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# A convenient method for the preparation of a variety of $^{13}C$-substituted D-fructose phosphates using readily available enzymes of the glycolytic pathway

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ABSTRACT

Methods are presented for the preparation of a variety of D-fructose phosphates, $^{13}C$-substituted at any single carbon site or at any two symmetrically disposed carbon sites, from either $^{13}C$-substituted pyruvate or L-alanine. It is demonstrated that millimole quantities of product can be obtaind in good yield following a "one-pot" incubation of $^{13}C$-substituted precursors with commercially available enzymes and cofactors of the glycolytic pathway. Since it has previously been shown that a wide variety of aldehydes serve as acceptable substrates for the final rabbit muscle aldolase-catalyzed condensation step, the method can potentially be applied to prepare a wide variety of $^{13}C$-substituted sugars and sugar phosphates.

## INTRODUCTION

Over the last decade, the cyanohydrin reduction has been used to successfully prepare a wide variety of (1-$^{13}C$)-substituted aldoses[1–4]. Using this method, a mixture of C-2 epimers of a (1-$^{13}C$)aldononitrile are produced from the condensation of ($^{13}C$) cyanide and the aldehyde moiety of a parent aldose. Subsequent catalytic hydrogenation of the nitriles gives C-2 epimeric (1-$^{13}C$)aldoses, which can be purified by column chromatography. (2-$^{13}C$)Aldoses may be prepared by molybdate-catalyzed epimerization of (1-$^{13}C$)aldose[5], while aldoses substituted at their ultimate carbon can be prepared by cyanohydrin condensation of ($^{13}C$)cyanide with a parent dialdose derivative[6]. $^{13}C$-Subsituted aldoses so produced may also be converted to their corresponding 2-ketoses either enzymatically or by hydroxide-catalyzed isomerization[7,8].

The $^{13}C$-substitution of sugar carbons other than those readily available through cyanohydrin condensation with ($^{13}C$)cyanide or by isomerization usually requires a combination of enzyme-catalyzed reactions using any one of a number of chemically prepared $^{13}C$-substituted substrates. For example, D-(4-$^{13}C$)fructose-6-phosphate and L-(4-$^{13}C$)sorbose-6-phosphate have been prepared by using rabbit muscle aldolase to catalyze the aldol condensation between 1,3-dihydroxy-2-propanone (dihydroxyacetone) phosphate (DHAP) and DL-(1-$^{13}C$)glyceraldehyde prepared by the cyanohydrin

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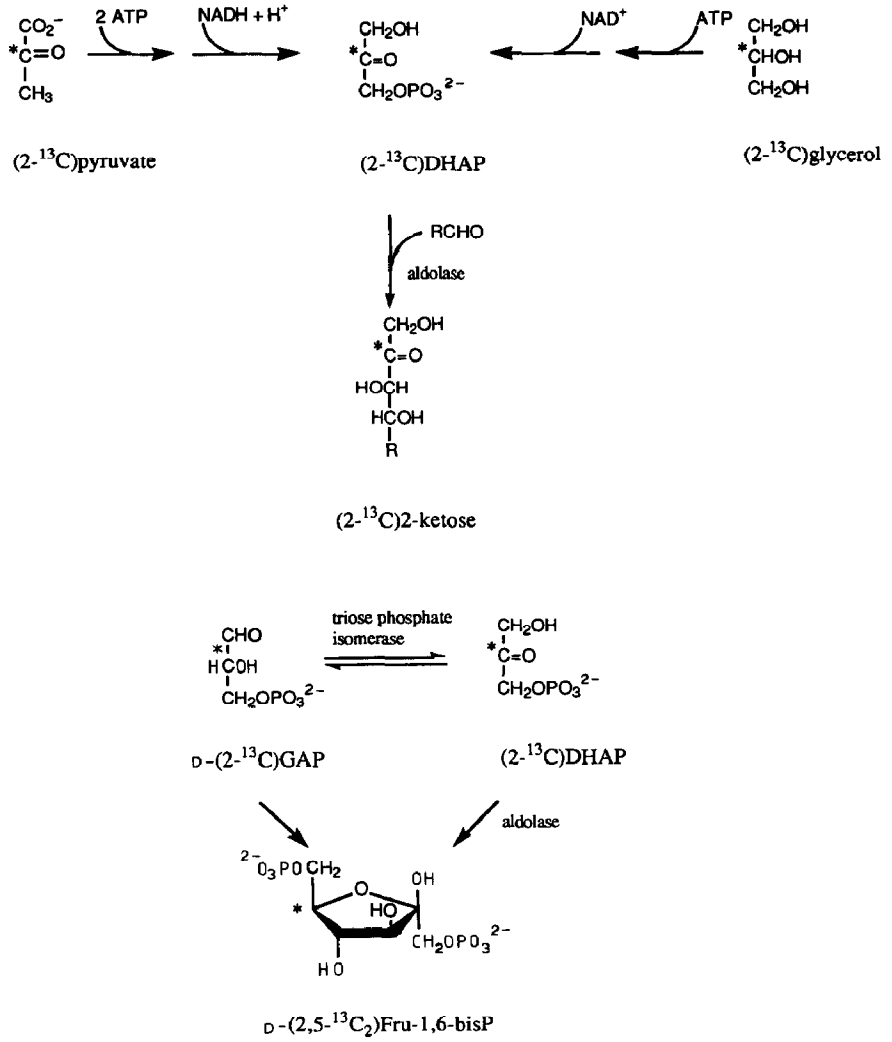
reduction method[9]. Since rabbit muscle aldolase is capable of accepting a wide variety of aldehyde substrates[10–14], ketoses $^{13}C$-substituted at any single carbon site or a variety of multiple carbon sites may be prepared if the appropriate $^{13}C$-substituted aldehyde or DHAP is available[15–16].

