Product analysis and yields were obtained by VPC. Compounds were isolated by preparative VPC and identified as follows. **34**: NMR δ 3.53 (dd, 2, CH_2Cl, J_{H-F} = 16.2, J_{34} = 5.5 Hz), 4.98 (d of d of t, 1, CHF, J_{H-F} = 46, J_{34} = 5.5, J_{23} = 4.5 Hz), 5.13–6.07 (m, 3, $CH=CH_2$). Anal. Calcd for C_4H_6ClF: C, 44.26; H, 5.57; F, 17.50. Found: C, 44.27; H, 5.56; F, 18.62. **35**: NMR δ 3.90–4.20 (m, 2, CH_2Cl), 4.88 (dd, 2, CH_2F, J_{H-F} = 45, J_{34} = 4.0 Hz), 5.77–6.18 (m, 2, CH=CH); additional evidence for the structure of **35** was obtained by its independent synthesis from *trans*-1,4-dichloro-2-butene and potassium fluoride.[21] Anal. Calcd for C_4H_6ClF: C, 44.26; H, 5.57; F, 17.50. Found: C, 44.38; H, 5.40; F, 14.06. **36** and **37**: NMR (agreement with lit.—see Heasley et al.[2a]).

Products from the reaction of **2** and **33** were separated on column B (40 °C) with retention times as follows (min): **38**, 1.5; **39**, 3.0; **30**, 4.1; and **41**, 10.4. Analysis and yields were obtained by VPC. Compounds were isolated by preparative VPC and identified as follows. **38**: NMR δ 3.43 (dd, 2, CH_2Br, J_{H-F} = 16.2, J_{34} = 5.5 Hz), 4.98 (d of d of t, 1, CHF, J_{H-F} = 45, J_{34} = 5.5, J_{23} = 4.5 Hz), 5.20–6.23 (m, 3, $CH=CH_2$). Anal. Calcd for C_4H_6BrF: C, 31.40; H, 3.95; F, 12.42. Found: C, 31.52; H, 3.95; F, 11.79. **39** NMR δ 3.8–4.0 (m, 2, CH_2Br), 4.83 (dd, 2, CH_2F, J_{H-F} = 45.0, J_{34} = 4.0 Hz), 5.75–6.12 (m, 2, CH=CH). The structure of the compound was further confirmed by its independent synthesis from *trans*-1-4-dibromo-2-butene and potassium fluoride.[21] Anal. Calcd for C_4H_6BrF: C, 31.40; H, 3.95; F, 12.42. Found: C, 30.58; H, 3.96; F, 11.01. **40**: IR (agreement with lit.[22] for CH_3O and $CH=CH_2$); NMR δ 3.33 (s, 3, CH_3O), 3.18–4.02 (multiplets, 3, $CHOMeCH_2Br$), 5.08–6.08 (m, 3, $CH=CH_2$). **41**: IR (agreement with lit.[22] for CH_3O and CH=CH); NMR δ 3.25 (s, 3, CH_3O), 3.75–4.07 (m, 4, CH_2Br and CH_2OMe), 5.73–6.07 (m, 2, CH=CH).

Products from Methyl Vinyl Ketone (42). Products from the reaction of **1** with **42** were reported on column A (70 °C) with retention times as follows (min): **43**, 4.8; and **44**, 9.6. Product compositions and yields were found directly by NMR after solvent removal. Samples isolated by preparative VPC were identified as follows. **43**: NMR δ 2.36 (s, 3, CH_3) and 5.3–3.9 (complex multiplet, 3, CH_2FCHCl); IR, 1732 cm^{-1} (C=O); further proof of the structure was obtained by elimination with triethylamine, which formed only 3-chloro-3-butene-2-one; elemental analysis was not possible because the compound darkened quickly at room temperature. **44**: n^{20}_D 1.4368, lit.[23] n^{20}_D 1.4368; NMR δ 2.11 (s, 3, $CH_3C=O$), 3.20 (s, 3, CH_3O), 3.52 (d, 1, CH_2OCH_3, J_{34} = 5.6), 4.02 (t, 1, CHCl, J_{34} = 5.6 Hz); IR, 1721 (C=O); treatment with triethyl amine caused no elimination, showing that the chlorine was exclusively in the β position.

Products from Styrene (45). Products from the reaction of **1** with **45** were separated on column C (120 °C) with retention times as follows (min): **48**, 9.9; **46**, 15; and **47**, 17. Compounds were isolated by preparative VPC and identified by comparison of NMR spectra to those reported (**46**,[10] **47**,[24] and **48**[25]). Products from the reaction of **2** with **45** were separated on column B (100 °C) with retention times as follows (min): **49**, 4.2; and **50**, 7.2. Compounds were isolated by a combination of liquid chromotography on silica gel and preparative VPC. The structure of **49** was confirmed by preparation of an authentic sample and comparison of the NMR spectra. The NMR spectrum of **50** agreed with the reported spectrum.[26]

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Registry No. **1**, 593-78-2; **2**, 28078-73-1; **3**, 110-83-8; **4**, 20421-40-3; **5**, 18424-56-1; **6**, 17170-96-6; **7**, 5927-93-5; **8**, 592-41-6; **9**, 51444-06-5; **10**, 55999-57-0; **11**, 55999-60-5; **12**, 1871-74-5; **13**, 24618-32-4; **14**, 24618-33-5; **15**, 156-60-5; **16**, 359-28-4; **17**, 86727-55-1; **18**, 96-33-3; **19**, 55900-27-1; **20**, 36997-02-1; **21**, 36997-03-2; **22**, 1537-52-6; **23**, 60456-17-9; **24**, 27704-96-7; **25**, 18707-60-3; **26**, 77332-67-3; **27**, 77332-69-5; **28**, 26839-90-7; **29**, 4358-59-2; **30**, 77332-68-4; **31**, 77332-70-8; **32**, 26849-33-2; **33**, 106-99-0; **34**, 54964-08-8; **35**, 86727-56-2; **36**, 7795-90-6; **37**, 57513-14-1; **38**, 86727-57-3; **39**, 86727-58-4; **40**, 22427-00-5; **41**, 22427-01-6; **42**, 78-94-4; **43**, 86727-59-5; **44**, 24474-37-1; **45**, 100-42-5; **46**, 20372-72-9; **47**, 3898-26-8; **48**, 622-25-3; **49**, 1786-36-3; **50**, 13685-00-2; *tert*-butyl hypochlorite, 507-40-4; *tert*-butyl hypobromite, 1611-82-1.

