Carbon-13-Enriched Carbohydrates: Preparation of Triose, Tetrose, and Pentose Phosphates†

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ABSTRACT: Three-, four-, and five-carbon aldononitrile phosphates were prepared, purified, and catalytically reduced with palladium–barium sulfate (5%) to the corresponding aldose phosphates in high yields at pH 1.7 ± 0.1 and atmospheric pressure. DL-Glyceraldehyde 3-phosphate and the tetrose 4-phosphates were prepared with carbon-13 enrichment at C-1, while the pentose 5-phosphates were prepared with enrichment at C-1 and C-2. Preparations of glycolaldehyde phosphate and D-glyceraldehyde 3-phosphate by lead tetraacetate oxidation of glycerol phosphate and fructose 6-phosphate, respectively, are described. The proportions of cyclic hemiacetals and linear *gem*-diol forms of the two- to five-carbon aldose phosphates in aqueous solution are reported. Carbon-13 chemical shifts and carbon–phosphorus and carbon–hydrogen coupling constants for the furanose phosphate ring and linear *gem*-diol phosphates are reported and discussed. D-[2-^{13}C]Ribulose 1,5-bisphosphate and L-[3,4-^{13}C]sorbose 1,6-bisphosphate were prepared enzymatically from D-[2-^{13}C]ribose 5-phosphate and DL-[1-^{13}C]glyceraldehyde 3-phosphate, respectively.

The advent of carbon-13 NMR (^{13}C NMR)[1] and developments in combined gas chromatography–mass spectrometry have increased the value of ^{13}C-enriched compounds for the study of their conversions and interactions in biological systems. Aldose phosphates such as D-ribose 5-P are important as intermediates in metabolic sequences and as components of more complex biological structures. The reported chemical syntheses of these compounds (Ballou & Fischer, 1955; Ballou et al., 1955; Klybas et al., 1959; Michelson & Todd, 1949; Stverteczky et al., 1973; Baer & Fischer 1943) are not suitable for the preparation of ^{13}C-enriched derivatives. In this paper we report a convenient method for the synthesis of aldose phosphates containing ^{13}C enrichment at various carbons in yields up to 85% based on the enriched carbon.

The procedure is based on the classical Kiliani synthesis (Kiliani, 1887). In that process, cyanohydrins (aldononitriles) are formed from the condensation of cyanide with an aldose in aqueous solution and are hydrolyzed in situ to the epimeric aldonic acids having one more carbon than the starting aldose. Fischer (1889) extended this sequence by reducing the aldonic acid lactones with sodium amalgam to form the corresponding aldoses. This synthetic sequence has been used to prepare ^{14}C-labeled aldoses (Isbell et al., 1952; Frush & Isbell, 1953; Isbell et al., 1954; Schaffer & Isbell, 1956) and several investigators have explored alternative methods for reducing the intermediate lactones (Bhattacharjee et al., 1975; Kohn et al., 1965).

The synthesis described in this paper was first applied to the preparation in high yields of simple aldoses enriched with ^{13}C at C-1 (Serianni et al., 1979a,b). The procedure avoids the intermediate formation of acids and lactones. Aldononitriles are formed at pH 8.5 ± 0.5, stabilized at pH values below 4, and hydrogenolyzed over a palladium catalyst at pH values between 1.0 and 4.0 to give the corresponding aldoses directly. The epimeric aldoses are purified by ion-exchange chromatography (Jones & Wall, 1960; Samuelson, 1972).

In this paper we describe the preparation of ^{13}C-enriched aldose phosphates. When an aldose phosphate is used as the parent aldose, the yield of the epimeric aldononitrile phosphates is high and the C-2 epimers can be separated by anion-exchange chromatography. The purified aldononitrile phosphates hydrogenolyze smoothly to the product aldose phosphates. Millimole amounts of the following compounds were prepared with 90 atom % carbon-13 enrichment: DL-[1-^{13}C]glyceraldehyde 3-P, D-[1-^{13}C]threose 4-P, D-[1-^{13}C]erythrose 4-P, DL-[1-^{13}C]xylose 5-P, DL-[1-^{13}C]lyxose 5-P, DL-[1-^{13}C]-arabinose 5-P, DL-[1-^{13}C]ribose 5-P, D-[2-^{13}C]xylose 5-P, D-[2-^{13}C]lyxose 5-P, D-[2-^{13}C]arabinose 5-P, D-[2-^{13}C]ribose 5-P, DL-[1,2-^{13}C]arabinose 5-P, DL-[1,2-^{13}C]ribose 5-P, L-[3,4-^{13}C]sorbose 1,6-P_2, D-[2-^{13}C]ribulose 1,5-P_2, DL-[1-^{13}C]glyceronitrile 3-P, D-[1-^{13}C]threononitrile 4-P, D-[1-^{13}C]erythrononitrile 4-P, DL-[1-^{13}C]xylononitrile 5-P, DL-[1-^{13}C]lyxononitrile 5-P, DL-[1-^{13}C]arabinononitrile 5-P, DL-[1-^{13}C]ribononitrile 5-P, D-[2-^{13}C]xylononitrile 5-P, D-[2-^{13}C]lyxononitrile 5-P, D-[2-^{13}C]arabinononitrile 5-P, D-[2-^{13}C]ribononitrile 5-P, DL-[1,2-^{13}C]arabinononitrile 5-P, and DL-[1,2-^{13}C]ribononitrile 5-P.

Materials and Methods

Compounds and Enzymes. D-Ribose, D-arabinose, D-xylose, D-lyxose, D-ribose 5-P, D-arabinose 5-P, D- and DL-glyceraldehyde 3-P diethyl acetal, D-fructose 1,6-P_2, and DL-glycerol 1-P were obtained from Sigma Chemical Co. and used without further purification. D-Erythrose and D-threose were prepared by methods described by Serianni et al. (1979b). D- and DL-glyceraldehyde 3-P were prepared from the acetal as described by Ballou & MacDonald (1963). Potassium [^{13}C]cyanide (K^{13}CN) was supplied by the Los Alamos Scientific Laboratory, University of California, Los Alamos, NM. Potassium [^{14}C]cyanide (K^{14}CN) was purchased from New England Nuclear with a specific activity of 46 mCi/mmol. Palladium–barium sulfate (5%) was purchased from Sigma Chemical Co. Lead tetraacetate was obtained from Aldrich Chemical Co.

D-Fructose-1,6-P_2 aldolase (EC 4.1.2.13) from rabbit muscle, alkaline phosphatase (EC 3.1.3.1), triosephosphate isomerase (EC 5.3.1.1) from yeast, D-ribose-5-P isomerase (EC

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[1] Abbreviations used: ^{13}C NMR, ^{13}C nuclear magnetic resonance spectroscopy; ^{1}H NMR, ^{1}H nuclear magnetic resonance spectroscopy; UMP, uridine 5′-phosphoric acid; AMP, adenine 5′-phosphoric acid; P, phosphate; P_2, bisphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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5.3.1.6) from yeast, and D-ribulose-5-P kinase (EC 2.7.1.19) from spinach were purchased from Sigma Chemical Co.