In the past $^{13}C$-substituted DHAP has been prepared from glycerol using commercially available enzymes[15–17] (Scheme 1). Because glycerol is achiral, this method is capable of producing DHAP $^{13}C$-substituted at either C-2 or at C-1 and C-3. Subsequent aldolase-catalyzed condensation with an unsubstituted aldehyde will then yield the corresponding (2-$^{13}C$) or (1,3-$^{13}C_2$)sugar phosphate. If, on the other hand, the $^{13}C$-substituted DHAP is concurrently treated with aldolase and triose phosphate




**Scheme 1**

isomerase* in the absence of unsubstituted aldehyde, an equilibrium mixture of DHAP and D-glyceraldehyde 3-phosphate will be established prior to condensation. The possible products of the condensation, D-(2,5-$^{13}C_2$)Fru-1,6-bisP or D-(1,3,4,6-$^{13}C_4$) Fru-1,6-bisP, can be chemically or enzymatically dephosphorylated and isomerized to corresponding $^{13}$C-substituted D-glucoses[15-17]. In previous work, we have shown that a wide variety of $^{13}$C-substituted sugar phosphates can be prepared from $^{13}$C-substituted pyruvate using readily available enzymes of the glycolytic pathway and the commercially unavailable enzyme, phospho(enol)pyruvate synthase[15,16,18,19]. Labeling of the sugar at C-1, C-2, or C-3 is limited only by the availability of the appropriately substituted pyruvate when the final aldol condensation is carried out in the presence of unsubstituted aldehyde. Aldol condensation in the absence of unsubstituted aldehyde and in the presence of TPI yields either D-(3,4-$^{13}C_2$)Fru-1,6-bisP, D-(2,5-$^{13}C_2$)Fru-1,6-bisP, or D-(1,6-$^{13}C_2$)Fru-1,6-bisP starting with (1-$^{13}$C), (2-$^{13}$C), or (3-$^{13}$C)pyruvate, respectively. Since an intermediate in the conversion of $^{13}$C-substituted pyruvate to DHAP is D-glyceraldehyde 3-phosphate, substitution at C-4, C-5, or C-6 of D-Fru-1,6-bisP can, in principle, be accomplished by adding unsubstituted DHAP to the reaction media. In practice, TPI is present as a contaminant of many commercial enzyme preparations so that rather than obtaining single site substitution in one of the three ultimate carbons of D-Fru-1,6-bisP, one obtains instead symmetrical substitution at two sites, each site having half the expected abduance of $^{13}$C.

In the present report we show that millimole quantities of D-fructose phosphates, $^{13}$C-substituted at a variety of single sites or doubly substituted with $^{13}$C at symmetrically disposed sites, may be prepared from either $^{13}$C-substituted pyruvate or $^{13}$C-substituted L-alanine. Conversion is carried out in a "one-pot" incubation of substrate with the commercially available enzymes of the glycolytic pathway. Preincubation of commercial enzyme preparations having significant TPI activity with 3-chloro-1-hydroxy-2-propanone-1-phosphate (a specific irreversible inhibitor of TPI[20,21]) made possible the preparation of D-Fru-1,6-bisP $^{13}$C-substituted at C-4, C-5, or C-6.

