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Note

Enzymic synthesis of ^{13}C-labeled ketose phosphates from three-carbon precursors. Specific assignment of resonances in ^{13}C-n.m.r. spectra of D-fructose 1,6-diphosphate

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In recent years, ^{13}C-n.m.r. spectroscopy has been used to study the structure and dynamic behavior of complex carbohydrates free in solution and existing as integral parts of glycoproteins[1,2]. In addition, ^{13}C-n.m.r. spectroscopy of carbohydrates isotopically enriched in ^{13}C at one or more structural positions has made possible the extrapolation of conformational information from geminal and vicinal ^{13}C–^{1}H and ^{13}C–^{13}C coupling-constants[3], and has minimized the signal-to-noise limitations often encountered in ^{13}C-n.m.r. studies of carbohydrates in complex biological systems[4]. Such ^{13}C-enriched carbohydrates may, ultimately, prove valuable as spectroscopic substrate-probes for studying lectins and other carbohydrate-binding proteins.

There is a need for fast and convenient methods for the introduction of ^{13}C into a variety of carbohydrate structures. The cyanohydrin synthesis with (^{13}C)cyanide has been used with aldoses for one-carbon extension, with enrichment at the reducing carbon atom[5–7]. The product, after lengthy purification *via* ion-exchange and adsorption chromatography, may be recycled through the reaction to produce a two-carbon extended aldose, ^{13}C-enriched at C-2 (50–60% yield).

Methods have been available for some time for the enzymic interconversions and condensation of isotopically enriched 1,3-dihydroxy-2-propanone ("dihydroxyacetone") phosphate and D-glyceraldehyde 3-phosphate to yield enriched D-fructose 1,6-diphosphate[7–10]. The enzyme effecting the initial condensation, muscle aldolase, also tolerates a wide variety of other C_1–C_4 aldehydes, as substrates, making feasible the specific isotopic enrichment of a variety of C_4–C_6 ketose phosphates from isotopically enriched 1,3-dihydroxy-2-propane 1-phosphate[11]. Purified enzyme-preparations are also readily available for the conversion of the resultant ketose phosphate into phosphate analogs of D-glucose, D-mannose, D-galactose, D-*erythro*-pentulose, and D-ribose, the latter *via* the pentose phosphate shunt. At the other end of the glycolytic pathway, ^{2}H- and ^{13}C-enriched enolpyruvate phosphate

has been prepared in relatively high yield from isotopically enriched pyruvate, by using enolpyruvate phosphate synthetase[12]. However, because of the large, positive free-energy change from enolpyruvate phosphate to 1,3-dihydroxy-2-propane phosphate, *via* the enzyme-catalyzed reaction of the glycolytic pathway, isotopic enrichment of aldose and ketose phosphates from enriched pyruvate has not been performed.

The present investigation shows that it is feasible to synthesize a variety of ketose phosphates, enriched with ^{13}C at any one of a number of single sites or synthetically related sites, starting from ^{13}C-enriched pyruvate and using enzymes of the glycolytic pathway. In order to overcome the large free-energy barrier, the adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio is kept high by enzymic rephosphorylation of the latter by phosphocreatine. Products, obtained in >95% yield from ^{13}C-enriched sodium pyruvate, may be readily purified in a single ion-exchange chromatography step. Comparison of ^{13}C-n.m.r. spectra of purified products ^{13}C-enriched at different sites allowed direct assignment of specific carbon resonances. Because of the relative ease of synthesis and high yields, enzymic synthesis of ^{13}C-enriched carbohydrates may have advantages over more-lengthy chemical methods.

EXPERIMENTAL

Materials. — Sodium (1-^{13}C)- and (2-^{13}C)-pyruvate (90% enrichment) were obtained from Merck and Co., Inc. (St. Louis, Mo). Enolpyruvate phosphate synthetase, 1.5 units/mL, was a gift from Dr. William A. Bridger, Department of Biochemistry, University of Alberta, Canada. All other enzymes and cofactors, used without further purification, were obtained from Sigma Chemical Co. (St. Louis, Mo).

