in buffer were taken and added to a 3-mL plastic cuvette containing 3 mL of buffer and the required substrates. The cuvette was stoppered with Parafilm and shaken for 3–4 s to mix the suspension. The absorption was read at 340 nm for 5 s. This process was repeated about 15 times. A linear response was obtained for the change of absorbance with time.

Acetyl phosphate was prepared as the diammonium salt via reaction of anhydrous H_3PO_4 with acetic anhydride.[8] Anhydrous H_3PO_4 was made by the slow addition of P_2O_5 (191.5 g) to stirred 85% H_3PO_4 (500 g) at −10 °C. The mixture was kept from moisture at room temperature with stirring for at least 1 h before use. Saturated NH_3/MeOH was prepared at −30 °C by passing anhydrous NH_3 over cooled MeOH for 30 min. Addition of acetyl phosphate to NH_3/MeOH was performed at −60 °C. The temperature during and after addition in the NH_3/MeOH to the reaction mixture should be −10 °C or below to prevent aminolysis of acetyl phosphate.

Rates of Phosphorolysis of Starches and Dextrin. The polysaccharide substances (2%, w/w) were added to water containing 0.2 M phosphate (pH 7.2). Of those examined, only glycogen and dextrin were completely soluble. For those substrates which were only partially soluble, the supernatant solution over the suspension was used. "Heat-treated" starch was prepared by bringing the starch suspension in water to 100 °C with stirring, letting stand for 1–2 min, and then cooling and adding an equal volume of 0.4 M phosphate buffer, pH 7.2. To those solutions (2.5 mL) were added EDTA (0.3 μmol), $MgCl_2$ (30 μmol), PGM (3 U), G-6-PDH (10 U), Pase (rabbit muscle, 1 U), and NADP (0.6 μmol). The rate of phosphorolysis was measured by following the absorbance change at 340 nm.

Kinetics of hydrolysis of glucose 6-phosphate were carried out with 1 mM solutions of glucose 6-phosphate. Aliquots (0.1 mL) were withdrawn periodically from solutions maintained under specified conditions, diluted to 3 mL with 0.1 M TRA buffer, pH 7.8, and assayed enzymatically for G-6-P.

Kinetics of Decomposition of NAD(P)(H). Solutions of NAD(P)(H) (0.1 mM) were prepared in 10 mL of 50 mM buffer and kept at 25 °C. Aliquots (3 mL) were withdrawn periodically, and the concentration of NAD(P)H was determined spectrophotometrically at 340 nm. Occasional points were checked enzymatically with *GluDH*. Decomposition in organic solvent/water (20% v/v) mixtures was carried out with the buffer concentration maintained at 0.1 M. The pH values were adjusted after addition of the organic solvent to the aqueous buffer at the chosen pH. For NAD(P) determinations, 1-mL portions were withdrawn and added to 2 mL of 0.1 M TRA, pH 7.8, containing G-6-P (2 mM). G-6-PDH (2 U) was added and the absorbance change at 340 nm was measured.

Immobilization of D-Lactate Dehydrogenase (D-LDH). The commercially available D-LDH in 1 mL of 3.2 M $(NH_4)_2SO_4$ (5 mg, 1725 U) was dissolved in 20 mL of 0.1 M phosphate buffer, pH 7.0, which had been cooled to 0 °C and deoxygenated with a stream of argon. The solution was concentrated by ultrafiltration under reduced pressure at 0 °C by using an Immersible-CX Molecular Separator Kit (Millipore Corp.). When the volume was reduced to about 1 mL, another 20-mL portion of cold phosphate solution was added and the mixture was again concentrated. Three repetitions of this process over 8 h resulted in an enzyme solution in 1 mL of phosphate buffer having 1380 U of activity (80%) which was ready for immobilization. To 0.6 g of PAN-1000 was added 2.4 mL of 0.2 M Hepes buffer containing 8.5 mM pyruvate and 1.0 mM NADH with vigorous stirring. After 1 min, 50 μL of 0.5 M DTT solution was added, followed by 300 μL of 0.5 M TET solution and the enzyme-containing solution. The mixture gelled within 3 min. It was kept at room temperature for 1 h. The gel was ground into small particles in a mortar and washed with 50 mL of 50 mM Hepes buffer, pH 7.5, containing 50 mM ammonium sulfate. The gel was separated by centrifugation and washed again with the same buffer without ammonium sulfate. A 20-μL aliquot of the gel suspension in 30 mL of TRA buffer (0.1 M, pH 7.5) was diluted in 3 mL of 0.2 M hepes buffer, pH 7.5, and the enzyme activity was measured (552 U, 40% yield).