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Synthesis of Sugars by Aldolase-Catalyzed Condensation Reactions[1]

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Dihydroxyacetone phosphate was prepared in 200-mmol scale from dihydroxyacetone by two procedures: reaction with phosphorus oxytrichloride and glycerol kinase catalyzed phosphorylation using ATP with in situ regeneration of ATP by phosphoenolpyruvate or acetyl phosphate. Dihydroxyacetone phosphate was converted to fructose 6-phosphate in 80% yield by exposure to a mixture of co-immobilized triosephosphate isomerase and aldolase followed by acid hydrolysis of the condensation product fructose 1,6-bisphosphate. Fructose 6-phosphate was subsequently converted by chemical and enzymatic schemes into fructose, glucose 6-phosphate, and glucose. Practical procedures are described for the preparation of D- and L-glyceraldehyde 3-phosphate and for several hexoses labeled with ^{13}C in the C-2 and C-2,5 positions.

Introduction

The work reported in this manuscript is part of a program to evaluate the potential of aldolase-catalyzed aldol reactions as a route to isotopically labeled and uncommon sugars on scales from 0.01 to 1 mol. The aldolase used in this work (from rabbit muscle, E.C. 4.1.2.13) is commercially available. It is inexpensive, and it has high specific activity and good stability. It requires dihydroxyacetone phosphate (DHAP)[2] as one substrate, but will accept a

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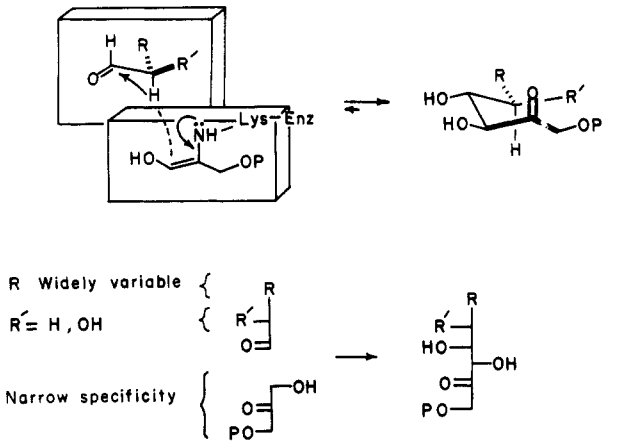
Table I. Kinetic Parameters of Aldolase from Muscle and Yeast[a]

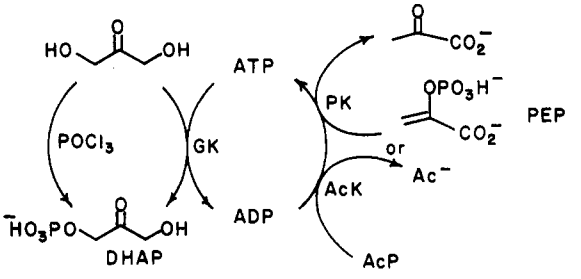
substrate	rabbit muscle K_m, mM	rabbit muscle V	yeast K_m, mM	yeast V
	Aldol Cleavage			
D-fructose 1,6-bisphosphate	0.006[b]	1[c]	0.36	1[d]
L-sorbose 1,6-bisphosphate	0.044	0.1	0.13	0.002
L-sorbose 1-phosphate	0.6	0.02	0.2	0.0002
D-fructose 1-phosphate	6.3	0.01	1.0	0.0004
D-fructose 6-phosphate		0.0001		
	Aldol Condensation			
dihydroxyacetone phosphate	1.0 (0.4)[e]		2.4	
+ D-glyceraldehyde-3-P	4.0	2.8	2.0	3.0
+ D,L-glyceraldehyde		0.1		
+ D,L-lactaldehyde		0.1[f]		
+ D,L-α-hydroxybutyraldehyde		0.1[f]		
dihydroxyacetone sulfate (3)				
+ D,L-glyceraldehyde-3-P		<0.0001[f]		
dihydroxyacetone				
+ D-glyceraldehyde-3-P		<0.0001		
acetol phosphate (2)				
+ D-glyceraldehyde-3-P		<0.0003		<0.0006
4-hydroxy-3-oxo-1-phosphonate (1)	0.4[e]			
+ D-glyceraldehyde-3-P		0.1[e]		

[a] The numbers cited are from: Richards and Potter (Richards, O. C.; Rutter, W. J. *J. Biol. Chem.* **1961**, *236*, 3185-92) unless otherwise indicated. The K_m of DHAP was determined in the presence of D-glyceraldehyde 3-phosphate. $V = V_{max}$. [b] Martensen, T. M.; Mansour, T. E. *Biochem. Biophys. Res. Commun.* **1976**, *69*, 844-51. [c] 42 μmol min^{-1} mg^{-1} of enzyme. [d] 180 μmol min^{-1} mg^{-1} of enzyme. [e] Stribling, D. *Biochem. J.* **1974**, *141*, 725-8. [f] Determined in this laboratory. The concentration of aldehyde acceptor was 50 mM and the concentration of donor (DHAP or dihydroxyacetone sulfate) was 20 mM, pH 7.0.

Scheme I. Stereochemistry of Reactions Catalyzed by Rabbit Muscle Aldolase



Scheme II. Chemical and Enzymatic Preparations of Dihydroxyacetone Phosphate



60% from $POCl_3$ (not isolated)
80% from ATP (isolated)

wide variety of aldehydes as the second.[3] The stereochemistry of the aldol condensation catalyzed by this enzyme is indicated in Scheme I; the two new chiral centers are formed enantiospecifically.[4] Aldolase has been used previously in small-scale (~2 mmol) syntheses of ^{13}C-labeled fructose and glucose.[5] Its utility for larger scale work has not been explored.

These methods should be useful in the synthesis of certain simple sugars and in the preparation of isotopically labeled sugars for use in biochemistry, metabolism, and physiology.

Results

The enzyme aldolase from rabbit muscle or yeast is highly specific for DHAP. Table I summarizes data on substrate specificity.[3] In general, any change in the structure of DHAP greatly reduces reactivity in the aldolase-catalyzed reaction. The phosphonate analogue 4-hydroxy-3-oxo 1-phosphonate, **1**, provides an exception: this substance is 10% as active as DHAP. Acetol phosphate **2** is about 0.01% as active as DHAP, while dihydroxyacetone monosulfate (**3**) is not a substrate for the muscle enzyme.

(2) Abbreviations: AcP, acetyl phosphate; DHA, dihydroxyacetone; FDP, D-fructose 1,6-bisphosphate; F-6-P, D-fructose 6-phosphate; F-1-P, D-fructose 1-phosphate; G-6-P, D-glucose 6-phosphate; Gd, glyceraldehyde; Gd-3-P, glyceraldehyde 3-phosphate; Gr-1-P, (*R*)-glycerol 1-phosphate; PEP, phosphoenolpyruvate; S-1-P, L-sorbose 1-phosphate; GK, glycerokinase; GPDH, glycerophosphate dehydrogenase; HK, hexokinase; GluDH, glutamic dehydrogenase; PGI, phosphoglucoisomerase; Pase, potato acid phosphatase; PK, pyruvate kinase; TPI, triosephosphate isomerase.