^{13}C-Enriched 1,3-dihydroxy-2-propanone . — To a mixture containing 10mM sodium (2-^{13}C)pyruvate or sodium (1-^{13}C)pyruvate, 20mM ATP, 30mM disodium phosphocreatine, and 10mM magnesium sulfate in aqueous 0.2M tris(hydroxymethyl)aminomethane (Tris), pH* (pH not corrected for D_2O content) 7.8 (12 mL total volume, 20% D_2O), were added 400 units of crystalline phosphocreatine kinase (130 units/mg), 50 units of adenylate kinase (25 μL, ammonium sulfate suspension), and 0.1 unit of enolpyruvate phosphate synthetase. The mixture was incubated in the n.m.r. probe for 6 h at 25°, whereupon it was determined, by means of signal intensities in the ^{31}P-n.m.r. spectrum, that nearly all of the original ^{13}C-enriched pyruvate had been converted, enzymically into enolpyruvate phosphate. The n.m.r. probe was then tuned to the ^{13}C-n.m.r. resonance frequency. Subsequent acquisition of the ^{13}C-n.m.r. spectrum showed no detectable signal arising from the enriched pyruvate, whereas a signal-to-noise ratio of >10:1 was observed for the signal arising from enolpyruvate phosphate. An enzyme assay for pyruvate, involving the reduction of pyruvate by lactic dehydrogenase, confirmed the absence of pyruvate in the mixture[13]. The sample was removed from the n.m.r.

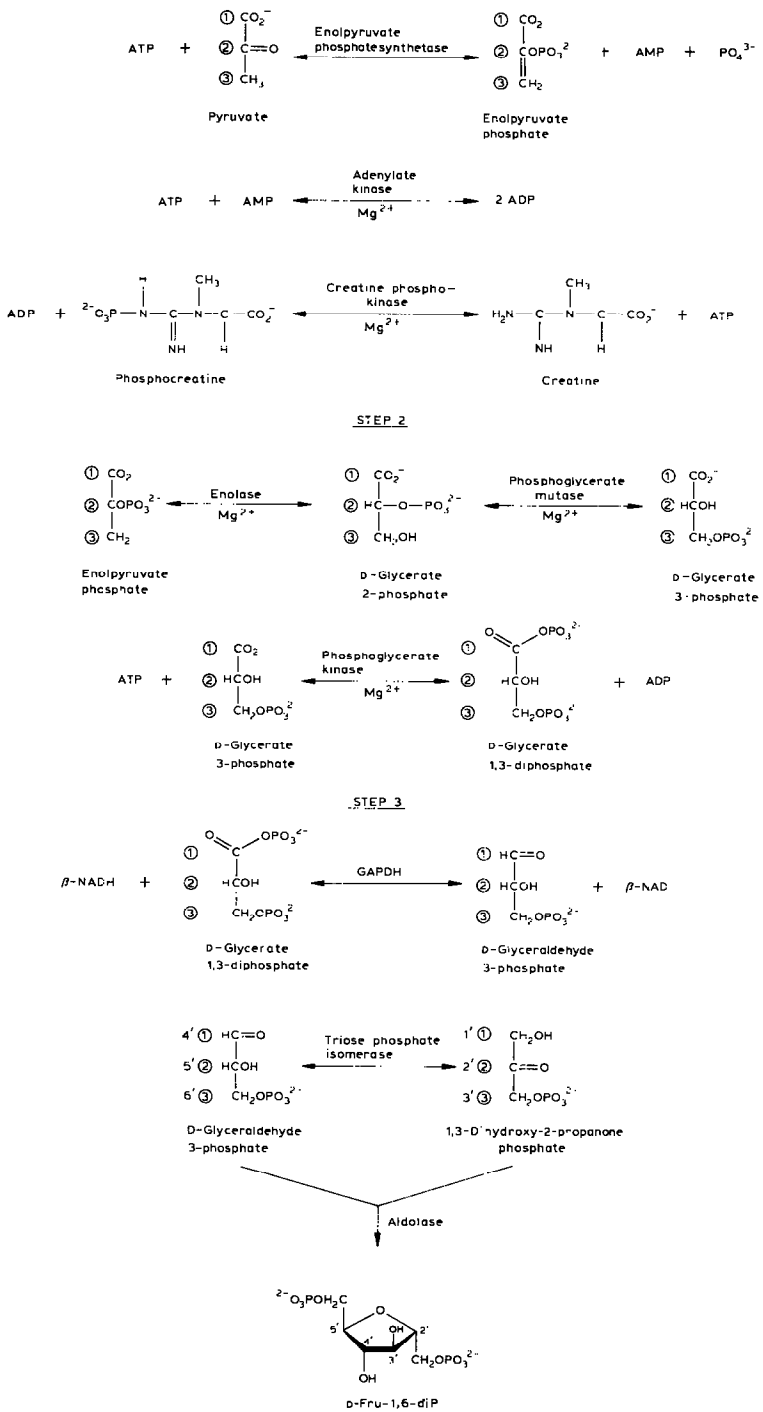
probe, the pH* readjusted to 6.65, and 150 units of crystalline yeast enolase (70 units/mg), 80 units of phosphoglycerate mutase (20 μL, ammonium sulfate suspension), and 60 units of phosphoglycerate kinase (20 μL, ammonium sulfate suspension) were added to the mixture. After incubation for another 2 h in the n.m.r. probe, it was ascertained by ^{31}P-n.m.r. spectroscopy that >90% of the enolpyruvate phosphate had been converted into an equilibrium mixture of glycerate 3-phosphate ("3-phosphoglycerate") and glycerate 1,3-diphosphate. The sample was again removed from the n.m.r. probe, and reduced β-nicotinamide dinucleotide (β-NADH) (to 0.13M) and 20 units of D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 10 μL, ammonium sulfate suspension) were added to the mixture. Within 10 min after addition of GAPDH, it was ascertained by ^{31}P-n.m.r. spectrosopy that essentially all of the D-glycerate 3-phosphate had been converted into 1,3-dihydroxy-2-propanone phosphate.