The $(NH_4)_2SO_4$ present in the original enzyme preparation could also be removed by centrifuging the enzyme suspension (5 mg in 1 mL of 3.2 M $(NH_4)_2SO_4$, 4 °C, 15000*g*, for 5 min). The precipitate was dissolved in 1 mL of 0.3 M Hepes buffer, pH 7.5, and immobilized as described above.

Immobilization of Isocitrate Dehydrogenase (ICDH). The commercial enzyme from pig liver suspended in 50% glycerol (100 mg/10 mL, 200 U) was dialyzed at 0 °C against 4 L of 0.05 M Tris/0.1 M Na_2SO_4 solution (pH 7.3) for 4 h, using a membrane tube with molecular cutoff of 6000–8000 (spectrum Medical Industries, Inc., Los Angeles), and then concentrated at 0 °C to 5 mL by using a molecular separator kit as described above. The resulting solution contained 180 U (90% yield) of enzyme. The procedure for immobilization was the same as that used for D-LDH. Buffer [20 mL, 0.2 M Hepes containing isocitrate (50 mM) and $NADP^+$ (1 mM)] was added to 5 g of PAN-1000, followed by DTT (0.5 mL), enzyme solution, and TET (3 mL). The gel was washed with 50 mM Tris/0.1 M Na_2SO_4/50 mM $(NH_4)_2SO_4$ (400 mL) and then with Tris/SO_4^{2-} buffer (400 mL). The total activity obtained was 102 U (56% yield).

Immobilization of Glucose-6-phosphate Dehydrogenase (G-6-PDH), Glutamate Dehydrogenase (GluDH), and Horse Liver Alcohol Dehydrogenase (HLADH). **G-6-PHD** from *L. mesenteroides* was immobilized according to the same general procedure: 10 mg of enzyme per g of PAN 1000 was used. G-6-P [(6 mM) and NADP (0.6 mM) were

(40) Bergmeyer, H. U. "Methods of Enzymatic Analysis"; Academic Press: New York, 1974.

present during the immobilization to protect the enzyme active site. Ater the gel had formed, it was washed with 50 mM $(NH_4)_2SO_4$ solution and 50 mM Hepes buffer (0.2 M, pH 7.5). The immobilization yield was 30%. GluDH was immobilized similarly (10 mg/g of PAN-1000) in the presence of ketoglutarate (15 mM), ADP (2 mM), NADH (5 mM), and NH_4Cl (20 mM): NH_4Cl was added together with the enzyme solution to minimize the reaction of NH_4^+ with PAN. The immobilization yield was 40%. For HLADH, 10 mL of 0.3 M Hepes buffer, pH 7.5, containing NAD^+ (2.5 mM) and ethanol (500 mM), was added to 2.5 g of PAN-1000 with stirring, followed by addition of enzyme solution (50 mg of enzyme in 2 mL of Hepes buffer) and TET (2.1 mL). DTT was not necessary in this case. Other procedures were the same as above. The immobilization yield was 60%.

Coimmobilization of Phosphorylase *a* (Pase) and Phosphoglucomutase (PGM). Pase (EC 2.4.1.1., 390 U/30 mg) and PGM (EC 2.7.5.1, 830 U/2 mg) were immobilized according to the general procedure by using PAN-1000 (4.12 g) in the presence of glycogen (1 mM), P_i (70 mM), G-1-P (2 mM), G-6-P (5 mM), glucose 1,6-diphosphate (1 mM), TET (2.7 mL), and DTT (0.3 mL). Other details were the same as those of the general procedure. The immobilized yields were as follows: Pase, 31%; PGM, 40%.