Instrumentation and Analytical Methods. ^{13}C NMR spectra were obtained using a Bruker WP-60 15.08-MHz Fourier transform spectrometer equipped with quadrature detection. All spectra were obtained with 4K spectral points. The spectrometer was locked to the resonance of D_2O in a capillary. Chemical shifts are given relative to external tetramethylsilane (Me_4Si) and are accurate to within ±0.1 ppm. Coupling constants in complex ^{13}C NMR spectra were obtained by comparison of the experimental data with the theoretical spectra generated by the ITRCAL program available from the Nicolet Computer Co., Madison, WI. This program permits the calculation of NMR spectra by first entering reasonable estimates of the chemical shifts and coupling constants for the nuclei in question and entering the actual frequency of the lines in the experimental spectrum. By changing the chemical shifts, the coupling constants, or both, iteration to a best fit of the theoretical with the real spectrum is obtained.

Catalytic reductions were performed using the apparatus described previously (Serianni et al., 1979b). pH measurements were made at 25 °C prior to NMR analysis at the reported temperatures.

Inorganic and organic phosphate assays were performed according to the procedure described by Leloir & Cardini (1957). Radioactivity was assayed on a Beckman LS-100 scintillation counter by using a Triton X-100 (1000 mL)–PPO (8 g)–POPOP (0.2 g)–toluene (2000 mL) cocktail. Aqueous sample (0.2 mL) was dissolved in 2.3 mL of the cocktail for analysis.

Glycolaldehyde Phosphate. Disodium DL-glycerol 1-P hexahydrate (8.6 g, 27 mmol) was moistened with 5 mL of H_2O and dissolved in 400 mL of glacial acetic acid with efficient stirring. Upon dissolution of the salt, 1.7 mL of 18 M sulfuric acid was added (Ballou, 1963), and addition of lead tetraacetate (24 g, 54 mmol) was made during 15 min. After 2 h, oxalic acid (4.5 g, 50 mmol) was added and stirring was continued for an additional 30 min. The suspension was filtered through Celite and the filtrate was concentrated at 30 °C in vacuo to approximately 300 mL. The filter cake was washed with 200 mL of H_2O, the concentrate and washings were combined, and barium acetate (13 g, 50 mmol) was added with efficient stirring at 4 °C for 15 min. The white suspension was filtered through Celite, the filter was washed with H_2O, and the filtrate and washings were treated with excess Dowex 50 (H^+). The suspension was filtered, the solution concentrated as before to about 200 mL, and the concentrate extracted overnight at 4 °C with diethyl ether in a continuous liquid–liquid extraction apparatus. The aqueous solution was recovered, concentrated as before to about 30 mL, and stored at −20 °C. Yield: 25 mmol (93%) by total P with a trace of inorganic P. Purity: at least 95% by ^{13}C NMR.

D-Glyceraldehyde 3-P. Disodium D-fructose 6-P dihydrate (3.4 g, 10 mmol) was treated with lead tetraacetate (18 g, 40.5 mmol) in the manner described for the preparation of glycolaldehyde P, except that 1.1 mL of 18 M sulfuric acid (20 mmol) was added prior to the addition of the oxidant. After Dowex 50 (H^+) treatment, the acidic solution of 2-*O*-glycoloyl-D-glyceraldehyde 3-P was concentrated to 10 mL and stored at 25 °C for 18 h to yield D-glyceraldehyde 3-P and glycolic acid. Alternatively, hydrolysis can be carried out by incubating the acidic solution at 40 °C for 6 h. The resulting solution was adjusted to pH 5.5 with 2 M NaOH and applied to a DEAE-Sephadex A-25 (40–120 mesh) column at 4 °C in the acetate form, washed with a small amount of H_2O, and eluted with a linear gradient of sodium acetate (1500 mL, 0.05–0.60 M, pH 5.5 ± 0.1). Glyceraldehyde 3-P eluted at 0.15 M sodium acetate and was preceded by glycolic acid. Fractions were assayed for D-glyceraldehyde 3-P by organic P analysis and for glycolic acid by the method of Lewis & Weinhouse (1957). Fractions containing D-glyceraldehyde 3-P were pooled, treated with excess Dowex 50 (H^+), and concentrated twice in vacuo at 30 °C to approximately 5 mL to remove acetic acid. Yield: 8.1 mmol (81%) by organic P analysis with a trace of inorganic P. Purity: at least 95% by ^{13}C NMR.

Table I: Purification and Epimeric Distribution of Aldononitrile Phosphates

parent aldose phosphates	chromatography on Dowex 1-X8 (formate)[a] peak 1[b]	peak 2[b]	ratio of epimers[c]
glyceraldehyde 3-P	threo	erythro	1.3:1 erythro
erythrose 4-P	arabino	ribo	1.4:1 ribo
threose 4-P	xylo	lyxo	1.5:1 lyxo

[a] A 2.2 × 51 cm Dowex 1-X8 (200–400 mesh) column in the formate form was employed. Solutions of aldononitrile phosphates were adjusted to pH 6.5–7.0 prior to application to the column bed. Gradients: for 4-carbon aldononitrile phosphates, 3000 mL, 0.2–0.9 M sodium formate, pH 3.9; for 5-carbon aldononitrile phosphates, 3000 mL, 0.05–0.8 M sodium formate, pH 3.9. Temperature = 4 °C; flow rate = 0.5 mL/min; 7 mL per fraction. [b] Aldononitrile P configurations were determined by reduction to aldose phosphates and incubation with alkaline phosphatase. The ^{13}C NMR spectra of the resulting aldoses were compared with those of standard pentoses. [c] Determined by computerized integration of ^{13}C NMR spectra of epimeric mixtures and by quantitation of organic P in purified preparations.

Aldononitrile Phosphates. Cyanide condensations were carried out according to the method of Serianni et al. (1979b). The $K^{13}CN$ solution (0.15 M) containing 10^7 cpm of $K^{14}CN$ was placed in a sealed flask and cooled to 5 °C with an ice bath prior to adjustment to pH 8.0 ± 0.1 with 2 M acetic acid. The solution of aldose phosphate at pH 7.5 was added while maintaining the pH of the reaction mixture between 7.5 and 8.0 with additions of 2 M acetic acid and/or 1 M NaOH. Stoichiometric amounts of aldose phosphate and cyanide were used, and the final concentration of reactants was 0.05–0.10 M. After 15 min at 5 °C and pH 7.5–8.0, the ice bath was removed and the reaction mixture allowed to warm to 25 °C over a period of 30–40 min. The pH was then adjusted to pH 4.0 ± 0.2 with 2 M acetic acid. Condensation was complete (>95%) when assayed by ^{13}C NMR using a short pulse width (10 μs, 55°) and long delay time (10 s) to facilitate aldononitrile detection.