D-(*3*-^{13}*C*)*fructose-1,6-diphosphate and* D-(*3*-^{13}*C*)threo-*2-pentulose 1-phosphate.* — An unfractionated mixture containing (1-^{13}C) 1,3-dihydroxy-2-propanone was brought to pH* 2.0 for 1 h in order to remove, by protein denaturation, contaminating triose phosphate isomerase. The pH* was readjusted to 7.0 and a 2-fold molar excess of either DL-glyceraldehyde 3-phosphate or D-glycolaldehyde and 2.0 units of rabbit muscle aldolase (100 μL, ammonium sulfate suspension) were added to the mixture. After 7 min of incubation, the pH* was brought to 4.0 and the products identified by ^{13}C-n.m.r. spectroscopy.

Chromatographic purification of ketose phosphates. — Following synthesis of ^{13}C-enriched ketose phosphates, the mixture was adjusted to pH* 7.0 and applied to a column (1.5 × 20 cm) of DEAE-Sephadex, pre-equilibrated with 0.05M ammonium formate at pH 7.0. The ketose phosphates were eluted from the column by using a linear gradient of increasing buffer concentration (0.05–0.5M ammonium formate, 500 mL total volume), and 10-mL fractions were collected at a flow rate of ~60 mL per h. Alternate fractions were enzymically assayed by the procedure of Mandl and Neuberg[14]. To the pooled fractions was added an equivalent excess of washed Dowex-50W (H^+ form, 200–400 mesh). The resin was filtered off and the product evaporated to 2–3 mL under diminished pressure (35°). The product was then lyophilized.

N.m.r.-spectral conditions. — ^{31}P-N.m.r spectra were acquired at 80.8 MHz with a JEOL-FX200 spectrometer. Spectra were collected by using a single-pulse sequence in which the proton decoupler was gated on during acquisition, and 4096 time-domain data points were collected by quadrature detection with a sweep width of 3000 Hz. The wait time between pulses was 20.7 s. Spectra were Fourier-transformed with 1-Hz digital broadening. Chemical shifts were measured digitally, without susceptibility correction, with respect to 85% phosphoric acid.