Coimmobilization of D-LDH, G-6-PDH, and Phosphoglucoisomerase (PGM). D-LDH (1000 U/3 mg), G-6-PDH (1380 U/2 mg), and PGI (EC 5.3.1.9, 1000 U/2 mg) were coimmobilized similarly by using 4 g of PAN-1000 in the presence of Hepes buffer (0.3 M, pH 7.5, 16 mL), DTT (0.2 mL), TET (3 mL), G-6-P (6 mM), F-6-P (1.5 mM), pyruvate (8.5 mM), NADP (0.6 mM), and NADH (1 mM). The immobilized yields were as follows: D-LDH, 40%; G-6-PDH, 38%; PGI, 41%.

Measurements of Enzyme Stability. Enzymes, either soluble or immobilized, in specified buffer solutions were incubated aerobically at 25 °C without stirring. Aliquots (20 μL) were withdrawn periodically, and the enzymatic activity was determined according to the method described in the assay section.

Glucose 6-Phosphate. Hexokinase-Catalyzed Phosphorylation of Glucose. G-6-P was prepared by HK-catalyzed phosphorylation and the ATP regeneration system.[9] A 3-L solution containing glucose (1.4 mol), ATP (10 mmol), $MgCl_2$ (98 mmol), EDTA (4.8 mmol), and 1,3-dimercapto-2-propanol (18 mmol) was deoxygenated and maintained under argon. Immobilized HK (1200 U) and AcK (1200 U) were added to this solution. Diammonium acetyl phosphate (1.4 mol, 90% purity) was added to the stirred reaction solution in 10 portions over 60 h, and the solution was maintained at pH 7.4 by addition of 4 M KOH solution by using a pH controller. The reaction was performed at 25 °C. After 3 days, 1.2 mol of G-6-P was formed (0.364 M in 3.3 L, yield 85%). The solution was separated from the polyacrylamide gels by decantation. Barium chloride (0.2 mol) was added to precipitate inorganic phosphate; this material was removed by filtration. The resulting solution (containing 0.364 M G-6-P) was used directly in the following synthesis of isocitrate, after dilution with water. The turnover number for ATP during the synthesis was 140, and the recovered enzyme activities were as follows: HK, 92%; AcK, 80%.

For the preparation of $Ba(G\text{-}6\text{-}P)\cdot 7H_2O$, a slight excess of $BaCl_2$ (0.37 mol) was added to 1 L of the above G-6-P solution (0.364 M) followed by slow addition of 400 mL of 95% ethanol. The precipitated solid (187 g) contained 96% $Ba(G\text{-}6\text{-}P)\cdot 7H_2O$ (0.33 mol). This salt could be stored in the refrigerator (4 °C) without decomposition in the absence of moisture.

For removal of barium, the barium salt (187 g) was suspended in 1.7 L of 0.2 M H_2SO_4 and stirred vigorously at room temperature for 30 min. The precipitated barium sulfate was removed by filtration or centrifugation. The free G-6-P acid solution was then neutralized with sodium hydroxide. A solution prepared by this procedure was used in the HLADH-catalyzed synthesis of (*S*)-benzyl-α-d_1 alcohol (see below).

For the preparation of a concentrated G-6-P solution, 1 L of the G-6-P solution (0.364 M) was acidified to pH 2.0 by using concentrated H_2SO_4 or HCl and concentrated under reduced pressure (aspirator, 20 mmHg) at 35 °C to 200 mL. The concentration of G-6-P in the resulting solution was 1.82 M. No decomposition of G-6-P was observed during the process, and most of the acetate produced from acetyl phosphate evaporated during concentration. The concentrated solution was stable in the refrigerator: No decomposition was observed over 1 month. This material was used in the D-lactate preparation described below.