## EXPERIMENTAL

*Materials.* — 1-Chloro-3-hydroxy-2-propanone dihydrogenphosphate (chloroacetol phosphate) and DHAP were prepared according to previously published procedures[20-22]. All other organic reagents and ion-exchange resins were obtained from Sigma

* Abbreviation used throughout the manuscript are as follows: TPI, triose phosphate isomerase; PK, pyruvate kinase; LDH, L-lactate dehydrogenase; CPK, creatine phosphokinase; PGlyM, phosphoglycerate mutase; 3PGPK, 3-phosphoglycerate phosphokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GluDH, glucose dehydrogenase; G6PDH, glucose 6-phosphate dehydrogenase; PEPS, phopho(enol)pyruvate synthetase; DHAP, 1,3-dihydroxy-2-propanone-3-phosphate; D-GAP, D-glyceraldehyde 3-phosphate; D-Fru-1,6-bisP, D-fructose 1,6-bisphospate; D-2-PGly, D-glyceric acid 2-phosphate; D-3-PGly, D-glyceric acid 3-phosphate; PEP, phospho (enol)pyruvic acid; D-DiPGly, D-glyceric acid 1,3-bisphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediamine tetraacetic acid.

Chemical Co. (St. Louis, MO) and used without further purification. Sodium (2-$^{13}$C) pyruvate and L-(1-$^{13}$C)alanine were purchased from Cambridge Isotope Laboratory (Woburn, MA). Two commercial preparations of rabbit muscle pyruvate kinase [EC 2.7.1.40] were used. The first obtained from Boehringer Mannheim (Indianapolis, IN) as a buffered solution in 50% glycerol was found to have significant lactate dehydrogenase (LDH) [EC 1.1.1.27] activity,as evidenced by the conversion of $^{13}$C-substituted pyruvate to lactate in some reaction mixtures. Another preparation, obtained in crystalline from from Sigma, was found to be comparatively free of LDH. All n.m.r. spectra shown are of reaction mixtures and purified compounds prepared using the Sigma enzyme. Porcine heart alanine aminotransferase (glutamate–pyruvate transaminase) [EC 2.6.1.2] obtained from Sigma as an ammonium sulfate suspension was also found to have significant LDH activity (5 units LDH activity per unit of transaminase activity). No efforts were made to further purify the transaminase or obtain the enzyme from other commercial sources. Rabbit muscle creatine phosphokinase (CPK) [EC 2.7.3.2], yeast enolase [EC 4.2.1.11], and potato acid phosphatase [EC 3.1.3.2] were obtained in either lyophilized or crystalline form from Sigma and were added as such directly to the reaction mixtures. Rabbit muscle phosphoglycerate mutase (PGlyM) [EC 5.4.2.1] was obtained from Boehringer Mannheim as an ammonium sulfate suspension. D-Glucose dehydrogenase (GluDH, from *Bacillus megaterium*) [EC 1.1.1.47], obtained from Sigma as a lyophilized solid, was dissolved in a pH 7.0 buffered solution containing 0.1M Tris, 2mM EDTA, 2mM $\beta$-mercaptoethanol, 0.1M KCl, 10mM $MgCl_2$ and 0.1% sodium azide (Buffer A). It was found that the dissolved enzyme could be stored at 4° for up to 6 months without significant loss of activity. All other enzymes including rabbit muscle phosphoglycerate kinase (3-PGPK) [EC 2.7.2.3], rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [EC 1.2.1.12], yeast triose-phosphate isomerase (TPI) [EC 5.3.1.1], rabbit muscle aldolase [EC 4.2.1.13], and glucose-6-phosphate dehydrogenase (G6PDH, from *Leuconostoc mesenteroides*) [EC 1.1.1.49]) were obtained from Sigma as ammonium sulfate suspensions.