^{13}C-N.m.r. spectra were acquired with broad-band decoupling and a recycle time of 20.3 s; 8192 time-domain data points were collected by using a sweep width of 12,000 Hz. Fourier transformation was performed with 2-Hz digital broadening. Chemical shifts were measured either with respect to 1,4-dioxane added as the internal reference, or with respect to 10% external 1,4-dioxane.



Scheme 1. Reactions relevant to the 3-step enzymic synthesis of D-fructose 1,6-diphosphate from ^{13}C-labeled pyruvate. Names of enzymes catalyzing each of the steps appear over the arrow connecting products with reactants. Chemical species, other than magnesium and adenine nucleotides, are given arbitrarily in their fully charged anionic forms. Circled numbers designate labels referred to in the text for the carbon atoms of three-carbon precursors and intermediates. Primed numbers are used to designate specific sites in the fructofuranose ring.

RESULTS AND DISCUSSION

^{13}C-Labeled ketose phosphates were prepared from ^{13}C-labeled sodium pyruvate by using the enzymes commonly found in the glycolytic and gluconeogenic pathways. In order to allow for the addition of reagents and the changes in pH needed to accommodate pH optima of some of the enzyme-catalyzed reactions, the synthetic scheme was conducted in three separate steps (see Scheme 1). The first step involved the synthesis of enolpyruvate phosphate from ^{13}C-labeled sodium pyruvate and ATP. Higher yields were obtained from this step by using the glyconeogenic enzyme, enolpyruvate phosphate synthetase, rather than the glycolytic enzyme, pyruvate kinase. In addition to generating ^{13}C-labeled enolpyruvate phosphate in 50–60% yield (under the conditions employed), the enolpyruvate phosphate synthetase-catalyzed reaction also yielded adenosine monophosphate (AMP) and inorganic phosphate as products[15]. The reaction could be driven further towards completion by regeneration of ATP in a coupled reaction-sequence, similar to that suggested by Krimsky for the synthesis of enolpyruvate phosphate by pyruvate kinase[16]. To this end, an excess of adenylate kinase was added in order to partially convert AMP and ATP present in the mixture into ADP (K_{eq} = 1)[17]. Concomitant addition of an excess amount of phosphocreatine and

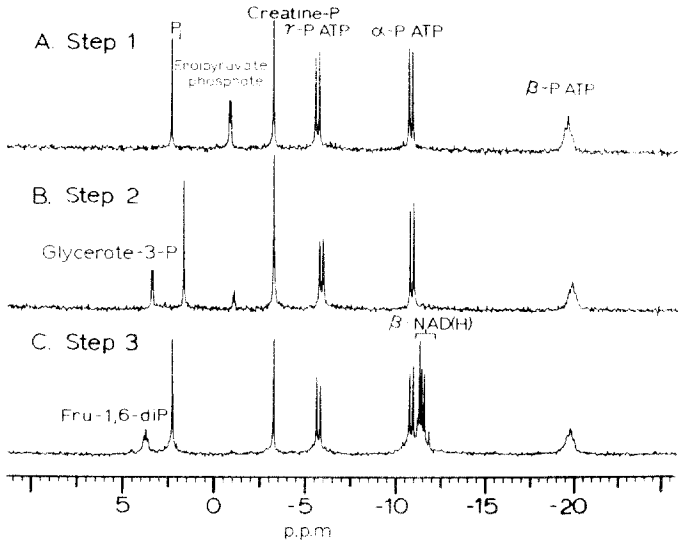


Fig. 1. ^{31}P-N.m.r. spectra (at 80.8 MHz) of equilibrium mixtures: (A) Mixture containing 10mM sodium (2-^{13}C)pyruvate, 20mM ATP, 30mM phosphocreatine, 10mM magnesium sulfate, 400 units of creatine phosphokinase, 50 units adenylate kinase, and an excess of enolpyruvate phosphate synthetase in 0.2mM Tris, pH* 7.8 (20% D_2O). The spectrum constitutes an average of 20 acquisitions, ~6 h after enzyme addition; (B) Mixture as in spectrum A, following addition of 150 units of yeast enolase, 80 units of phosphoglycerate mutase, and 60 units of phosphoglycerate kinase. The pH* was adjusted to 6.65; (C) Mixture as in spectrum B, following addition of β-NADH (to 0.13M), 20 units of GAPDH, and 20 units of muscle aldolase. The spectrum constitutes an average of 40 acquisitions, 10 min after addition of enzyme. Resonances labeled NAD(H) arise from β-NADH and its oxidized counterpart (β-NAD$^+$).