(3) In addition to the substrates described in Table I, the aldolase from rabbit muscle also accepts the following aldehydes using DHAP as reactant, but kinetic data are not available for the reactions: formaldehyde, glycolaldehyde, L-glycidaldehyde (O'Connell, E. L.; Rose, I. A. *J. Biol. Chem.* **1973**, *248*, 2225-31), L-threose (Jones, J. K. N.; Matheson, N. K. *Can. J. Chem.* **1959**, *37*, 1754-6), D-ribose, L-arabinose, D-lyxose, and D-xylose (Jones, J. K. N.; Stephton, H. H. *Ibid.* **1960**, *38*, 753-60). The enzyme from peas has been reported to accept: glycolic aldehyde (Hough, L.; Jones, J. K. N. *J. Chem. Soc.* **1952**, 4047-52), D,L-lactaldehyde (Hough, L.; Jones, J. K. N. *Ibid.* **1952**, 4052-5), and D-erythrose (Hough, L.; Jones, J. K. N. *Ibid.* **1952**, 342-5).

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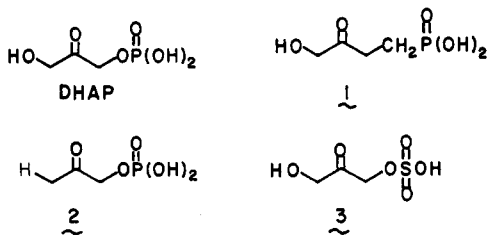


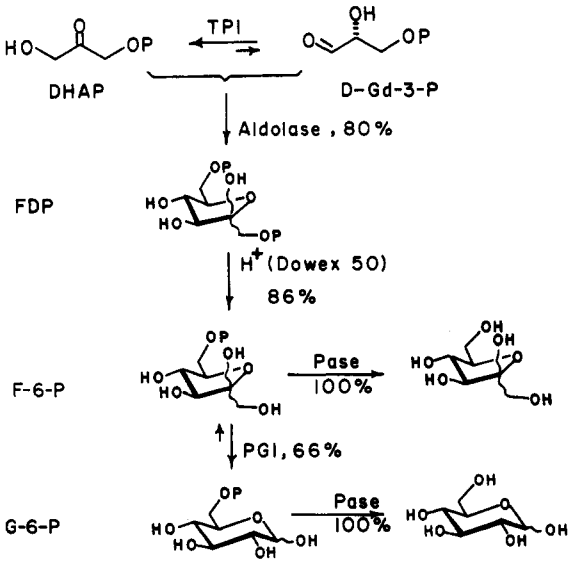
Dihydroxyacetone Phosphate. We have examined two routes from dihydroxyacetone (DHA) to DHAP (Scheme II). For most preparations carried out on scales up to several moles, the procedure based on chemical phosphorylation is the more convenient (especially for laboratories in which preparation of PEP or AcP are not routine). The deficiencies of the chemical procedure are that it generates relatively dilute solutions of product and that impurities remaining from the chemical steps may complicate workup. Further, because the product solution from this chemical route contains phosphate, isolation and purification of the DHAP from it are inconvenient. It is best used directly in subsequent transformations without isolation. The purity of the DHAP prepared by this procedure seems, however, to be perfectly acceptable in the enzymatic reactions we have explored.

The biochemical route to DHAP is based on the enzymatic phosphorylation of DHA by ATP with in situ cofactor regeneration. This procedure gives a product that can either be isolated in relatively high purity (80–90%) or used in situ. The enzymatic procedure using PEP as the ultimate phosphorylating agent is somewhat more convenient than that based on AcP, because PEP has greater stability in solution than AcP.[6,7] The procedures are otherwise comparable.

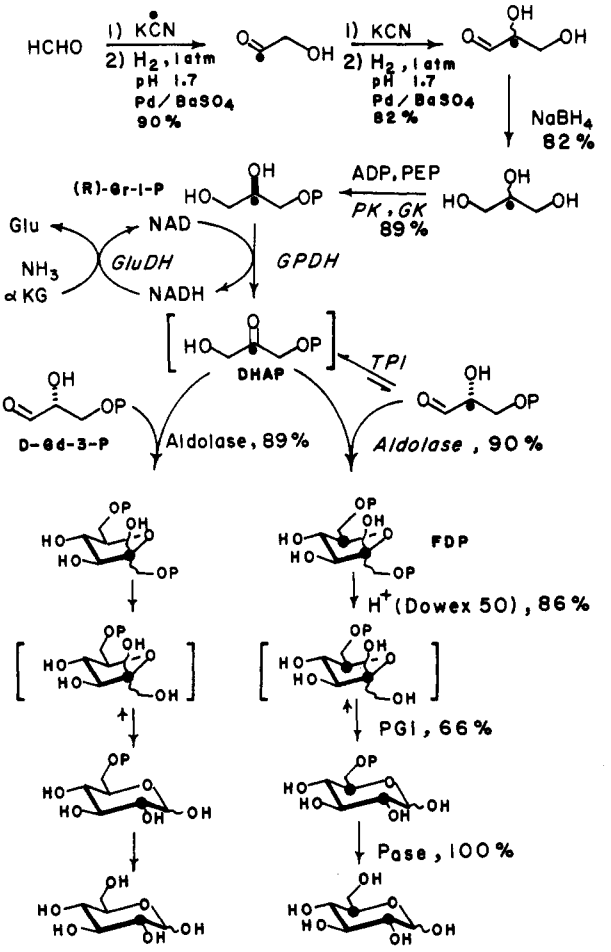
D- and L-Glyceraldehyde and D- and L-Glyceraldehyde 3-Phosphate. These three-carbon substances condense with DHAP in aldolase-catalyzed reactions and yield hexose phosphates; the stereochemistry at C-5 is defined by the chirality of the glyceraldehyde. Procedures for preparing each are summarized in Table II. D-Glyceraldehyde (D-Gd) can be prepared in 82% enantiomeric excess (ee) by selective phosphorylation of L-Gd in a mixture of D-Gd and L-Gd using glycerokinase (GK). It can also be prepared by treatment of D-glyceraldehyde 3-phosphate (D-Gd-3-P) with acid phosphatase (Pase, E.C. 3.1.3.1). L-Gd is best prepared in 100% ee by Pase-catalyzed hydrolysis of L-Gd-3-P prepared enzymatically by using GK. D-Gd-3-P is easily obtained by isomerization of DHAP with triosephosphate isomerase (TPI, E.C. 5.3.1.1) and used in situ, although the equilibrium mixture contains 96% DHAP.[8] If a separate preparation is required, it can be obtained by oxidative cleavage of fructose 6-phosphate with lead tetraacetate. L-Gd-3-P is most easily obtained by enantiospecific phosphorylation of the L-Gd present in D,L-Gd using glycerokinase.