*Preparation of* D-*(2,5-$^{13}C_2$)fructose 1,6-bisphosphate from (2-$^{13}$C)pyruvate.* — Sodium (2-$^{13}$C)pyruvate (1.44 g, 13 mmol), D-glucose (2.1 g, 15 mmol), ATP (0.36 g, 0.5 mmol), $\beta$-NAD (0.36 g, 0.5 mmol), and disodium phosphocreatine ( 9.0 g, 32 mmol) were dissolved in 270 mL of Buffer A. The pH of the solution was carefully adjusted to pH 6.80 with 0.1M HCl.To the reaction mixture was added PK, (5000 units), CPK (2880 units), yeast enolase (600 units), PGlyM (400 units), 3PGPK (600 units), GAPDH (1440 units), GluDH (20 units), and rabbit muscle aldolase (40 units). TPI was not added but was present as a contaminant of commercial phosphocreatine kinase and enolase. During the course of the reaction an argon purge was kept on the reaction vessel, and the pH was controlled by a pH monitor/controller. By addition of N HCl the pH was maintained between pH 6.7 and 7.25. After 24 h the yield was assessed by $^{13}$C-n.m.r. spectroscopy (68%) and the enzymes were recovered by ultrafiltration. D-(2,5-$^{13}$C) fructose-1,6-bisphosphate was then precipated as its calcium salt by addition of a ten-fold excess of calcium acetate.

*Preparation of* D-*(2,5-$^{13}C_2$)fructose.* — The calcium salt of D-(2,5-$^{13}C_2$)Fru-1,6-bisP was suspended in distilled water, and Dowex-50 ($H^+$) resin was slowly added to the stirred suspension until the solution turned clear. The resin was removed by filtration, and solid sodium acetate was added until the solution was 0.1N in acetate. Potato acid phosphatase (200 units) was then added to the solution at pH 5, and the reaction mixture was allowed to stand for 48 h at room temperature at the end of which time the enzyme was removed by ultrafiltration. The reaction mixture was then titrated to neutral pH with dilute NaOH and made 40% by volume in ethanol. Barium acetate (2 equiv.) added to the reaction mixture, and the precipitated barium phosphate was removed by centrifugation. Further purification of D-(2,5-$^{13}C_2$) fructose was carried out on a Dowex-50 ($Ba^{2+}$) column[1].

*Preparation of* D-*(3,4-$^{13}C_2$)fructose-1,6-bisphosphate from* L-*(1-$^{13}$C)alanine.* — The $^{13}$C-substituted pyruvate described in the above experimental procedure may be generated *in situ* from $^{13}$C-substituted L-alanine and unsubstituted α-ketoglutarate. In an example of the methodology, L-(1-$^{13}$C)alanine (1.2 g, 13.1 mmol) and α-ketoglutarate (10.1 g, 65.5 mmol) were used as substrates in place of sodium (2-$^{13}$C)pyruvate in the above description of the preparation of D-(2,5-$^{13}C_2$)Fru-1,6-bisP. The reaction was carried out as described with the addition of pyruvate–glutamate transaminase (1000 units). The overall yield of D-(3,4-$^{13}C_2$)fructose-1,6-bisphosphate as determined by $^{13}$C-n.m.r. spectroscopy was ~ 62%.

*Inhibition of triose phosphate isomerase.* — 1-Chloro-3-hydroxy-2,2-dimethoxypropane dihydrogenphosphate, biscyclohexylammonium salt was prepared according to published procedures[20,21]. A solution of the active form of the inhibitor, 1-chloro-3-hydroxy-2-propanone dihydrogenphosphate (chloroacetol phosphate), was prepared by dissolving the dimethyl ketal salt (94 mg, 0.21 mmol) in water (3 mL). A small amount of Dowex-50 ($H^+$) was added to render the solution pH 2.0, and the resin was filtered off by suction. The solution was made to 4 mL, incubated for 40 h at 40°, and finally, adjusted to pH 5.5 with solid sodium hydrogencarbonate. The solution (4.4mM in inorganic phosphate, 33.1mM in base-labile phosphate as determined spectrophotometrically) was stored frozen. It was found that when TPI (17 units) was incubated with this inhibitor solution (5 μmol) according to published procedures, less than 0.1% of the original activity remained.

*TPI activity in enzymes.* —The 8 enzymes, PK, CPK, enolase, PGlyM, 3PGPK, GAPDH, G6PDH* and aldolase, were assayed for their enzyme activity and for TPI activity according to standard procedures[23]. Only CPK and enolase showed TPI activity which was a significant fraction of the enzyme activity (0.4 and 235% for CPK and enolase, respectively).