creatine phosphokinase resulted in phosphorylation of ADP to ATP (K_{eq} = 10^{10})[18]. The ^{31}P-n.m.r. spectra (acquired at 47 kG) of the Step 1 equilibrium mixture are shown in Fig. 1A. The doublet resonance at 0.8 p.p.m. in the ^{31}P-n.m.r. spectrum ($J_{^{13}C-^{31}P}$ = 7.3 Hz) has an average chemical-shift identical to the phosphorus resonance of purified enolpyruvate phosphate obtained commercially.

Fig. 1B shows the ^{31}P-n.m.r. spectrum of the mixture following 2 h of incubation in the presence of an added excess of yeast enolase, phosphoglyceromutase, and phosphoglycerate kinase (Step 2). The product of the coupled reaction, D-glycerate 1,3-diphosphate, is expected to be quickly hydrolyzed in aqueous solution to the more stable D-glycerate 3-phosphate[19]. In agreement with these expectations, the average chemical-shift of the doublet resonances at 3.4 p.p.m. in the ^{31}P-n.m.r. spectrum ($J_{^{13}C-^{31}P}$ = 7.3 Hz) is equal to the chemical shift observed for the resonance of D-glycerate 3-phosphate under identical conditions.

^{13}C-Labeled 1,3-dihydroxy-2-propanone phosphate could be prepared from the Step 2 mixture by adding a 2-fold excess of β-NADH and GAPDH. It was not possible to isolate the intermediate product of the reaction, glyceraldehyde 3-phosphate, because of the high contamination by triose phosphate isomerase of the commercial preparations of creatine phosphokinase (Sigma). Subsequent addition of muscle aldolase to the ^{13}C-labeled 1,3-dihydroxy-2-propanone phosphate yielded D-fructose 1,6-diphosphate (D-Fru-1,6-diP) enriched at C-2 and C-5 or C-3 and C-4 (Step 3). Ketose phosphates exclusively labeled with ^{13}C at C-2 or C-3 could be obtained from the mixture containing ^{13}C-labeled 1,3-dihydroxy-2-propanone phosphate by pH denaturation of contaminating triose phosphate isomerase (pH 2.0 for 1 h), followed by addition of a 2-fold excess of the appropriate unlabeled aldehyde and muscle aldolase. In a typical mixture, following synthesis of ketose phosphate, none of the original pyruvate could be detected by enzymic assay with lactic dehydrogenase[13]. In addition, the total amount of ketose phosphates prepared by this method was assayed to be within 10% of the amount of ^{13}C-labeled pyruvate initially added[14]. The absence of significant amounts of reaction intermediates was confirmed by the absence of detectable resonances arising from such intermediates in either the ^{13}C-n.m.r. (not shown) or ^{31}P-n.m.r. spectra.

Fig. 1C shows the ^{31}P-n.m.r. spectrum of D-(2,5-^{13}C)Fru-1,6-diP prepared by adding an excess of β-NADH, GAPDH, and muscle aldolase to the mixture prepared in Step 2. Resonances in the ^{31}P-n.m.r. spectrum previously assigned[20] to the two phosphate groups of the β-furanose anomer of D-Fru-1,6-diP appear to be split about equally by nearby ^{13}C-enriched carbon atoms (C-2 and C-5). Because the coupling constant (8.8 Hz) is nearly equal to the chemical-shift difference between the two phosphate resonances, the overlapping signals appear as a triplet at ~3.8 p.p.m.