Fructose 6-Phosphate and Derivatives. The conversion of DHAP to fructose 1,6-bisphosphate (FDP) and fructose 6-phosphate (F-6-P) and from this latter intermediate to glucose 6-phosphate (G-6-P) and glucose is outlined in Scheme III. The initial step is the equilibrium conversion of DHAP to D-glyceraldehyde 3-phosphate (D-Gd-3-P) by TPI. The two are then condensed to FDP

Scheme III. Syntheses of Fructose 1,6-Bisphosphate, Fructose 6-Phosphate, Fructose, Glucose 6-Phosphate, and Glucose



Scheme IV. Syntheses of (2-$^{13}C_1$)Glucose and (2,5-$^{13}C_2$)Glucose and Intermediates (Heavy Circles Indicate Positions of ^{13}C Label)



by aldolase. The FDP was converted to F-6-P by acid-catalyzed hydrolysis. The conversion of this substance to fructose, to G-6-P, and (by way of G-6-P) to glucose follows enzymatic and acid-catalyzed routes outlined in the scheme.

These conversion are all relatively straightforward. The enzymes required are commercially available, inexpensive,

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(8) Oesper, P.; Meyerhof, O. *Arch. Biochem. Biophys.* **1950**, *27*, 223–33.

Table II. Preparation of Glyceraldehydes

reactant	reagent, catalysts	product: yield, % (ee, %) glyceraldehyde D	glyceraldehyde L	glyceraldehyde-3-P D	glyceraldehyde-3-P L
D,L-glyceraldehyde	GK, ATP	45[a] (82)			41[a] (100)
dihydroxyacetone phosphate	TPI			4 (100)	
D-fructose 6-phosphate	$Pb(OAc)_4$, 4 equiv			85 (100)	
D-glyceraldehyde-3-P	Pase	85 (100)			
L-glyceraldehyde-3-P	Pase		85 (100)		
D-fructose	$Pb(OAc)_4$, 4 equiv	96 (100)[b]			
L-sorbose	$Pb(OAc)_4$, 4 equiv		96 (100)[b]		

[a] Based on D,L-glyceraldehyde. [b] Perlin, A. S. *Methods Carbohydr. Chem.* 1962, *1*, 61-3.

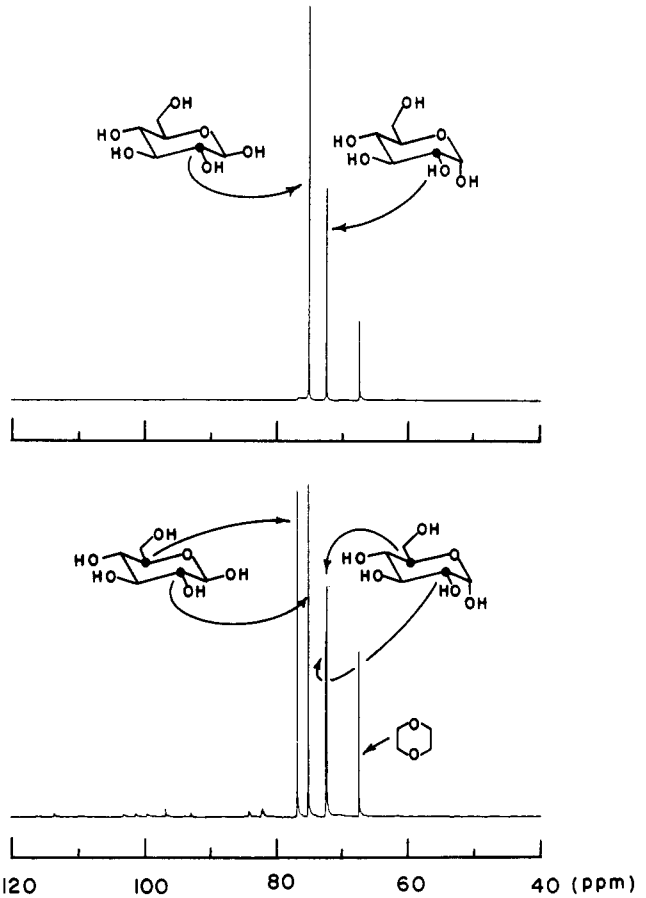

Figure 1. ^{13}C NMR spectra of glucose with ^{13}C enrichment at C-2 (A) and at C-2 and C-5 (B).

and stable in the immobilized forms. No inhibition has been observed in a reaction with reactants present at 0.1 M concentrations. One technical problem in the preparation of G-6-P is that the equilibrium mixture achieved in the PGI-catalyzed reaction contains 70% G-6-P and 30% F-6-P;[9] this mixture complicates isolation of the pure sugars. To separate these two species, barium acetate or barium chloride was added to the solution. G-6-P precipitated as its barium salt; F-6-P remained in the solution.

Preparation of Isotopically Labeled Fructose and Glucose Derivatives. In preparing isotopically labeled sugars, it is useful to differentiate between the two C_3 pieces which are assembled to make the C_6 sugar. We take advantage of the ability of aldolase to accept a range of aldehydes as reactants and use DHAP as only one component of the aldol reaction. Scheme IV outlines a synthesis of glucose (and intermediates) labeled with ^{13}C at

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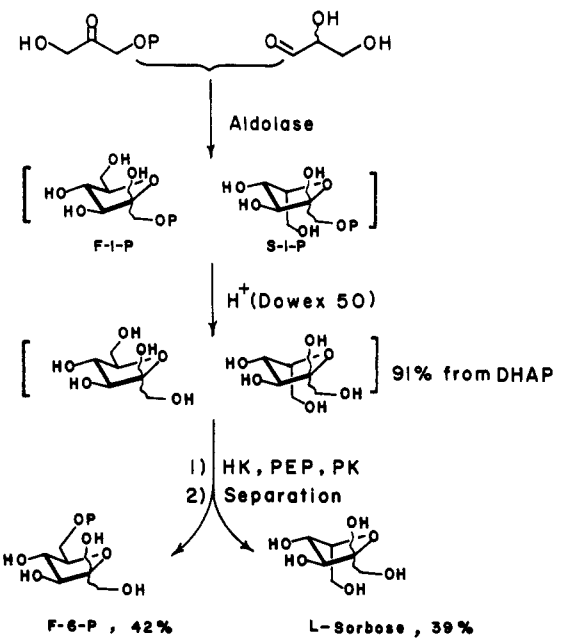
Scheme V. Preparation of D-Fructose 6-Phosphate and L-Sorbose



the C-2 and C-2,5 positions. Figure 1 contains the ^{13}C NMR spectra of glucose prepared by using these procedures.