*Inhibition of TPI in CPK and enolase.* — TPI activity of these two crystalline enzymes were inhibited by the same general procedure used to inhibition TPI. Specifi-

* Glucose 6-phosphate dehydrogenase was used in place of glucose dehydrogenase for NADH regeneration when it was found that CAP inhibition of TPI also resulted in a significant inactivation of glucose dehydrogenase.

cally, 25 units of CPK was dissolved with 0.1M $NaHCO_3$, 1mM EDTA, pH 8, buffer (99 mL). An aliquot (1 mL) was removed for enzymatic assay for CPK activity. The remaining 98 mL was added with of chloroacetol phosphate solution (1 mL, 33.1mM), mixed and incubated for 5 min at room temperature. Following incubation, M $\beta$-mercaptoethanol solution (1 mL) was added to make the solution 0.01M in $\beta$-mercaptoethanol, and the final solution was dialyzed overnight against 0.01M sodium phosphate, pH 6. The dialyzed solution was assayed for both CPK and TPI activities. It was found that the ratio (expressed as percent) of TPI/CPK activity dropped from 0.4 to less than 0.01%.

For TPI inhibition in enolase, enolase (1.5 mg, 76 units) was dissolved with 0.3M $NaHCO_3$, 3mM EDTA, pH 8,buffer (37 mL). An aliquot (1 mL) was removed for enzymatic assay for enolase activity. The remaining 36 mL was added with chloroacetol phosphate solution (63 mL, 33.1mM), mixed, and incubated for 60 min at room temperature. As was the case for TPI inhibition in the enolase preparation, M $\beta$-mercaptoethanol (1 mL) was added following incubation, and the enzyme mixture was dialyzed overnight. The dialyzed solution was assayed for both enolase and TPI activities. It was found that the ratio (expressed as percent) of TPI/enolase activity dropped from 235 to less than 0.02%.

*Synthesis of* D-*(5-$^{13}$C)fructose-1,6-bisphosphate.* — Buffer A (2 mL) containing 50 mM (2-$^{13}$C)sodium pyruvate (0.01 mmol), 150mM phosphocreatine (0.3 mmol), 4mM ATP (8 $\mu$mol), 4mM $\beta$-NADH (8 $\mu$mol), 60mM D-glucose 6-phosphate (0.12 mmol), and 50mM DHAP (0.10 mmol) was brought to pH 7.0. To this solution was added PK, (162 units), G6PDH (90 units), GAPDH (49 units), aldolase (5 units), PGlyM (3 units), 3PGPK (19 units), and the dialyzed chloroacetal phosphate-treated CPK and enolase solutions (see above). The formation of D-(5-$^{13}$C)Fru-1,6-bisP was monitored by $^{13}$C-n.m.r. spectroscopy every 2 h. The reaction was stopped when the C-2 resonance of the $\beta$-furanose anomer appeared at 102.0 p.p.m. (19 h). At this point the yield from pyruvate was estimated by peak integration to be about 54%. A solution of 10% (v/v) heptanol–ethanol (0.5 mL) was added to the reaction mixture, and the mixture was heated for 1 min in a boiling water bath. The mixture was cooled in ice and then filtered to remove the coagulated proteins. The solution at pH 7.3 was raised to pH 8.4 with a dilute NaOH solution, and saturated $CaCl_2$ solution (1.0 mL) was added. The precipitated calcium salt of mainly D-(5-$^{13}$C)Fru-1,6-bisP was collected by filtration and washed twice with a little warm water. It was redissolved in water containing Dowex-50 ($H^+$), and the resin was filtered off to give a solution (2.2 mL) which contained the product, D-Fru-1,6-bisP (45.4 mmol, 45.4%, based on (2-$^{13}$C)pyruvate, as determined by enzymatic assay). The solution was comprised of 94% D-(5-$^{13}$C)Fru-1,6-bisP and 6% D-(2-$^{13}$C)Fru-1,6-bisP as estimated from the $^{13}$C-n.m.r. spectrum (without n.O.e.) by integration.