The racemic mixture of glyceronitrile phosphate was adjusted to pH 1.7 ± 0.1 with 2 M HCl and hydrogenolyzed directly to DL-glyceraldehyde 3-P without further purification. Epimeric tetrono- and pentononitrile phosphates were purified by ion-exchange chromatography on Dowex 1-X8 (200–400 mesh) in the formate form at 4 °C by employing linear gradients of sodium formate (Table I and Figure 1A). The nitrile phosphate with *cis*-2,3-hydroxyl groups is the major product and is eluted last under these conditions. Column capacity exceeds 6 mmol of aldononitrile phosphate. Yields of the glycero-, tetrono-, and pentononitrile phosphates after cyanide condensation and chromatography are 85%.

Fractions containing the aldononitrile phosphates were pooled and adjusted to pH 1.5 with Dowex 50-X8 (H^+). After

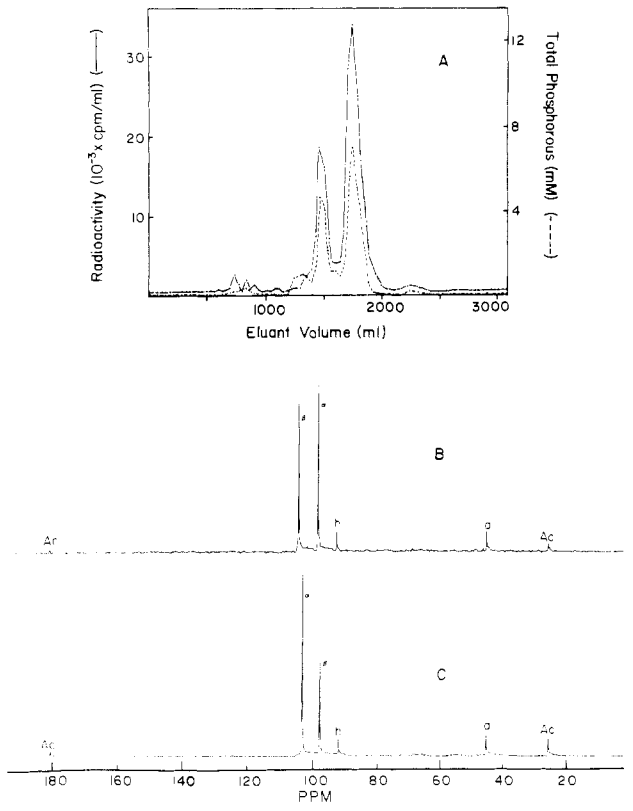

FIGURE 1: Separation of DL-[1-^{13}C]xylononitrile 5-P and DL-[1-^{13}C]lyxononitrile 5-P and ^{13}C NMR analyses of the products after hydrogenolysis over palladium. (A) Chromatography of the 2-epimeric pentononitrile phosphates on a 2.2 × 51 cm Dowex 1-X8 (200–400 mesh) column in the formate form at 4 °C developed with a linear gradient of sodium formate (3000 mL, 0.05–0.8 M, pH 3.9). Column effluent was assayed for radioactivity and total phosphate. The xylo epimer was eluted before the lyxo epimer. (B and C) ^{13}C NMR analyses showing resonances due to the enriched carbons of the reduction products from DL-[1-^{13}C]xylononitrile 5-P (B) and DL-[1-^{13}C]lyxononitrile 5-P (C); C-1 resonances of the 1-^{13}C-enriched α- and β-furanose and hydrated forms of DL-aldose 5-P appear at approximately 100 ppm, [1-^{13}C]-1-amino-1-deoxyalditol 5-P (a) appears at approximately 43 ppm, and resonances due to natural abundance ^{13}C of acetic acid (Ac), used to adjust pH prior to hydrogenolysis, appear at approximately 23 and 180 ppm. Spectra were obtained at 13 ± 1 °C with a sweep width of 3000 Hz and a filter width of 2400 Hz.

filtration, the acidic solutions were concentrated to 100 mL in vacuo at 30 °C and extracted continuously with diethyl ether overnight at 4 °C to remove formic acid. The aqueous acidic solutions were recovered, concentrated in vacuo at 30 °C to approximately 10 mL, and adjusted to pH ≤2 with Dowex 50-X8 (H^+) prior to hydrogenolysis.

Aldose Phosphates. Palladium–barium sulfate (5%, 62 mg per mmol of nitrile) was weighed into a side-arm flask, 5–10 mL of H_2O was added, and the suspension was reduced for 15–20 min at atmospheric pressure and 25 °C with efficient stirring. During this period, the catalyst changed from a brown to a whitish gray color. The solution of aldononitrile phosphate adjusted to pH 1.7 as described below was added from an addition funnel into the reduction vessel which was filled and evacuated three times prior to a final charging with hydrogen. The concentration of aldononitrile phosphate solution during hydrogenolysis varied between 50 and 100 mM.

Purified four- and five-carbon aldononitrile phosphate solutions were treated with 1 mL of glacial acetic acid per mmol of nitrile phosphate and then with 2 M HCl or 2 M NaOH to pH 1.7 ± 0.1. Reductions were carried out as described for the three-carbon homologue.

Aldononitrile phosphates were typically reduced for 6–8 h at 25 °C. In a few instances, incomplete reduction was noted. In these cases, the spent catalyst was removed by filtration through Celite and a second reduction was performed to complete the conversion to the aldose phosphate.

Hydrogenolysis products were assayed by ^{13}C NMR (Figure 1B and 1C) to determine the extent of reduction to 1-amino-1-deoxyalditol phosphates and the amount of unreacted aldononitrile phosphates. Yields of the three-, four-, and five-carbon aldose phosphates based on the analysis of ^{13}C NMR spectral peak areas in reactions with [1-^{13}C]aldononitrile phosphates are 85–95%.

After hydrogenolysis, the catalyst was removed by filtration through Celite and the solution treated with excess Dowex 50-X8 (H^+). After filtration, the solution was concentrated to 10 mL. Typically, the reaction mixture contains product aldose phosphate, 1-amino-1-deoxyalditol phosphates, and a small amount of aldononitrile phosphate. This solution was adjusted to pH 4.5 ± 0.1 with dilute NaOH and applied to a 1.2 × 50 cm DEAE-Sephadex A-25 (OAc^-) column at 4 °C which had been equilibrated with 0.05 M sodium acetate at pH 4.5 ± 0.1. The column was developed with a linear acetate gradient (1500 mL, 0.05–0.8 M sodium acetate, pH 4.5 ± 0.1). Fractions (6 mL) were collected with a flow rate of 0.5 mL per min. The 1-amino-1-deoxyalditol phosphate eluted at the void volume, followed in order by aldose phosphate and the aldononitrile phosphate. Fractions containing aldose phosphate were pooled, treated with excess Dowex 50 (H^+), and concentrated in vacuo at 30 °C to approximately 10 mL. Recovery from DEAE-Sephadex chromatography based on phosphate assay was greater than 95%. Aldose phosphate solutions were stored at pH 4.0 and −15 °C.