We note that these sequences of reactions are also directly applicable to the synthesis of glucose and fructose having different labeling patterns and (using L-glyceraldehyde and L-glyceraldehyde 3-phosphate) to sorbose derivatives. For example, introduction of the ^{13}C by the *second* cyanohydrin reduction (to generate (1-$^{13}C_1$)-D,L-Gd) would yield glucose containing label in the C-1, C-3, C-4, and C-6 positions (or the C-1 and C-3 positions, if D-Gd-3-P is supplied exogenously). D- or L-Gd labeled at C-1 (prepared from D,L-Gd, enzymatically as described above), after aldolase-catalyzed condensation with DHAP, would yield glucose or L-sorbose, respectively, containing label in the C-4 position. (1-$^{13}C_1$)-D,L-Gd would react with DHAP and generate a mixture containing F-1-P and sorbose-1-P labeled at the C-4 positions. After acid-catalyzed hydrolysis to remove the phosphate moiety from both sugars, the fructose in the mixture can be converted to F-6-P with hexokinase (HK, E.C. 2.7.1.1) and thus separated from the unreacted L-sorbose (Scheme V).

Conclusion

Aldolase-catalyzed condensation provides a practical route to a wide variety of simple sugars and sugar derivatives, and especially to those (glucose, fructose, and derivatives) centrally important in intermediary metabolism. The procedure has the advantage that it is applicable to

unprotected sugars in aqueous solution at pH 7. Epimeric mixtures can, in favorable cases, be separated without chromatography by taking advantage of substrate-selective enzymatic phosphorylation. The sugar phosphates prepared here are of 80–90% purity. The impurities are mainly inorganic phosphates, and are of no consequence in most further enzymatic transformations. Using immobilized enzymes and in situ cofactor regeneration, these methods should be applicable to preparations carried out on mole quantities. Although the enantioselectivity of aldolase-catalyzed reactions has been examined explicitly with only a few unnatural substrates, it is probably high: we have, for example, prepared 6-deoxy-D-fructose and 6-deoxy-L-sorbose[10] with high enantioselectivity using D- and L-lactaldehyde, respectively, as substrates.

A limitation of the enzymatic method is that dihydroxyacetone phosphate is required as one reactant in the aldol condensation, although the second component can be varied over a range of structures. A second disadvantage is that there is no way of changing the stereochemistry of the centers formed in the aldol condensation.

Overall, these reactions seem to have genuine value as a method of synthesis for polyhydroxy compounds and complement methods based on asymmetric epoxydation developed by Sharpless and co-workers and regioselective aldol reactions developed by a community of synthetic chemists.[11] We will describe further applications of these reactions in future reports.

Experimental Section

Materials and Methods. ^{13}C labeled KCN was from Stohler Isotope Chemicals. Enzymes were from Sigma. $POCl_3$ was distilled before use. Dihydroxyacetone was obtained from Sigma and was ~95% pure. All other reagents were reagent grade. UV spectra were taken with a Perkin Elmer 552 spectrophotometer, equipped with a constant temperature cell. HPLC analyses were carried out by use of a Waters Model 6000A equipped with a μBondapack carbohydrate column (0.4 × 30 cm), aqueous acetonitrile (H_2O:CH_3CN = 15:85 v/v; flow rate = 2 mL/min) as solvent, and refractive index detection. Enzyme immobilizations in PAN gel were carried out following the procedure described.[12] Enzymatic analyses were carried out according to standard methods[13] using horse liver alcohol dehydrogenase for glyceraldehyde; glycerophosphate dehydrogenase (GPDH) for DHAP and (*R*)-glycerol 1-phosphate (Gr-1-P); aldolase and GPDH for FDP; PGI and G-6-PDH for F-6-P; hexokinase (HK) and G-6-PDH for glucose; glycerokinase (GK) and GPDH for glycerol; DHAP, aldolase, and GPDH for D-glyceraldehyde; aldehyde dehydrogenase for glycolaldehyde; and glyceraldehyde 3-phosphate dehydrogenase (Gd-3-PDH) for D-glyceraldehyde 3-phosphate (D-Gd-3-P). The proton-decoupled C-13 NMR (68 MHz) spectra were obtained with a JEOL instrument at ambient temperature. Samples were dissolved in D_2O (final concentration ~0.2 M, 5-mm tubes). Ten transients were accumulated at a sweep width of 15 KHz with 30° pulses. Chemical shifts were expressed in ppm with dioxane (67.4 ppm) as internal standard.

Dihydroxyacetone Phosphate (DHAP); Enzymatic Phosphorylation. To a 1-L solution containing dihydroxyacetone (18 g, 0.2 mol), ATP (2 mmol), $MgCl_2$ (5 mmol), and PEP (as its monopotassium salt, K^+PEP^-, 41.2 g, 0.2 mol);[6] pH 7.0, was added PAN-immobilized GK (150 U, 8 mL of gel) and pK (1000 U, 15 mL of gel). The mixture was stirred at room temperature under argon and the pH was automatically controlled at 7.0 by adding aqueous KOH (1 N) using peristaltic pump. Enzymatic analysis indicated that the reaction was complete in 12 h. After recovery of the enzyme-containing gels, the solution was treated with active charcoal (10 g) and filtered. The colorless filtrate was treated with Dowex 50 (H^+ form) until the pH reached 4.2 (~2 g of resin), then filtered, and concentrated to a volume of 200 mL. Ethanol (1 L) was added to precipitate DHAP as a monopotassium salt. After being dried in vacuo over KOH, the solid (37 g) contained 90% by weight $DHAP^-K^+$ (160 mmol, 80% yield), as determined enzymatically by using GPDH and NADH. Turnover numbers (and recovered activities for the components of the system) were as follows: GK, 3×10^7 (86%); PK, 2×10^7 (88%); ATP, 100 (88%).