The ^{13}C-n.m.r. spectra of chromatographically purified D-(3,4-^{13}C)Fru-1,6-diP, D-(3-^{13}C)Fru-1,6-diP, D-(2,5-^{13}C)Fru-1,6-diP, and D-(3-^{13}C)*threo*-2-pentulose 1-phosphate (D-xylulose 1-phosphate) are shown in Fig. 2A–D, respectively. Resonances at 82.5 and 76.7 p.p.m. in the spectrum of D-(3-^{13}C)Fru-1,6-diP (Fig. 2B)

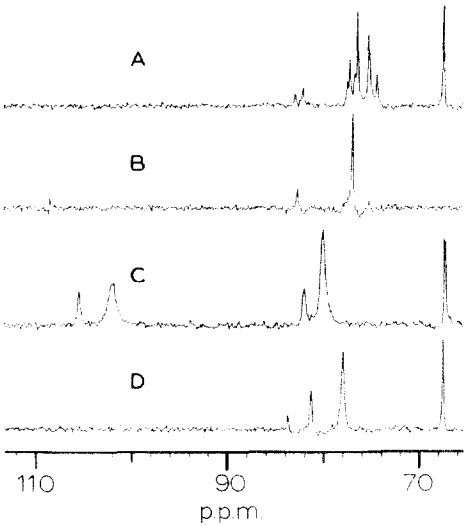


Fig. 2. ^{13}C-N.m.r. spectra of chromatographically purified ketose phosphates, pH* 7.2; (A) 9mM D-(3,4-^{13}C)Fru-1,6-diP, after 500 scans; (B) 3mM D-(3-^{13}C)Fru-1,6-diP, after 4000 scans; (C) 9mM D-(2,5-^{13}C)Fru-1,6-diP, after 500 scans; (D) 3mM D-(3-^{13}C)*threo*-pentulose 1-phosphate, after 4000 scans. The resonance appearing at 67.4 p.p.m. in spectra A, B, and D arises from 1,4-dioxane added as the internal reference.

may readily be assigned to C-3 of the α- and β-furanose anomers by comparing the integrated peak-intensities with intensities expected on the basis of the tautomeric populations determined for D-fructose phosphates[21]. Split by the neighboring, ^{13}C-enriched C-4, each of these resonances occurs as doublets ($J_{^{13}C-^{13}C} = 41 \pm 3$ Hz) in the spectrum of D-(3,4-^{13}C)Fru-1,6-diP (Fig. 2A). By elimination, the doublet resonances observed as downfield shoulders on the C-3 doublet, and the more-intense doublet centered at ~74.8 p.p.m. in the spectrum of D-(3,4-^{13}C)Fru-1,6-diP, may be assigned to C-4 of the α- and β-furanose anomers. Based upon integrated peak-intensities and observed chemical-shifts for anomeric carbon atoms of other furanoses, resonances at 105.5 and 102.1 p.p.m. in the spectrum of D-(2,5-^{13}C)Fru-1,6-diP (Fig. 2C) may be assigned to C-2 of the α- and β-furanose forms. The remaining resonances in the spectrum (at 82.1 and 80.1 p.p.m.) may then be assigned to C-5 of the α and β anomers. The broadening of some of the C-2 and C-5 resonances in spectrum 3C most probably results from a combination of ^{13}C–^{31}P and ^{13}C–^{13}C long-range couplings. The assignment of resonances to specific carbon atoms in spectra of ^{13}C-enriched analogs of D-Fru-1,6-diP confirms assignments previously made by Koerner *et al.*[21] in natural-abundance spectra of D-Fru-1,6-diP, but differ from those made by Que and Gray[22] or Doddrell and Allerhand[23].

The assignment of resonances in the spectrum of D-*threo*-pentulose 1-phosphate to C-3 of the α- or β-furanose anomers are based on the expected anomeric composition and the observations made by Que and Gray that cisoid substitution of diols on the tetrahydrofuran ring leads to shielding of the hydroxylated carbon

atoms relative to transoid substitution[22]. The assigned chemical-shift also agrees with those made previously for D-*threo*-pentulose (77.1 and 81.4 p.p.m. for C-3 of the β-furanose and α-furanose anomers[24]). The low-intensity resonance at 83.6 p.p.m. most probably arises from C-3 of the open-chain keto or *gem*-diol form[5–8]. The relative amounts of tautomers were measured by peak integration as α-D-*threo*-pentulose 1-phosphate, 22%, β-D-*threo*-pentulose 1-phosphate, 73%, and open chain keto or *gem*-diol form, 5%.

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