Characterization of Aldose Phosphates. Purified 1-^{13}C-enriched aldose phosphates (100 μmol) in 2 mL of 50 mM Tris-HCl buffer at pH 9.0 were incubated with alkaline phosphatase for 1 h at 36 °C. Carbon-13 NMR spectra of the resulting 1-^{13}C-enriched aldoses were compared with standard spectra (Table I).

D-[*2-*^{13}*C*]*Ribulose 1,5-*P_2 (D-[*2-*^{13}*C*]*Erythropentulose 1,5-*P_2). D-[2-^{13}C]Ribose 5-P (0.2 mmol) was incubated with 245 units of D-ribose 5-P isomerase for 30 min at pH 7.5 and 36 °C. The resulting mixture containing 28% D-[2-^{13}C]-ribulose 5-P was converted to D-[2-^{13}C]ribulose 1,5-P_2 by the method of Horecker et al. (1956) and purified by the method of Byrne & Lardy (1954).

L-[*3,4-*^{13}*C*]*Sorbose 1,6-*P_2. DL-[1-^{13}C]Glyceraldehyde 3-P (75 mM) was incubated for 30 min at pH 7.5 and 34 °C with triosephosphate isomerase, producing a mixture of L-[1-^{13}C]glyceraldehyde 3-P and [3-^{13}C]dihydroxyacetone phosphate. D-Fructose-1,6-P_2 aldolase was added and the mixture incubated for 4 h at 34 °C. The reaction mixture was made 50% in hot ethanol and centrifuged to remove protein. The supernatant was concentrated in vacuo at 30 °C to 2 mL, adjusted to pH 7.5 ± 0.1, and analyzed by ^{13}C NMR.

Results and Discussion

The high yields and relative simplicity of the manipulations involved in the syntheses reported in this paper make many of the biologically important aldoses with terminal phosphate esters more readily available for use in biological systems. Our principal interest in these compounds is in the ^{13}C-enriched derivatives, but the synthesis can be applied equally well to the preparation of unenriched compounds such as D-erythrose 4-P which are not easily prepared by other methods (Ballou et al., 1955; Klybas et al., 1959). The synthesis can be applied using [^{13}C]- and/or [^{14}C]cyanide to afford the respective

Table II: ^{13}C Chemical Shifts of Aldose Phosphates and Related Compounds

compound	pH[b]	carbon position (ppm)[a] C-1	C-2	C-3	C-4	C-5
glycolaldehyde P, hydrate[c]	7.5	90.7[d]	68.2[d]			
D-glyceraldehyde 3-P, hydrate[c]	7.5	91.3	74.9[d]	66.0[d]		
DL-erythrose 4-P, hydrate	1.5	90.3	73.8	71.4[d]	68.0[d]	
DL-threose 4-P, hydrate	4.5	90.8	74.1	70.6[d]	67.0[d]	
D-arabinose 5-P						
α-furanose	5.5	102.2	82.2	76.7	83.1[d]	65.1[d]
β-furanose	5.5	96.3	77.0	75.1	81.1[d]	66.2[d]
hydrate	5.5	91.2				
D-ribose 5-P						
α-furanose	5.5	97.5	71.9	71.3	83.6[d]	65.8[d]
β-furanose	5.5	102.4	76.4	71.7	82.5[d]	66.6[d]
hydrate	5.5	90.7				
DL-xylose 5-P						
α-furanose	5.5	96.9	76.8			
β-furanose	5.5	103.0	81.3			
hydrate	5.5	90.9				
DL-lyxose 5-P						
α-furanose	5.5	101.9	78.2			
β-furanose	5.5	96.5	72.5			
hydrate	5.5	90.4				
DL-glyceronitrile 3-P[c]	2.5	120.3	62.0[d]	67.1[d]		
DL-erythrononitrile 4-P	1.7	120.3	63.1	71.8[d]	65.9[d]	
DL-threononitrile 4-P	1.7	120.2	62.6	71.6[d]	65.4[d]	
D-arabinononitrile 5-P	4.6	122.6	62.7[e] (3.2)			
D-ribononitrile 5-P	4.6	121.2	64.2[e] (0.8)			
DL-xylononitrile 5-P	4.3	120.5	63.3[e] (3.2)			
DL-lyxononitrile 5-P	4.3	121.4	63.1[e] (3.8)			

[a] Chemical shifts were determined at 13 ± 1 °C with a sweep width of 1500 Hz and a filter width of 2400 Hz. Chemical shifts which are not entered were not measured. [b] Solutions were adjusted to the given pH (±0.1 unit) at 25 °C prior to NMR analysis. [c] These determinations were made at 34 ± 1 °C. [d] Resonance appears as a doublet arising from ^{31}P–^{13}C coupling. [e] Chemical shifts were measured at the pH value shown in parentheses.

^{13}C-enriched and/or ^{14}C-labeled aldose phosphates. It has previously been applied serially to introduce enrichment at one or more positions in the three- and four-carbon aldoses (Serianni et al., 1979b). In the present study, we have prepared the four diastereoisomeric pentose 5-phosphates with ^{13}C enrichment at C-2, starting with D-glyceraldehyde 3-P and using two cycles of condensation and hydrogenolysis. The overall yield based on D-glyceraldehyde 3-P or $K^{13}CN$ was approximately 55%. Thus, serial application of the synthesis starting with glycolaldehyde phosphate can presently be used to prepare aldose phosphates with enrichment at all but the penultimate and terminal carbons. Syntheses of [1-^{13}C]- and [2-^{13}C]glycolaldehyde phosphate are presently under investigation to permit the preparation of aldose phosphates with ^{13}C and/or ^{14}C enrichment at any carbon.

Solution Structures of Aldose Phosphates. The structures of the aldose and ketose phosphates in aqueous solution are of considerable interest (Benkovic & Schray, 1976; Gray, 1971; Midelfort et al., 1976; Swenson & Barker, 1971). It is useful to compare the solution structures of aldose phosphates with those of the simple aldoses from which they are derived and with those having one fewer carbon atom. The terminal phosphate eliminates the possibility for hemiacetal formation at the primary hydroxyl group, leaving opportunities for intra- or intermolecular reactions of the carbonyl group similar to those of the smaller nonphosphorylated aldoses.