Glucose 6-Phosphate. Phosphorolysis of Dextrin. To a 1-L solution containing 0.4 M phosphate buffer, pH 7.0, and 40 g of dextrin were added phosphorylase *a* (Pase, 240 U) and phosphoglucomutase (PGM, 600 U) coimmobilized in polyacrylamide gels and 3 mmol of DTT. The reaction mixture was kept under argon with stirring at 25 °C. Enzymatic assay indicated the presence in solution of 78 mmol of G-6-P after 5 days. $MgCl_2$ (0.4 mol) was added to the solution. Aqueous ammonia (40 mL of 14% solution) was added slowly to the cooled, stirred solution. The resulting precipitate of $MgNH_4PO_4$ was removed by filtration. Ethanol (95%, 400 mL) was added to the filtrate, and the precipitate of unreacted dextrin was discarded. Barium chloride (0.1 mol) was added to the solution and stirred for 30 min at 0.4 °C. The precipitated solid (35 g) was isolated. This material contained 82% of $Ba(G\text{-}6\text{-}P)\cdot 7H_2O$ (29% yield based on the number of glucose unit in the starting dextrin). The recovered enzyme activities were as follows: Pase, 80%; PGM, 86%.

Glucose 6-Phosphate: Phosphorolysis of Soluble Starch. A suspension of 40 g of soluble starch in 1 L of water was stirred and heated to boiling (it took about 30 min to bring the solution to a boil). The opaque solution was allowed to remain at 100 °C for 1–2 min. The mixture was cooled to room temperature. To the resulting solution was added 0.4 mol of NaH_2PO_4. It was adjusted to pH 7.0 with 5 N NaOH. The solution was deoxygenated and maintained under argon. DTT (3 mmol) and the enzymes recovered from the dextrin phosphorolysis described above (Pase, 190 U; PGM, 490 U) were added to the solution. The reaction was performed by using the same conditions described for dextrin phosphorolysis. After 5 days, no further reaction was observed, and the G-6-P (96 mmol) formed was isolated as barium salt in the same way as described above: 43 g of solid was obtained which contained 70 mmol of $Ba(G\text{-}6\text{-}P)\cdot 7H_2O$ (86% purity), corresponding to a 32% yield based on the number of glucose units in the starch. The recovered enzyme activities were as follows: Pase, 76%; PGM, 78%.

Preparation of Fructose 6-Phosphate from Fructose 1,6-Diphosphate. F-1,6-P_2Ca_2 (260 g, 0.47 mol; practical grade from Sigma, 75% purity) was dissolved in 1.5 L of 2 N HCl. The solution was kept at 85–90 °C (using a steam bath or water bath) with stirring for 1.5 h. The brown solution was treated with 60 g of activated charcoal and filtered to remove colored materials. To the colorless solution were added 107 g of potassium oxalate (0.94 mol) and 200 g of $Ba(OH)_2$ (0.64 mol). The resulting solution was adjusted to pH 6 by the addition of 5 N NaOH with stirring, and the precipitated calcium oxalate and barium phosphate were removed by filtration. The filtrate (1.5 L) contained 0.33 mol of F-6-P (70% yield). This solution was stored in the refrigerator (4 °C) for use in subsequent reactions.

Determination of Optical Purity. Benzyl-α-d_1 alcohol and D-lactate were converted to their MTPA esters[41] by using freshly prepared (+)-(2*R*)-α-methoxy-(2-(trifluoromethyl)phenyl)acetyl chloride. Zinc D-lactate (0.5 g) was first converted to its methyl ester by using anhydrous methanol (18 mL) and dried Dowex 50W (H^+ form, 1 g) as catalyst. Type 3A molecular sieves were added, and the mixture was refluxed for 4 h. After filtration, methanol was removed by evaporation and the methyl lactate was converted to the MTPA ester. The enantiomeric purity was determined by ^1H NMR examination of the protons of the methoxyl group at 250 MHz. The MTPA ester of the racemic alcohols were used as references for determining the shift values of each pair of diastereotopic methoxyl group.

The optical purity of D-lactate was also determined enzymatically. The zinc D-lactate (90 mg) was dissolved in 10 mL of glycine–hydrazine buffer (0.4 M, pH 9.0). A 3-mL aliquot was taken, and L-lactate dehydrogenase (10 U) and NAD^+ (2 mM) were added to the solution. The increase of absorbance at 340 nm (read against a reference consisting of the same solution, omitting only the enzyme) was recorded until no change of absorbance (~5 h) was observed. The concentration of L-lactate in the sample was determined by using 6.22 mM^{-1} cm^{-1} as absorbance coefficient of NADH.