A similar reaction on the same scale was carried out by using acetylphosphate diammonium salt (38 g, 90% purity, 0.2 mol)[7] as the phosphorylating agent and acetate kinase (AcK, 500 U, 10 mL of gel) as catalyst for ATP regeneration. Addition of dithiothreitol (2 mM) to the reaction was necessary to protect the AcK from oxidation. The reaction was conducted under argon and was complete in 8 h. After separation of the reaction solution from the enzyme-containing gels, the solution was mixed with barium acetate (7.7 g, 30 mmol) and the precipitated barium phosphate was removed by filtration. The solution remaining after removal of the barium phosphate was subject to the same workup described in the preceding paragraph and yielded K^+-$DHAP^-$ (38 g, 82% purity, 150 mmol of DHAP) in 75% yield. Turnover numbers (and recovered activitie) were as follows: GK, 3×10^7 (84%); AcK, 3×10^7 (72%); ATP, 100 (86%).

Chemical phosphorylation of dihydroxyacetone was carried out on a 0.2-mol scale. To a stirred (0–5 °C) suspension of DHA (18 g, 0.2 mol) and $POCl_3$ (18.7 mL, 0.2 mol) in acetonitrile (300 mL) was added pyridine (16.2 mL, 0.2 mol) dropwise over a period of 20 min with the temperature controlled in the range 0–5 °C. The mixture was stirred continuously at room temperature for another 20 min. Water (860 mL) was added and the solution was heated on a steam bath for 30 min to hydrolyze P–Cl bonds. Enzymatic analysis of the mixture showed that 60% of the DHA had been converted to DHAP. This solution was adjusted to pH 4.0 and stored in a refrigerator;[14] the concentration of DHAP retained 90% of its original value after 1 month. The presence of acetonitrile and pyridine in the solution did not interfere with the subsequent enzymatic steps.

D-Fructose 1,6-Bisphosphate (FDP). To the DHAP solution prepared above from DHA and $POCl_3$ (1 L of solution containing 0.1 mol of DHAP) were added $MgCl_2$ (2 mmol) and mercaptoethanol (0.1 mL). The mixture was neutralized with 5 N NaOH to pH 7.0. Co-immobilized TPI (104 U) and aldolase (60 U) in 5 mL of gel was added, and the mixture was kept at room temperature under argon with stirring for one day. Enzymatic analysis indicated that the reaction was complete and that 45 mmol of FDP was present in the solution, corresponding to a yield of 90% based on DHAP. Barium chloride (26.3 g, 120 mmol) was added to the solution with stirring, and the precipitated material was separated by filtration and discarded. More $BaCl_2$ (17.4 g, 100 mmol) was mixed with the filtrate, followed by addition of ethanol (600 mL) to precipitate the desired product. The solid (28.3 g) contained 40 mmol of Ba_2·FDP (86% purity, 80% yield based on DHAP). This material was easily converted to F-6-P by treatment with Dowex 50 and hydrolysis at 90 °C for 5 h (see below). Conversion of F-6-P to G-6-P using PGI as catalyst was carried out according to the procedure described previously[9] and 66% of G-6-P was isolated as its barium salt.

Glycolaldehyde and D,L-glyceraldehyde (labeled or unlabeled) were prepared on scales of 30 mmol according to Barker's procedures[5] with the following modifications. After hydrogenation, the mixture containing the aldoses was filtered through Celite

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(14) DHAP is not stable at values of pH higher than 6. The half-life of DHAP (10 mM) in triethanolamine buffer (0.1 M, pH 7.0) is 65 h.

and treated with Dowex 50-X8 (H^+) until the solution had pH ~2. The resin was removed by filtration, and the filtrate was concentrated to an oily residue under reduced pressure aroung 40 °C; this procedure removed acetic acid. The residue, after dilution with water, was used directly in further reactions without additional purification: the ion-exchange chromatography[5] was eliminated. The progress of hydrogenations was monitored by enzymatic determination of the aldose produced by use of aldehyde dehydrogenase and NAD.[13]

Dihydroxyacetone monosulfate was prepared in a procedure similar to that for glucose 6-sulfate.[15] Pyridine–sulfur trioxide (1.4 g, 43 mmol) in DMF (7 mL) was added with stirring to a solution of dihydroxyacetone (3.9 g, 43 mmol) dissolved in DMF (10 mL) over 3 min at 25 °C. The mixture was stirred for another hour and concentrated (0.1 mm Hg, 35 °C) to remove DMF and pyridine. The oily residue was dissolved in water, adjusted to pH 7.5 (2 N NaOH), and concentrated again to remove pyridine. The residue was dissolved in 10 mL of water. Enzymatic analysis using glycerol dehydrogenase indicated that the solution contained no dihydroxyacetone. The product was a good substrate for GPDH, indicating the dihydroxyacetone monosulfate was produced. The concentration of dihydroxyacetone monosulfate (determined by complete conversion to (*R*)-glycerol 1-sulfate using GPDH and NADH) was 2.1 M, corresponding to an overall yield of 49%. This solution was used directly without further purification.

Aldolase-Catalyzed Condensation of DHAP and D,L-Glyceraldehyde. To a solution of D,L-glyceraldehyde prepared as described above (20 mL, 1.4 mmol, pH 7.0) was added DHAP (1.4 mmol) and immobilized aldolase (10 U, 1 mL of gel). The mixture was kept at room temperature under argon with stirring for 5 h. After separation of the enzyme-containing gel, the solution was treated with 10 g of Dowex 50 (H^+ form) and the resulting solution (pH 1.7) was heated on a steam bath for 10 h to hydrolyze the phosphate esters. HPLC analysis indicated that the resulting solution (20 mL) contained 0.64 mmol each of D-fructose (retention time 8 min) and L-sorbose (retention time 7 min).

Hexokinase-Catalyzed Selective Phosphorylation of D-Fructose in the Presence of L-Sorbose. To the solution prepared above were added ATP (0.01 mmol) PEP (0.7 mmol), and $MgCl_2$ (0.1 mmol). The pH was adjusted to 7.0 and HK added (40 U, 1 mL of gel). The mixture was allowed to react for 2 h, at which time no fructose remained. The F-6-P generated by this phosphorylation was isolated as its barium salt (0.27 g of solid contained 0.6 mmol of Ba F-6-P, 88% purity). This solid contained F-6-P as the only component that could be detected by HPLC. The unreacted L-sorbose was still in the solution (0.5 mmol) as determined by HPLC; no fructose was detected. No further purification was carried out.