In dilute solution, glycolaldehyde P and glyceraldehyde 3-P appear to exist predominantly as monomeric hydrates (linear *gem*-diol) with C-1 resonances at approximately 91 ppm (Table II). Resonances in this region of the ^{13}C spectrum are characteristic of linear *gem*-diol carbons (Grindley et al., 1977; Serianni et al., 1979b). When the acid form of DL-[1-^{13}C]glyceraldehyde 3-P is concentrated to dryness, a mixture of dimers and oligomers with C-1 chemical shifts at 105.2, 103.8, 102.9, 97.3, 93.1, 92.8, 89.7, and 89.0 ppm is formed. These revert rapidly to the hydrate in dilute aqueous solution (0.1 M) at pH 1–2. Dilute aqueous solutions of DL-glyceraldehyde 3-P contain about 5–10% of these higher structures at pH 5.5. Dimers and oligomers of [1-^{13}C]-glycolaldehyde and DL-[1-^{13}C]glyceraldehyde have also been observed (Serianni et al., 1979b) in dilute aqueous solution at pH 6.

Table III: Structural Forms of the Pentose 5-Phosphates in Aqueous Solution[a]

pentose 5-P	% composition, ±3%[b] β	α	hydrate
arabinose 5-P	40	58	2
ribose 5-P	64	34	≃1
xylose 5-P	45	51	4
lyxose 5-P	37	58	5

[a] Determined at 13 ± 1 °C and pH 5.5 ± 0.1. [b] Determined by computer integration of ^{13}C NMR spectra of 1-^{13}C-enriched pentose 5-phosphates. Aldehydo forms were not observed.

The tetrose 4-phosphates exist principally as monomeric hydrates in dilute aqueous solution at pH 2–5, but chemical shifts at 97.9 and 98.4 ppm, typical of hemiacetals, indicate the presence of up to 15% of dimers and/or oligomers in solutions of [1-^{13}C]erythrose 4-P. In concentrated solutions of D-[1-^{13}C]erythrose, C-1 resonances at 98.5, 97.9, 92.5, and 92.1 ppm have been attributed to dimeric or higher order structures (Serianni et al., 1978b).

Aqueous solutions of the [1-^{13}C]pentose 5-phosphates at pH 5.5 contain predominantly α- and β-furanose forms, with a small proportion (1–5%) of linear *gem*-diol (Table III). In comparison, solutions of [1-^{13}C]tetroses contain approximately 12% hydrate (Serianni et al., 1979b). Assignment of the chemical shifts to the α- and β-furanose forms of the pentofuranose phosphates was made by analogy to those assigned

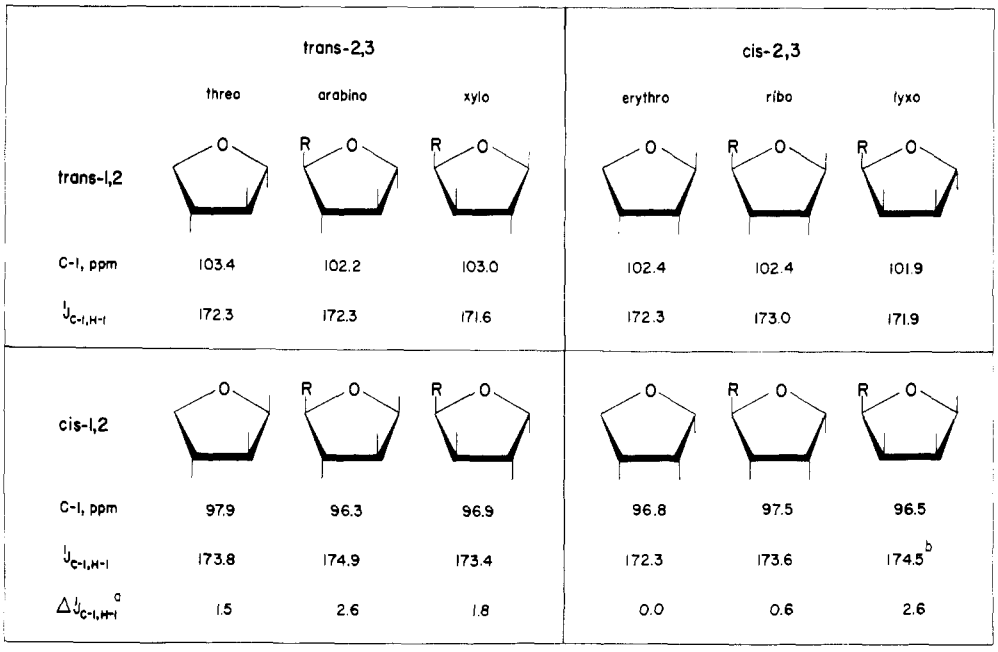

FIGURE 2: Structural and ^{13}C NMR spectral relationships between the furanose forms of the four pentose 5-phosphates and the two tetroses. [a]$\Delta^1J_{C-1,H-1} = {}^1J_{C-1,H-1}$(cis-1,2) $- {}^1J_{C-1,-H-1}$(trans-1,2). [b]The error in this coupling constant is ±1.5 Hz due to the complexity of the coupled signals. All other C–H couplings are accurate to ±0.7 Hz. Chemical shifts are accurate to ±0.1 ppm. R = CH_2OP.

to the α- and β-methyl furanosides (Ritchie et al., 1975; Gorin & Mazurek, 1976).

Furanose rings having OH-1 trans to OH-2 (Figure 2) are generally more stable than those having the corresponding cis arrangement (Angyal, 1969), and the α-lyxo-, α-arabino-, and β-ribofuranose phosphates which have this arrangement are the predominant anomers in aqueous solution at pH 5.5 (Table III). D-*xylo*-Pentofuranose phosphate is an exception to this rule, indicating that destabilization arising from two cis-1,3 interactions in the β anomer (Figure 2) is equivalent to that arising from a single cis-1,2 interaction in the α anomer.

As observed by Ritchie et al. (1975), a useful relationship exists between the chemical shift of C-1 and the anomeric configuration in the furanose ring. As in the methyl furanosides, the resonances for C-1 of anomers of the aldofuranose phosphates having OH-1 trans to OH-2 are typically downfield ($\simeq$105 ppm) from those with the cis arrangement ($\simeq$97 ppm) (Figure 2).

It is well established that the relative positions of hydroxyl groups in pyranoses and furanoses determine the relative stabilities of anomers and various ring forms (Angyal, 1969). Although the conformations of the furanose rings in the aldose phosphates are not established, certain relative configurations of hydroxyl groups appear to be preferred leading to the observed anomeric preferences listed in Table III. Specifically, the pentose phosphates with OH-2 trans to OH-3 generally have a greater proportion of the anomer with OH-1 cis to OH-2 than do those pentose phosphates with OH-2 cis to OH-3 (Figure 2).