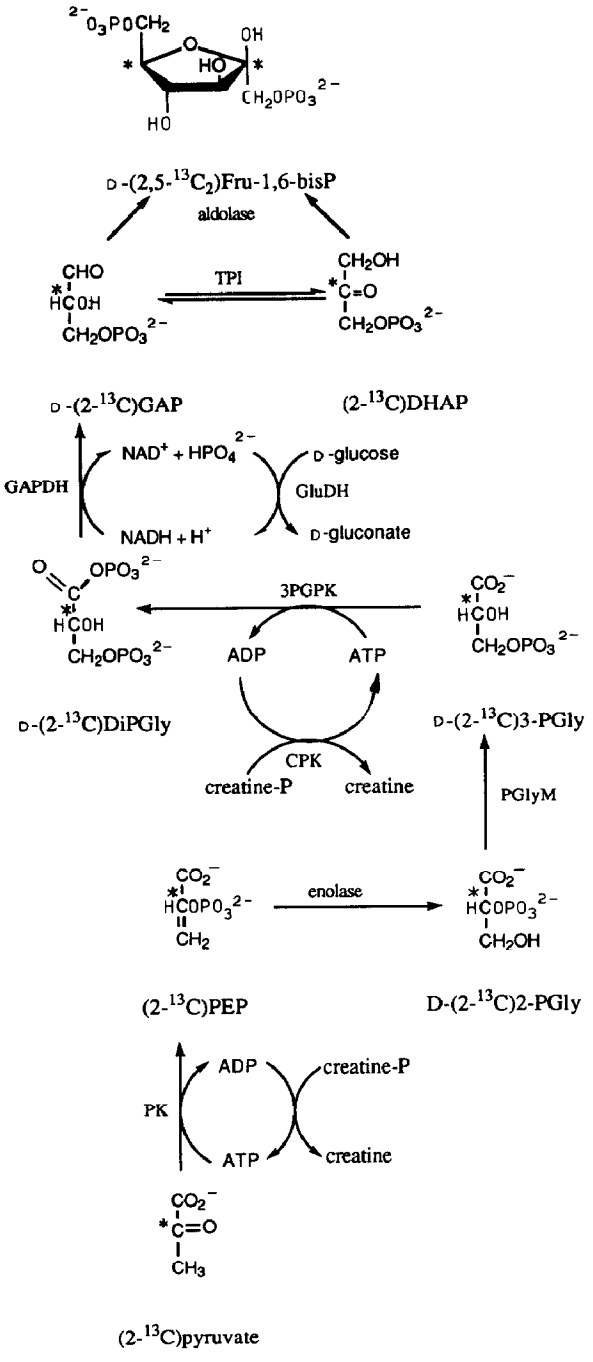
*$^{13}$C-N.m.r. spectral parameters.* — $^{13}$C-N.m.r. spectra were acquired at either 50.1 or 125.25 MHz using a 20-degree sampling pulse and a 5-s delay time between consecutive acquisitions. The proton decoupler was gated on only during acquisition. Spectra of the reaction mixtures were processed with 8 or 10 Hz digital broadening. Chemical

shifts are reported using the $^{13}$C resonance of 1,4-dioxane (67.4 p.p.m.) as the external standard.

## RESULTS AND DISCUSSION

*Preparation of symmetrically $^{13}$C-substituted* D-*fructose 1,6-bisphosphates from $^{13}$C-substituted pyruvate or $^{13}$C-substituted* L-*alanine.* — The conversion of D-fructose 1,6-bisphosphate to pyruvate is generally carried out *in vivo* using the enzymes of the glycolytic pathway. The first step in the reaction sequence, a reverse aldol condensation to yield products glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, is thermodynamically unfavorable[24] ($\Delta G^{\circ\prime}$ = 23.4 kJ/mol D-Fru-1,6-bisP). It is the large negative free-energy changes associated with the ensuing reactions responsible for producing four equivalents of ATP from ADP ( − 50 kJ/mol), which are ultimately responsible for driving the overall equilibrium towards pyruvate. Any scheme intent on preparing $^{13}$C-substituted D-Fru-1,6-bisP from substituted pyruvate must be specifically designed to overcome these large negative free-energy changes in the reverse direction. In previous work it was demonstrated that $^{13}$C-substituted PEP could be prepared from substituted pyruvate in nearly quantitative yield using the gluconeogenic enzymes PEPS in place of the more common glycolytic enzyme, pyruvate kinase[18,19]. In two additional separate reactions, $^{13}$C-substituted PEP was converted first to D-glycerate 1,3-diphosphate and finally, following the addition of two equivalents of NADH, to $^{13}$C-substituted D-Fru-1,6-bisP. However, the difficulty in the preparation of PEPS[25,26] and the expense of NADH make routine use of the procedure to prepare millimole quantities of $^{13}$C-substituted sugars unfavorable both from a practical and an economic standpoint.