Selective Glycerokinase-Catalyzed Phosphorylation of L-Glyceraldehyde to L-Glyceraldehyde 3-Phosphate in a Mixture of D- and L-Glyceraldehyde. To 20 mL of a solution containing D,L-glyceraldehyde (D,L-Gd, 0.1 M, 2 mmol), PEP (0.1 M, 2 mmol), ATP (0.02 mmol, 1 mM), and $MgCl_2$ (1 mM, 0.02 mmol), pH 7.0, were added GK (20 U, 0.5 mL of gel) and PK (40 U, 1 mL of gel). The reaction was allowed to proceed for 6 h to complete the reaction. L-Gd-3-P and D-Gd were separated according to the procedure described elsewhere.[5] L-Gd-3-P barium salt (0.2 g, 0.82 mmol) was obtained in 41% yield based on D,L-Gd. The D-Gd obtained (20 mM in 5 mL solution) was allowed to react with an equal quantity of DHAP with aldolase catalysis until D-Gd was not detected and yielded a mixture containing D-fructose 1-phosphate (91%) and L-sorbose 1-phosphate (9%) (determined by HPLC analysis of the phosphate-free sugars obtained by Pase-catalyzed hydrolysis), while treatment of L-Gd-3-P with DHAP and aldolase under the same conditions gave L-sorbose 1,6-bis(phosphate) as the only product.

To prepare L-Gd, the L-Gd-3-P·Ba prepared above (0.2 g, 0.82 mmol) was suspended in 10 mL of water and treated with Dowex 50 (H^+) to remove the barium ions. After filtration and washing with water (10 mL), the combined filtrates were adjusted to pH ~3–4 by adding NaOH solution (2 N). Acid phosphatase (Pase, 40 U in 1 mL of gel) was added and the mixture was stirred under argon for 4 h until no further increase in the concentration of L-Gd was observed. After separation of enzyme-containing gel, the final solution (24 mL) contained 0.70 mmol of L-Gd (85% yield) determined enzymatically.

D-Fructose 6-Phosphate. To a deoxygenated solution (1 L) containing D-fructose (200 mmol, 0.2 M), ADP (5 mM), $MgCl_2$ (10 mM), and mercaptoethanol (5 mM) was added 4 mL of gel containing co-immobilized HK (600 U) and AcK (450 U). Solid diammonium acetyl phosphate (90% purity, 210 mmol) was added in ten equal portions to the mixture with stirring over a period of 20 h. The solution was automatically controlled at pH 7.2 by addition of 2 N NaOH. Enzymatic analysis indicated that it contained 196 mmol (98%) of F-6-P. After separation of the enzyme-containing gel, the solution was treated with 5 g of activated carbon and filtered, and the filtrate was concentrated under reduced pressure at 40 °C to a volume of ~100 mL.

D-Glyceraldehyde 3-Phosphate. The F-6-P solution (2 M) obtained above was used directly for the preparation of D-Gd-3-P according to the procedure described previously[16] with slight modification. In a representative procedure, 5 mL of the F-6-P solution containing 10 mmol of F-6-P was mixed with glacial acetic acid (400 mL) followed by addition of concentrated H_2SO_4 (1.1 mL, 20 mmol). Lead tetraacetate (18 g, 40.5 mmol) was added with stirring over a period of 10 min. After 2 h, oxalic acid (3.6 g, 40 mmol) was added to precipitate lead ion. The solution (containing 8.5 mmol of D-Gd-3-P) was filtered and lyophilized, and the powder obtained was used directly for the aldolase-catalyzed condensation.

(2-$^{13}C_1$)Glycerol. Starting from ^{13}C labeled KCN (2 g, 30 mmol) and formaldehyde (2.5 mL of solution containing 37% HCHO, 30 mmol), (1-$^{13}C_1$)glycolaldehyde was prepared in 90% yield according to the procedure described above for the synthesis of glycolaldehyde and glyceraldehyde. The material was used in another cycle of the reductive cyanohydrin reaction, and (2-$^{13}C_1$)-D,L-Gd was obtained as an oily residue in an overall yield of 74% (22 mmol) based on formaldehyde as starting material. This compound was dissolved in 100 mL of water and added dropwise over a period of 30 min to a stirred, cold, aqueous solution (100 mL) containing $NaBH_4$ (2.3 g, 60 mmol). The mixture was stirred for another 10 h and then treated with Dowex 50 (H^+) until the solution had pH 4.0. The resin was removed by filtration and the filtrate was mixed with an equal volume of 20 mL. Another portion of methanol (100 mL) was added and the mixture concentrated again. The procedure was repeated 3–4 times to remove borate as trimethyl borate. The final residue obtained contained 18 mmol of glycerol (82% yield) as determined by HPLC (retention time 2.4 min) and by enzymatic analysis.

(2-$^{13}C_1$)-(*R*)-Glycerol 1-Phosphate. The labeled glycerol prepared above was dissolved in a solution (100 mL, pH 7.2) containing PEP (19 mmol, 0.19 M), ADP (4 mM), $MgCl_2$ (6 mM), and co-immobilized GK (EC 2.7.1.30) and PK (EC 2.7.1.40) in PAN (110 U of GK and 400 U of PK in 2 mL of gel). The mixture was kept under argon with stirring and the pH automatically controlled at 7.6. Enzymatic analysis indicated that the reaction was complete in 6 h. After separation of the enzyme-containing gel, the solution was treated with activated carbon (2 g) and filtered and the filtrate treated with Dowex 50 (H^+) until the solution had pH ~2. After filtration to remove the resin, the solution was extracted 3 times with ether (50 mL each time) to remove pyruvic acid. The aqueous solution was neutralized by addition of barium carbonate until the solution had pH ~6. An equal volume of ethanol was added. The barium salt of (2-$^{13}C_1$)-(*R*)-Gr-1-P was obtained in 89% yield (5.6 g of solid contained 16 mmol of the title compound with 88% purity determined enzymatically). The residual enzyme activities recovered were GK, 99%; PK, 94%.

(2,5-$^{13}C_2$)-D-Fructose 1,6-Bisphosphate ((2,5-$^{13}C_2$)-FDP) and (2-$^{13}C_1$)-D-Fructose 1,6-Bisphosphate ((2-$^{13}C_1$)-FDP). The barium salt of the (2-$^{13}C_1$)-(*R*)-Gr-1-P prepared above (3.5 g, 10 mmol) was suspended in 50 mL of water. Dowex 50 (H^+) (~5 g) was added with stirring until the compound dissolved. The resin was separated by filtration and washed with 10 mL of water,

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and the combined filtrates were neutralized to pH 6.8 by addition of 2 N NaOH solution. The solution was diluted to ~100 mL, followed by adding α-ketoglutarate monosodium salt (11 mmol), NAD (0.1 mmol), and aqueous ammonium hydroxide until the solution had pH 7.0–7.2. The following enzymes co-immobilized in PAN were then added to the solution: GPDH (EC 1.1.1.8, 80 U) and GluDH (EC 1.4.1.3, 84 U) in 5 mL of gel; TPI (EC 5.3.1.1, 200 U) and aldolase (80 U) in 4 mL of gel. The reaction was allowed to proceed for 24 h. Enzymatic analysis indicated that 4.6 mmol of FDP was produced, and no further reaction was observed. The solution, after separation of gel, was treated with Dowex 50 (H^+) until the pH had reached 4.0 to remove most of the glutamic acid and concentrated below 35 °C to a volume of ~10 mL. Cold ethanol (50 mL) was added to the mixture to precipitate FDP as its disodium salt (4.5 mmol, 90% yield with 88% purity determined enzymatically) while the unreacted α-keto acid still remained in the solution.