Isocitrate was determined directly by enzymatic methods with ICDH and NADP. The increase of absorbance at 340 nm was measured and the content of isocitrate was determined.

Synthesis of D-Lactate. A 5-L solution containing sodium pyruvate (0.12 M), G-6-P (0.12 M), $MgCl_2$ (5 mM), EDTA (1 mM), and 1,3-dimercapto-2-propanol (2.5 mM) was deoxygenated with argon. The immobilized G-6-PDH (400 U), D-LDH (300 U), and NAD^+ (0.5 mmol) were added. The reaction was conducted under argon at 25 °C with stirring and the pH maintained at 7.8 by addition of 2 N KOH solution by using an automatic pH controller. After 4 days, the reaction was complete. Enzymatic assays indicated that the summed concentration of NAD and NADH was 85% of the original value of NAD. The polyacrylamide gels were allowed to settle, and the solution was decanted. $BaCl_2$ (0.95 mol) was added to the decanted solution with stirring, followed by 1 L of ethanol. The precipitated solid was collected by filtration and washed with 50% ethanol. The precipitated solid was collected by filtration and washed with 50% ethanol. After being dried, the product (250 g) contained 92% of barium 6-phosphogluconate, corresponding to a 80% yield based on G-6-P added (see below). The filtrate and washings were collected and concentrated under reduced pressure at 45 °C to a volume of 2 L and then acidified to pH 2.5 with concentrated HCl. Some

(41) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543–9.

inorganic salt was removed by addition of 2 L of ethanol and filtration of the resulting precipitate. The filtrate was concentrated again under reduced pressure to a volume of 500 mL and neutralized to pH 6.0 by addition of solid $ZnCO_3$. Ethanol was added to the hot (70 °C) solution until turbidity appeared. After the solution was cooled, the precipitates were removed by filtration and discarded. The filtrate was concentrated to a volume of 300 mL and some zinc lactate precipitated. Ethanol (100 mL) was added to the suspension. After the suspension was cooled to 4 °C, the precipitated zinc lactate was collected by filtration and dried. This material (53 g) contained 95% of zinc D-lactate and 1.5% of zinc L-lactate by enzymatic assays. These values correspond to a 97% enantiomeric excess and 70% yield based on pyruvate. ^1H NMR spectra (250-MHz) of the MPTA ester of D-lactate methyl ester showed a 95% ee based on the signals of the methoxyl groups (3.660 ppm for the D-lactate ester and 3.560 ppm for the L-lactate ester). The turnover number for NAD in this experiment was 1200. The G-6-PDH and LDH activities in the residual gels were 92% and 94%, respectively, of the original values.

Conversion of Barium 6-Phosphogluconate (6-PG) to 6-Phosphogluconic Acid and Assay Procedure. Concentrated HCl (18 mL) was added to a stirred suspension of 0.2 mol of barium 6-phosphogluconate (Ba_3(6-PG)$_2$, 106 g, 90% purity) in H_2O (1 L). After the solution was stirred for 10 min, Na_2SO_4 (31.2 g, 0.22 mol) was added to the clear solution and the precipitated $BaSO_4$ was removed by centrifugation or filtration. An aliquot of the solution (5 μL) was withdrawn and added to 3 mL of TRA buffer (0.1 M, pH 7.6) containing $MgCl_2$ (5 mM), NADP (0.4 mM), and 6-PGDH (5 U). The concentration of 6-PG was determined from the absorbance change at 340 nm. This assay showed 0.19 mol of 6-PG in 1.1 L of solution (0.173 M; yield, 95%).

For the preparation of NaCl-free 6-PG, the same quantity of Ba_3(6-PG)$_2$ was mixed with 1.1 L of 0.2 M H_2SO_4 and the mixture was stirred for 30 min. The precipitated $BaSO_4$ was removed and the solution (1.2 L) assayed: it contained 0.188 mol of 6-PG (0.157 M; yield, 95%).