Assignment of Chemical Shifts (Table II). Chemical shifts for C-1 have been discussed above in terms of the various forms present in aqueous solution. Assignments of carbons bearing the phosphate group and adjacent carbons have been made on the basis of comparison with standards and on the predictable coupling pattern of phosphorus to carbon (Lapper et al., 1973; Lapper & Smith, 1973). C-2 resonances were assigned on the basis of ^{13}C–^{13}C coupling to C-1 in 1-^{13}C-enriched compounds. The 2-^{13}C-enriched compounds prepared also permit unequivocal assignment of C-2 and of C-3 chemical shifts by difference (the close proximity of C-2 and C-3 resonances causes the C-3 resonance to be obscured in several of the 2-^{13}C-enriched compounds). As observed by Ritchie et al. (1975) for glycosides and Serianni et al. (1979b) for the tetrofuranoses, the C-2 and C-3 chemical shifts for the pentofuranose 5-phosphates in which OH-2 and OH-3 are trans (arabino, xylo) are downfield from those of the cis compounds (ribo, lyxo) (Table II and Figure 2). The relative configuration at C-2 and C-3 does not apparently affect the chemical shifts of C-1, C-4, and C-5. Ritchie et al. (1975) have observed that substitution of CH_2OH for H at C-4 of a tetrofuranoside to produce a pentofuranoside causes a downfield shift of the C-4 resonance of approximately 10 ppm. The C-4 resonance in the tetrofuranoses (Serianni et al., 1979b) also shifts downfield approximately 10 ppm when a 4-H is replaced by CH_2OP to produce the pentose 5-P, suggesting that phosphorylation at the primary alcohol may not significantly alter electron density at C-4. Alternately, the CH_2OP group may alter the through-bond effect on a neighboring group and, at the same time, propagate a through-space effect that cancels the through-bond effect.

The nitrile group shields the α carbon in the aldononitrile phosphates, producing an upfield shift of approximately 10 ppm from the α carbon of the aldose phosphates. This effect is expected and is analogous to proton shielding observed in the acetylene system (Pople, 1957).

Carbon–Phosphorus Coupling Constants. Two-bond coupling between carbon and phosphorus is not very sensitive to changes in structure of the phosphate ester. Two-bond ($^2J_{POC}$) coupling constants observed in this study (Table IV) are typical of those observed in other phosphate esters, ranging from 4.4 Hz for threose 4-P (pH 4.5) to 5.5 Hz for α-ribose and α-arabinose 5-phosphates (pH 5.5). The measured $^2J_{POC}$ of 4.8 Hz for β-D-ribose 5-P at pH 5.5 is similar to the reported $^2J_{POC}$ value of 4.7 Hz for the β-D-ribose 5-P moieties in UMP and AMP (Alderfer & Ts'o, 1977) at pH 6.3.

Three-bond ($^3J_{POCC}$) coupling (Table IV) is dihedral-angle dependent and can be used to determine molecular orientations in solution. For β-D-ribose 5-P and the β-D-ribose 5-P moieties in 5′-UMP and 5′-AMP, $^3J_{POCC}$ = 8.4 Hz (pH 5.5), 8.5 Hz (pH 6.3), and 8.7 Hz (pH 6.3), respectively. The magnitude

Table IV: ^{13}C-P and ^{13}C-H Coupling Constants of Aldose Phosphates and Related Compounds

compound	pH	^{13}C-P coupling constant (Hz)[a]		^{13}C-H coupling constant (Hz)[a]		
		$^2J_{POC}$	$^3J_{POCC}$	$^1J_{C1,H1}$	$^1J_{C2,H2}$	$^2J_{C1,H2}$
glycolaldehyde P, hydrate[d]	7.5	5.1	7.7			
DL-glyceraldehyde 3-P, hydrate[d]	7.5	5.1	6.2	159.8		*b*
DL-erythrose 4-P, hydrate	1.5	5.1	8.1	163.9		*c*
DL-threose 4-P, hydrate	4.5	4.4	8.1	162.6[e] (2.1)		3.5[e] (2.1)
DL-arabinose 5-P						
α-furanose	5.5	5.5	8.1	172.3	149.6	*c*
β-furanose	5.5	4.8	8.4	174.9	147.4	*c*
DL-ribose 5-P						
α-furanose	5.5	5.5	8.4	173.6	152.9	1.6
β-furanose	5.5	4.8	8.4	173.0	153.6	*b*
DL-xylose 5-P						
α-furanose	5.5			173.4	150.7	*b*
β-furanose	5.5			171.6	153.6	*b*
DL-lyxose 5-P						
α-furanose	5.5			171.9	145.2	*b*
β-furanose	5.5			174.5	147.4	4.8
DL-glyceronitrile 3-P[d]	2.5	4.7	8.8			
DL-erythrononitrile 4-P	1.7	5.1	8.4			
DL-threononitrile 4-P	1.7	5.5	8.4			
DL-arabinononitrile 5-P	3.2				152.9	
DL-ribononitrile 5-P	0.8				153.6	
DL-xylononitrile 5-P	3.2				153.2	*c*
DL-lyxononitrile 5-P	3.8				154.5	4.6

[a] Coupling constants were measured with a sweep width of 1500 Hz and a filter width of 2400 Hz at 13 ± 1 °C at the pH values (±0.1 unit) indicated and are accurate to within ±0.7 Hz. Couplings which are not entered were not measured. [b] Broadening observed. [c] No coupling observed. [d] These determinations were made at 34 ± 1 °C. [e] Couplings were measured at the pH value shown in parentheses.

of these couplings indicates that the preferred position of the phosphate group is trans to C-4 and gauche to H-5′ and H-5″ (Alderfer & Ts'o, 1977). The values of $^3J_{POCC}$ for both cyclic and acyclic aldose phosphates (Table IV) indicate that in these compounds a trans arrangement is preferred. It should be noted that $^3J_{POCC}$ couplings for several α-glycosyl 1-phosphates indicate that the preferred position of the phosphate is trans to C-2 and gauche to C-1 and to O-5, but substantial amounts of other rotamers may be present since the maximum value for $^3J_{POCC}$ may be 12 Hz or more when the trans arrangement is fixed (O'Connor et al., 1978).

Carbon–Hydrogen Coupling Constants. Carbon–hydrogen coupling constants can provide useful information about carbohydrate structures as shown by Bock et al. (1973), Walker et al. (1976), and Schwarcz & Perlin (1972). These couplings are particularly easy to observe in ^{13}C-enriched compounds, and some useful correlation of magnitude of coupling constants to structure emerge from this study. The ^{13}C–H couplings observed in the ^{13}C-enriched aldose phosphates and their derivatives are listed in Table IV.

$^1J_{C1,H1}$ couplings for the *gem*-diolic triose and tetrose phosphates range from 160 to 164 Hz. Formation of the furanose phosphate ring increases the value of $^1J_{C1,H1}$ by about 10 Hz. This increase upon cyclization has been observed for erythro- and threofuranoses (Serianni et al., 1979b) and provides another parameter, in addition to chemical shift, for the identification of linear hydrates in solution by ^{13}C NMR and ^1H NMR.