Scheme 2 shows how D-(2,5-$^{13}C_2$)Fru-1,6-bisP can be prepared from (2-$^{13}$C) pyruvate using commercially available pyruvate kinase to catalyze the first step in the reverse glycolysis reaction sequence. As was the case in past work, ATP is regenerated *in situ* by means of a creatine kinase catalyzed phosphoryl transfer from phosphocreatine to ADP[18,19]. This particular form of ATP regeneration allows the total ATP–ADP concentration to remain low in comparison to the total concentration of $^{13}$C-substituted substrates while at the same time providing an additional 12.5 kJ/mol of free-energy change in favor of product formation. NADH can also be regenerated *in situ* by coupling the reduction of D-glycerate 1,3-diphosphate to the GluDH catalyzed oxidation of D-glucose to $\delta$-gluconolactone and subsequent hydrolysis to D-gluconate. This regeneration system is similar to the glucose-6-phosphate dehydrogenase NADH regeneration system commonly used in a wide variety of enzyme-catalyzed reductions carried out on a preparative scale[27]. However, the GluDH system has the advantage that it uses more readily available and less expensive glucose as its substrate, and it allows for the selective isolation of D-Fru-1,6-bisP as its insoluble calcium or barium salt without co-precipitation of other unsubstituted sugar phosphates. Additionally, the GluDH-catalyzed reaction carried out at neutral pH provides an additional large, negative, standard free-energy change, making the preparation of D-Fru-1,6-bisP from pyruvate thermodynamically favorable[28].

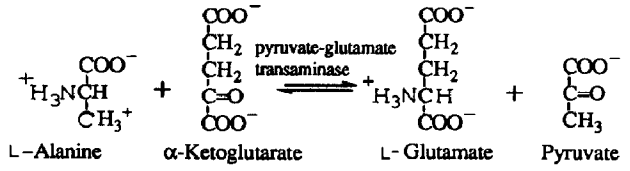

D-(2,5-13C2)Fru-1,6-bisP
aldolase
TPI
D-(2-13C)GAP
(2-13C)DHAP
NAD+ + HPO4 2-
NADH + H+
GAPDH
GluDH
D-glucose
D-gluconate
3PGPK
ADP
ATP
D-(2-13C)DiPGly
D-(2-13C)3-PGly
CPK
creatine-P
creatine
PGlyM
enolase
(2-13C)PEP
D-(2-13C)2-PGly
PK
ADP
ATP
creatine-P
creatine
(2-13C)pyruvate

**Scheme 2**

In practice, the reaction was carried out by incubating (2-$^{13}$C)pyruvate (50 mM) with glucose (1 equiv.) phosphocreatine (2 equiv.), and ATP and NAD (0,05 equiv.) with the mixture of nine enzymes in a buffered aqueous solution. Because the activity of phosphoglycerate mutase decreases markedly above neutral pH[29], the expected pH increase arising from removal of pyruvate from the reaction media and the buffering effect of the product inorganic phosphate is countered by the addition of dilute HCl. Fig. 1A shows the $^{13}$C-n.m.r. spectrum of a typical reaction mixture following an overnight incubation at room temperature. Resonances in the spectrum arising from $^{13}$C-substituted material can be assigned to the C-2 of pyruvate (205.8 p.p.m.) and its hydrate (94.9 p.p.m.) and the C-2 (105.5 and 102.0 p.p.m.) and the C-5 (82.1 and 80.1 p.p.m.) of the α- and β-furanose anomers of D-Fru-1,6-bisP[18,19,30]. Additionally, resonances are also present which, on the basis of their known chemical shift, can be assigned to the $^{13}$C-substituted C-2 of DHAP (212.2 p.p.m.) and its corresponding hydrate (96.0 p.p.m.)[15,31]. Integration of the resonances shows the distribution of $^{13}$C-enrichment in the final reaction mixture to be 24% (2-$^{13}$C)pyruvate (12mM), 68% D-(2,5-$^{13}C_2$)Fru-1,6-bisP (17mM), and 8% (2-$^{13}$C)DHAP (4.4mM). The final concentration of (2-$^{13}$C)DHAP is in reasonable agreement with that calculated on the basis of free-energy changes associated with the aldolase- and TPI-catalyzed steps in the reaction sequence (5.3mM). It is unclear, however, why the free-energy change for the overall reaction calculated from the estimated concentrations of $^{13}$C-substituted substrates and the stoichiometry of the overall reaction ( − 9.1 kJ/mol) is significantly less than the sum of free-energy changes for the separate constituent reactions taken from the literature ( − 23.4 kJ/mol)[24,28]. As would be expected for a reaction mixture at equilibrium, longer reaction times, even with the addition of fresh enzymes, failed to increase the yield.