Benzaldehyde-α-*d* was prepared from benzaldehyde-1,3-dithiane by exchange with D_2O and hydrolysis.[42] Dry hydrogen chloride was bubbled into a solution of 1,3-propanedithiol (101 mL, 1 mol) and benzaldehyde (102 mL, 1 mol) in chloroform (750 mL) at 0 °C until the mixture saturated (~5 min). After standing at 25 °C for 0.5 h, the mixture was washed with two 200-mL portions of water, three 200-mL portions of 10% KOH solution, and two 200-mL portions of water, dried over anhydrous Na_2SO_4 (50 g), and evaporated under reduced pressure. The solid obtained was treated with 10 g of activated charcoal and crystallized from methanol (500 mL) to give 180 g (94%) of 2-phenyl-1,3-dithiane, mp 69–70 °C. This material was dissolved in 1.6 L of anhydrous tetrahydrofuran (distilled over CaH_2) and cooled to −60—70 °C. The system was deoxygenated with argon. A solution of *n*-butyllithium in hexane (625 mL, 1 mol) was then introduced over a period of 1 h into the dithiane solution by using a stainless-steel cannula under a slight positive argon pressure. The temperature of the reaction mixture was maintained between −60 and −70 °C. After the solution stood at the same temperature for 6 h, D_2O (>99.9% *d*, 130 mL) was added slowly with stirring and the solution was warmed to room temperature. The solution was neutralized at 0 °C with 1 N HCl (~1 L) and tetrahydrofuran was removed on a rotary evaporator. The solution was extracted with two 800-mL portions of CH_2Cl_2/pentane (1:1, v/v). The organic phase was washed with 1 N $NaHCO_3$ (400 mL), water (400 mL), and saturated NaCl solution (400 mL). The residue after evaporation of the solvent was recrystallized from methanol to give 145 g (0.85 mol) of 2-phenyl-1,3-dithiane-*2-d*, mp 69–70 °C. The deuterium incorporation was near 100%, based on the virtual absence of the C-2 proton (δ ($CDCl_3$) 5.1).

A solution of $HgCl_2$ (326 g, 1.2 mol) in methanol/H_2O (9:1, v/v) (600 mL) was added to a stirred warm mixture (~40 °C) of deuteriodithiane prepared above (119 g, 0.6 mol) and HgO (118 g, 0.54 mol) in the same solvent mixture (3 L) in a 5-L flask. The mixture was refluxed with stirring for 4 h under argon and then cooled to room temperature. The white precipitate was removed by filtration, and the filtrate was distilled through a 40-cm Vigreux column until about 1 L remained. CH_2Cl_2/pentane (1:1, v/v) (1 L) was added to the residue, and the solution was washed twice with 500-mL portions of 4 M NH_4OAc solution and twice with 500-mL portions of saturated NaCl solution and dried over anhydrous Na_2SO_4 (80 g). Solvent was removed by distillation as before to furnish 300 mL of liquid, which was transferred to a 500-mL flask and distilled again through a 20-cm Vigreux column under reduced pressure (27 mmHg). The fractions of benzaldehyde-α-*d* with boiling point 77–79 °C were collected (51 g, 80%). NMR analysis (in $CDCl_3$) showed no aldehyde proton absorption at 9.95 ppm.

Synthesis of (*S*)-Benzyl-α-d_1 Alcohol. To a 3-L solution contained 0.5 mol of G-6-P (generated from the barium salt by treatment of equivalent amount of 0.2 M H_2SO_4 as described in the G-6-P preparation), 10 mmol of $MgSO_4$, 0.48 mmol of NAD, and 400 U each of G-6-PDH and HLADH (these activities were based on benzaldehyde as substrate), benzaldehyde-α-*d* (0.48 mol, 51.4 g) was added dropwise over 2 days. The solution was kept under argon and the pH was controlled at 7.5 by adding 4 N KOH using a pH controller. After 4 days, the solution was decanted from the gel and extracted continuously with four 250-mL portion of ether. 6-PG was recovered from the aqueous phase as its barium salt in 82% yield as described previously. The ether solution was washed once with saturated aqueous NaCl solution, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting liquid was distilled through a 10-cm Vigreux column to obtain 44 g of benzyl-α-d_1 alcohol (80%), bp 110 °C (30 mmHg). The compound was pure and the deuterium incorporation was almost 100% by ^1H NMR (60 MHz, CCl_4): δ 2.1 (s, 1 H), 4.6 (t, 1 H, J_{HCD} = 1.8 Hz), 7.33 (s, 5 H). The optical purity of the (*S*)-benzyl-α-d_1 alcohol was determined by 250-MHz ^1H NMR spectroscopy with the MTPA ester: the methoxy group was monitored (3.501 ppm for (*S*)-benzyl-α-d_1 alcohol in $CDCl_3$, 3.535 ppm for (*R*)-benzyl-α-d_1 alcohol); ee = 95%. The turnover number of NAD^+ was 1000, and its residual activity was 80%. The reisolated G-6-PDH and HLADH retained 90% and 86% of their original activities, respectively.