Bock et al. (1973), Bock & Pedersen (1974, 1975), and Walker et al. (1976) have shown that $^1J_{C1,H1}$ in the pyranoses is dependent on the configuration at C-1 and has been used to assign anomeric configuration to carbohydrates. In these rings, $^1J_{CH}$ for an axial H-1 is approximately 10 Hz smaller than $^1J_{CH}$ for an equatorial H-1. One-bond C-1 to H-1 coupling in the pentose 5-phosphates and tetroses is also sensitive to the configuration at C-1. The cis-1,2 anomers of threofuranose and arabino-, xylo-, and lyxofuranose phosphates have larger C-1 to H-1 coupling constants than the respective trans-1,2 anomers (Table IV and Figure 2), although differences between anomers are smaller ($\Delta^1J_{C1,H1}$ = 1.5–2.6 Hz) than those observed for the pyranoses. $^1J_{C1,H1}$ for erythrofuranose and ribofuranose phosphate is not as sensitive to configuration at C-1. Differences in $^1J_{C1,H1}$ for the furanose ring probably reflect conformational preferences (Bishop & Cooper, 1963) which must be determined before a full interpretation of ^{13}C–H coupling with respect to furanose configuration can be made.

$^1J_{C2,H2}$ couplings are typically 20–25 Hz less than $J_{C1,H1}$ couplings for both the linear and furanose forms of phosphorylated (Table IV) and simple aldoses (Serianni et al., 1979b). Examinations of $^1J_{CH}$ couplings in the pyranoses have shown similar differences between $^1J_{C1,H1}$ and $^1J_{C2,H2}$ (Bock & Pedersen, 1974, 1975).

Several geminal $^2J_{C1,H2}$ coupling constants for the [1-^{13}C]aldose phosphates were observable (Table IV). Threose 4-P and threose hydrate (Serianni et al., 1979b) show $^2J_{CCH}$ couplings of 3.5 and 4.4 Hz, respectively, whereas erythrose 4-P and erythrose hydrate do not exhibit coupling. Proton-coupled spectra of [1-^{13}C]- and [2-^{13}C]pentofuranose 5-phosphates vary in complexity with the furanose configuration. For example, geminal and longer range ^{13}C–H coupling is not apparent in α- and β-[1-^{13}C]arabinofuranose 5-P, whereas α- and β-[1-^{13}C]lyxofuranose 5-P show complex coupling patterns. Analysis by ^1H NMR will be required to identify specific ^{13}C–H and H–H couplings and relate the value of these couplings to furanose conformation and configuration.

Enzymatic Conversions of ^{13}C-Enriched Aldose Phosphates. The principal goals in undertaking the preparation of ^{13}C-enriched carbohydrates are to study their structures and behavior in solution, to use them as NMR probes of enzyme–substrate interactions, and to follow their enzymic conversions by NMR spectroscopy. We report two examples of the latter in the preparation of ^{13}C-labeled ketose phosphates.

The ^{13}C NMR spectrum of D-[2-^{13}C]ribose 5-P has strong resonances at 71.9 and 76.5 ppm due to C-2 of the α- and

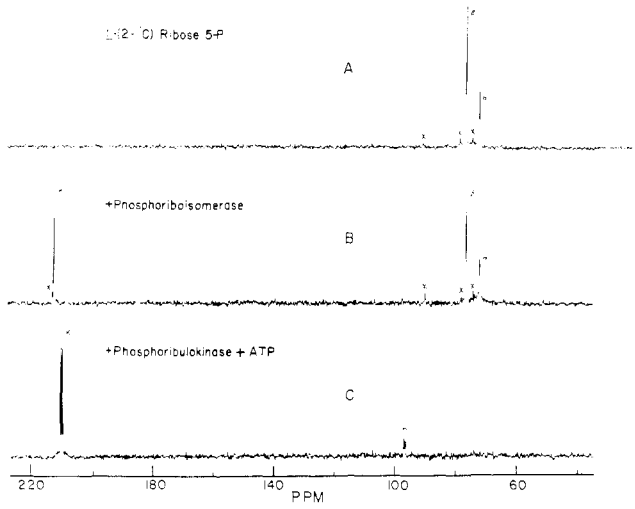

FIGURE 3: The enzymatic conversion of D-[2-^{13}C]ribose 5-P to D-[2-^{13}C]ribulose 1,5-P_2 as followed by ^{13}C NMR. Peaks designated by "X" are unidentified components. Only resonances of the enriched nuclei are shown. (A) The ^{13}C NMR spectrum of D-[2-^{13}C]ribose 5-P at 36 °C, showing α- and β-furanose forms. (B) The addition of phosphoriboisomerase to A produces a downfield resonance (k) originating from the keto form of D-[2-^{13}C]ribulose 5-P. (C) The addition of phosphoribulokinase and Mg^{2+}–ATP to B produces D-[2-^{13}C]ribulose 1,5-P_2, whose ^{13}C NMR spectrum is shown after purification. The doublet at approximately 210 ppm originates from the keto form, whereas the doublet at approximately 95 ppm originates from the keto–hydrate form. Splitting of these resonances is caused by ^{31}P–^{13}C coupling. Spectra were obtained at 36 ± 1 °C with a sweep width of 3000 Hz and a filter width of 6000 Hz.

β-furanose forms, respectively (Figure 3A). Addition of D-ribose-5-P isomerase causes the appearance of a resonance downfield (213.7 ppm) (Figure 3B) which is characteristic of the free keto form of D-[2-^{13}C]ribulose 5-P. The equilibrium favors the aldose phosphate (72%) at 36 °C as previously observed by Axelrod & Jang (1954). Addition of D-ribulose-5-P kinase and Mg^{2+}–ATP converts the downfield singlet into a doublet arising from carbon–phosphorus coupling and shifts the equilibrium toward the product, D-[2-^{13}C]ribulose 1,5-P_2. The ^{13}C NMR spectrum of purified D-[2-^{13}C]ribulose 1,5-P_2 (Figure 3C) at pH 7.6 shows doublets centered at 211.7 ppm (88%, $^3J_{POCC}$ = 7.3 Hz) and 97.6 ppm (12%, $^3J_{POCC}$ = 6.6 Hz) indicating that aqueous solutions of D-ribulose 1,5-P_2 at pH 7.6 contain 88% keto and 12% hydrated forms. This result compares favorably with a determination made by infrared spectroscopy (Gray & Barker, 1970). D-[2-^{13}C]-Ribulose 1,5-P_2 provides a standard for the chemical shift of a keto hydrate and will serve as a useful probe of the active site of D-ribulose-1,5-P_2 carboxylase/oxygenase.