Another reaction similar to that described above was carried out except that unlabeled D-Gd-3-P (10 mmol) was added and TPI omitted.[17] The (2-$^{13}C_1$)-FDP produced was isolated as the disodium salt (8.6 mmol, 86% yield with 89% purity determined enzymatically). The enzyme activities were recovered at the end of the reaction: GPDH 89%; GluDH, 86%; TPI, 84%; aldolase, 86%.

(2,5-$^{13}C_2$)-D-Fructose 6-Phosphate ((2,5-$^{13}C_2$)-F-6-P) and (2-$^{13}C_1$)-D-Fructose 6-Phosphate ((2-$^{13}C_1$)-F-6-P). The labeled FDP prepared above was converted to ^{13}C-labeled F-6-P via acid-catalyzed hydrolysis. In a representative procedure, the labeled FDP (5 mmol) was dissolved in water (20 mL) and treated with Dowex 50 (H^+ form) until the solution had pH 1.0–1.5. The ion-exchange resin was removed and washed. To the combined solutions was added 1 mL of concentrated HCl, and the solution was incubated at 85–90 °C for 5 h. Enzymatic analysis indicated that 86% of the FDP had been converted to F-6-P.

(2,5-$^{13}C_2$)-D-Glucose 6-Phosphate ((2,5-$^{13}C_2$)-G-6-P) and (2-$^{13}C_1$)-D-Glucose 6-Phosphate ((2-$^{13}C_1$)-G-6-P). The solutions of labeled fructose 6-phosphate obtained above were adjusted to pH 7.0 by adding aqueous NaOH (2 N) at 10–15 °C. The final, combined, neutralized solutions (60 mL) contained 4.3 mmol of ^{13}C-labeled F-6-P. Immobilized PGI (EC 5.3.1.9, 102 U in 1 mL of gel) and $MgCl_2$ (0.1 mmol) were added to the solution, and the mixture was kept under argon with the pH automatically controlled at 7.0. After 5 h, the solution contained 3 mmol of G-6-P as determined enzymatically; no further increase in the quantity of this product was observed at longer reaction times. The G-6-P was isolated as its barium salt in 66% yield according to the procedure described previously.[9] The solution could be used directly for the following conversion without further purification.

(2,5-$^{13}C_2$)-D-Glucose and (2-$^{13}C_1$)-D-Glucose. The labeled BaG-6-P prepared above was treated with Dowex 50 in water to remove barium ion. The resin was removed, and the solution was adjusted to pH 3.5 by addition of NaOH (2 N). Immobilized potato acid phosphatase (Pase, EC 3.1.3.1, 105 U in 2 mL of gel) was added to the solution (70 mL containing 3 mmol of ^{13}C-labeled G-6-P), and the mixture was kept under argon with stirring at room temperature for 24 h. Enzymatic analysis indicated that 3 mmol of glucose had been produced. After the gel was separated, the solution was concentrated to a volume of 10 mL; ethanol (60 mL) was added to precipitate most of the inorganic salt. The supernatant was concentrated again to a solid (0.7 g containing 3 mmol of glucose, 81% purity) having ^{13}C NMR (ppm) 72.5 (αC-2), 72.3 (αC-5), 75.1 (βC-2), 76.8 (βC-5). These values are consistent with those reported for glucose.[18] For further purification, the material was passed through a Dowex 50-X8 (Ba form)[19] column (3 × 110 cm) and eluted with water. The fractions (290–340 mL) corresponding to glucose were collected and lyophilized. A powder (0.45 g) containing 2.4 mmol of glucose (100% purity based on HPLC and enzymatic analysis) was obtained.

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Registry No. 3, 17302-80-6; DHAP, 57-04-5; DHA, 96-26-4; PEP, 138-08-9; AcP, 590-54-5; ATP, 56-65-5; D-Gd-3-P, 591-57-1; L-Gd-3-P, 20283-52-7; aldolase, 9024-52-6; D-Gd, 453-17-8; L-Gd, 497-09-6; GK, 9030-66-4; Pase, 9001-77-8; TPI, 9023-78-3; F-6-P, 643-13-0; lead tetraacetate, 546-67-8; FDP, 488-69-7; G-6-P, 56-73-5; glucose, 50-99-7; fructose, 57-48-7; [2-$^{13}C_1$]-D-glucose, 70849-17-1; [2,5-$^{13}C_2$]-D-glucose, 86595-19-9; $POCl_3$, 10025-87-3; acetylphosphate diammonium salt, 55660-58-7; G-6-P barium salt, 58823-95-3; glycolaldehyde, 141-46-8; D,L-glyceraldehyde, 56-82-6; pyridine–sulfur trioxide, 42824-16-8; L-sorbose, 87-79-6; hexokinase, 9001-51-8; F-6-P barium salt, 6035-54-7; L-Gd-3-P·1/2Ba, 86595-20-2; [2-$^{13}C_1$]glycerol, 82425-96-5; [1-$^{13}C_1$]glycolaldehyde, 71122-42-4; [2-$^{13}C_1$]-D,L-Gd, 71122-43-5; [2-$^{13}C_1$]-(*R*)-glycerol 1-phosphate, 86595-21-3; [2,5-$^{13}C_2$]-FDP, 84270-12-2; [2-$^{13}C_1$]-FDP, 84270-11-1; GPDH, 9075-65-4; GluDH, 9029-12-3; [2,5-$^{13}C_2$]-F-6-P, 86595-22-4; [2-$^{13}C_1$]-F-6-P, 86595-23-5; [2,5-$^{13}C_2$]-G-6-P, 86595-24-6; [2-$^{13}C_1$]-G-6-P, 86595-25-7; PGI, 9001-41-6.

(17) The aldolase from Sigma is contaminated with triosephosphate isomerase (TPI) (~0.05%, w/w). To prepare TPI-free aldolase, the enzyme should be treated with the active-site directed irreversible inhibitor bromohydroxyacetone phosphate (De La Mare, S.; Coulson, A. F. W.; Knowles, J. R.; Priddle, J. D.; Offord, R. E. *Biochem. J.* **1972**, *129*, 321–31). The PAN-immobilized aldolase we used here did not show significant contamination by TPI activity. The reason for preferential exclusion (or destruction) of the TPI activity during immobilization is not known.

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