Synthesis of *threo*-D(+)-Isocitrates. A 2-L solution containing G-6-P (0.15 M), ketoglutarate (0.15 M), NH_4HCO_3 (0.4 M), $MgCl_2$ (5 mM), $MnCl_2$ (1 mM), and 1,3-dimercapto-2-propanol (3 mM) was kept under CO_2 and the pH was automatically controlled at 7.5. The immobilized G-6-PDH (100 U), ICDH (100 U), and NADP (0.1 mM) were then added, and the mixture was kept at the same pH under CO_2 with stirring. After 4 days, the concentration of isocitrate was 0.102 M (68% reacted) and no further reaction occurred. The total concentration of NADP and NADPH at the conclusion of the reaction was 50% of the original value. The gels were separated by centrifugation. GluDH (100 U), G-6-PDH (100 U), and EDTA (4 mM) were added to the supernatent solution, and the solution was stirred under argon to consume the remaining G-6-P and ketoglutarate (~3 days at pH 8.0). The solution, after separation of gels, was adjusted to pH 4.0 with concentrated HCl to remove bicarbonate (liberated as CO_2), which would precipitate with Mn^{2+} and influence the following isolation step. Manganese(II) dichloride (63 g, 500 mmol) was added to the solution and the pH adjusted to 7.5 by adding 5 N NaOH. Ethanol (300 mL) was added to precipitate 6-PG as its manganese(II) salt (96 g). This solid contained 84% 6-PG (corresponding to an overall yield of 75%). Barium chloride (78.2 g, 320 mmol) was then added to the mother liquid to precipitate isocitrate as the barium salt (78 g): this material contained 88% of isocitrate by enzymatic assay, corresponding to an overall yield of 58%. The turnover number for the synthesis of isocitrate was 1020 and 1500 for the total reaction. The residual activities of the recovered immobilized enzymes were as follows: G-6-PDH, 82%; ICDH, 86%; G-6-PDH (second addition), 92%; GluDH, 91%. These three enzymes could be used repeatedly or stored in the refrigerator at 4 °C under argon.

Synthesis of D-Lactate from Fructose 6-Phosphate. Sodium pyruvate (36 g, 0.33 mol), $MgCl_2 \cdot 6H_2O$ (1.2 g, 6 mmol), and NAD (152 mg, 0.2 mmol) were dissolved in the F-6-P solution (0.33 mol in 1.5 L) prepared above. The solution was adjusted to pH 7.8 with 5 N NaOH and deoxygenated with argon. DTT (0.92 g, 6 mmol) and enzymes coimmobilized in 4 g of PAN-1000 (D-LDH, 400 U; PGI, 410 U; G-6-PDH, 520 U) were added to the solution. The total volume was 1.6 L. The reaction was carried out in the same way as described for the G-6-P/G-6-PDH system for D-lactate synthesis. After 2 days, the reaction was complete and zinc lactate was isolated (30 g) in 67% yield with 92% of purity (determined enzymatically). Barium 6-phosphogluconate was isolated (131 g) in 75% yield with 90% purity. The turnover number of NAD was 1650 and the recovered enzyme activities were as follows: PGI, 92%; G-6-PDH, 90%; D-LDH, 90%.

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(42) Seebach, D.; Erickson, B. W.; Singh, G. *J. Org. Chem.* **1966**, *31*, 4303–4.