Another ketose phosphate of biological interest was prepared by incubating DL-[1-^{13}C]glyceraldehyde 3-P with triosephosphate isomerase to produce a mixture of L-[1-^{13}C]-glyceraldehyde 3-P (91.3 ppm) and [3-^{13}C]dihydroxyacetone P (66.7 and 65.6 ppm) (Figure 4A). The two chemical shifts for [3-^{13}C]dihydroxyacetone P arise from the keto (65%) and hydrated keto (35%) forms in agreement with earlier estimations based on ^{1}H NMR spectroscopy (Gray & Barker, 1970). The addition of D-fructose-1,6-P_2 aldolase to this mixture causes the rapid formation of both L-[3,4-^{13}C]sorbose 1,6-P_2 (77.9 and 77.5 ppm) and D-[3,4-^{13}C]fructose 1,6-P_2 (76.7 and 76.2 ppm) (Figure 4B). After incubation for an additional 4 h and removal of protein, four resonances are observed at 77.8, 77.2, 77.0, and 76.5 ppm (Figure 4C) which are due to the major equilibrium product, L-[3,4-^{13}C]sorbose 1,6-P_2 (Figure 4C). The chemical shifts for C-3 and C-4 of sorbose 1,6-P_2 are expected to be similar to those of fructose

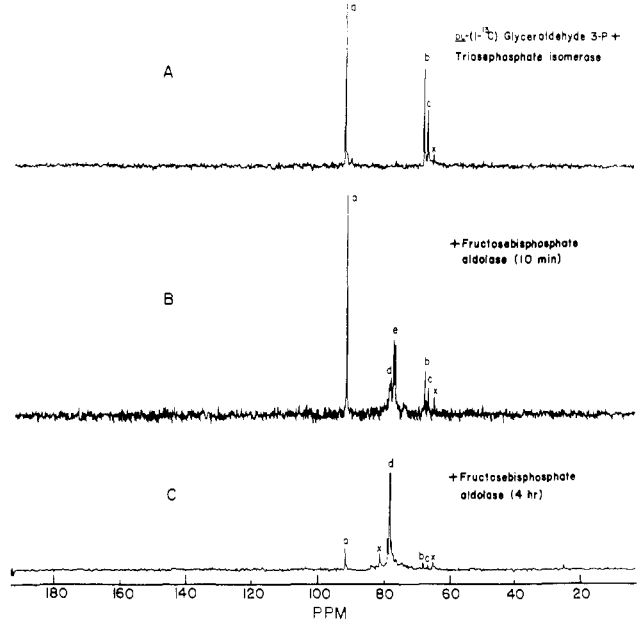

FIGURE 4: The enzymatic conversion of DL-[1-^{13}C]glyceraldehyde 3-P to L-[3,4-^{13}C]sorbose 1,6-P_2 as followed by ^{13}C NMR. Peaks designated by "X" are unidentified components. Only the resonances of enriched nuclei are shown. (A) The ^{13}C NMR spectrum of DL-[1-^{13}C]glyceraldehyde 3-P hydrate (a) after the addition of triosephosphate isomerase, producing resonances due to C-3 of the keto (b) and keto–hydrate (c) forms of [3-^{13}C]dihydroxyacetone P. (B) The addition of D-fructose-1,6-P_2 aldolase to A causes the appearance after 10 min of resonances due to C-3 and C-4 of D-[3,4-^{13}C]fructose 1,6-P_2 (e), with a smaller amount of L-[3,4-^{13}C]-sorbose 1,6-P_2 (d). (C) The same reaction mixture as analyzed by ^{13}C NMR after 4 h shows little unreacted [1-^{13}C]glyceraldehyde 3-P and [3-^{13}C]dihydroxyacetone P and the major product, L-[3,4-^{13}C]sorbose 1,6-P_2. Spectra were obtained at 34 ± 1 °C with a sweep width of 3000 Hz and a filter width of 2400 Hz.

1,6-P_2 reported by Koerner et al. (1973). At 15.08 MHz, the difference in frequency between C-3 and C-4 is smaller (approximately 30 Hz) than the expected ^{13}C–^{13}C coupling constant (40–50 Hz) and a complex spectrum is observed from which the coupling constant cannot be estimated readily (Figure 4C). Using computer simulation, we estimated $^1J_{C3,C4}$ to be 47.7 ± 1.0 Hz. It should be noted that this parameter can only be measured in doubly enriched compounds. In singly enriched compounds, the resonance due to the enriched carbon will obscure the resonance due to the unenriched adjacent carbon. The fundamental resonances of coupled carbons must be at least 100 Hz apart to permit easy estimation of coupling in singly enriched compounds.

Glycolaldehyde and Glyceraldehyde Phosphates. For the preparation of millimolar quantities of aldose phosphates, glycolaldehyde P and D-glyceraldehyde 3-P were prepared from DL-glycerol 1-P and D-fructose 6-P, respectively, by lead tetraacetate oxidation. Sodium metaperiodate oxidation was also examined, but traces of iodate interfere with hydrogenolysis of the aldononitrile phosphates, and careful chromatographic purification was required. On the other hand, aldononitrile phosphates prepared from lead tetraacetate oxidation products hydrogenolyze smoothly.

Ion-Exchange Chromatography and Stability of Products. Epimeric tetrose and pentose phosphates are difficult to separate by ion-exchange chromatography (Bartlett, 1959). For this reason, the epimeric aldononitrile phosphates were separated on Dowex 1-X8 (formate) at 4 °C by using linear formate gradients at pH 3.9 ± 0.1. Epimeric mixtures of these aldononitrile phosphates can also be separated on Dowex 1-X8 (chloride) using linear chloride gradients, but hydrogenolysis

in the presence of chloride ion consistently yielded larger amounts of 1-amino-1-deoxyalditol phosphates (≃45%).

It is important to maintain acidic conditions during the separation and handling of aldononitrile phosphates since the reaction between the parent aldose and cyanide is reversible, and, at pH >8, purified aldononitrile phosphates revert to epimeric mixtures.

Aldose phosphates, particularly the triose and tetrose phosphates, should be handled at low pH to avoid base-catalyzed isomerizations and β elimination. The acyclic triose and tetrose phosphates isomerize to give mixtures which include keto compounds when chromatographed on Dowex 1-X8 (formate). Purification of the alkali-sensitive aldose phosphates and the pentose phosphates can be achieved by anion-exchange chromatography on DEAE-Sephadex A-25 at 4 °C by using linear gradients of acetic acid at pH 4.5 ± 0.1. The tetrose 4-phosphates consistently yielded skewed peaks with notable tailing, whereas triose and pentose phosphates yielded symmetric peaks. Isomerization to keto compounds on DEAE-Sephadex was not observed under the conditions used.

It is usual to prepare aldose phosphates having four or fewer carbons as acetals to protect the base-sensitive aldehydic function (Ballou & Fischer, 1955; Ballou et al., 1955). We find, however, that the free aldose phosphates are stable during long-term storage at pH 1.0–2.0. When stored at −15 °C as 50 mM solutions, no detectable changes occur over a 2-month period as determined by ^{13}C NMR analysis of the 1-^{13}C-enriched compounds and by inorganic phosphate analysis. Storage at higher temperatures, however, results in degradation of these compounds even in acidic solution.

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