Following the preparation of D-(2,5-$^{13}C_2$)Fru-1,6-bisP, the enzymes were removed from the reaction mixture by ultrafiltration, and their residual enzyme activities were measured. The residual enzyme activities reported as the percent of original activity were: PK, 92%; CPK, 85%; enolase, 90%; PGlyM, 75%; 3PGPK, 88%; GAPDH, 91%; aldolase, 20%; GluDH, 80%. The isolated enzyme mixture, made saturated with $NH_4SO_4$, can be stored at 4° for use in later reactions. The product, D-(2,5-$^{13}C_2$)Fru-1,6-bisP, isolated from the reaction mixture as its insoluble calcium salt, was dephosphorylated by acid phosphatase to yield D-(2,5-$^{13}C_2$)fructose[17]. Fig. 1B shows the $^{13}$C-n.m.r. spectrum of the final, chromatographically purified product. Resonances appearing in the spectrum can be assigned to the C-2 of the open-chain keto form (215.1 p.pm.), the β-pyranose form (99.1 p.p.m.), and the α- and β-furanose forms of the sugar (105.4 and 102.5 p.p.m.), while resonances for the C-5 of the β-pyranose, α- and β-furanose forms appear at 70.1, 82.2, and 81.5 p.p.m.[18,32–36], respectively. A small resonance, which can be assigned to the C-2 of contaminating (2-$^{13}$C)pyruvate, also appears in the spectrum.

The pyruvate used as the initial $^{13}$C-substituted substrate in the preparation of symmetrically substituted fructose 1,6-bisphosphates may be formed *in situ* from $^{13}$C-substituted L-alanine *via* an enzyme-catalyzed transamination catalyzed by L-glutamate–pyruvate transaminase (Scheme 3).




**Scheme 3**

Since the equilibrium constant for the reaction is near unity[24], the initial reaction mixture is charged with a five-fold excess of α-ketoglutarate in order to increase product formation. Fig. 1C shows the $^{13}$C-n.m.r. spectrum of a reaction mixture for the preparation of D-(3,4-$^{13}C_2$)Fru-1,6-bisP from L-(1-$^{13}$C)alanine following a 48 h incubation at room temperature. Doublets appearing in the spectrum at 76.7 and 74.8 p.p.m. ($J_{^{13}C-^{13}C}$ = 41 ± 3 Hz) have previously been assigned to the C-3 and the C-4 carbons of the β-furanose anomer[18,32–36]. Corresponding resonances for the α-furanose anomer occur at 82.5 p.p.m. and as a shoulder of the C-3 β-furanose doublet at 76.7 p.p.m. Other resonances in the spectrum arising from $^{13}$C-substituted carbons include those assignable to the C-1 of pyruvate (170.7 p.p.m.), the C-1 of L-alanine (176.7 p.p.m.), the C-1 of L-lactate (183.3 p.p.m.), and the C-1 of DHAP and its hydrate (66.3 and 65.1 p.p.m.)[15]. The L-(1-$^{13}$C)lactate present in the mixture is formed from pyruvate in a reduction catalyzed by LDH present as a contaminant in the commercial preparation of transaminase used. $^{13}$C-N.m.r. peak integration shows that the respective percentages of label distribution among $^{13}$C-substituted species to be: L-alanine, 9.8% (4.9mM); pyruvate, 12.8% (6.4mM); L-lactate, 9.8% (4.9mM); D-Fru-1,6-bisP, 62.1% (15.5mM) and DHAP, 5.3% (2.65mM). From these relative percentages, it may be determined that the yield of $^{13}$C-substituted D-Fru-1,6-bisP from pyruvate, taking into account the L-lactate which occurs as a byproduct of the reactions, is 72%. This is in reasonable agreement with the overall 68% yield of $^{13}$C-substituted product obtained when only pyruvate is used as the $^{13}$C-substituted substrate.

*Preparation of carbohydrates $^{13}$C-substituted at a variety ofsingle carbon sites.* — Rabbit muscle aldolase is able to accept a variety of substrates having the aldehyde functionality, but it is quite specific for DHAP[10–14]. Therefore, it should be possible to prepare a variety of 2-ketose using a slightly modified version of Scheme 2 in which a slight excess of unsubstituted aldehyde is initially added to the reaction mixture,$^{13}$C-substituted pyruvate or L-alanine will then be converted to $^{13}$C-substituted DHAP and condense with the unsubstituted aldehyde substrate. Because DHAP contributes to C-1 through C-3 of the product, single-site substitution at any of these first three carbons is possible from the appropriately $^{13}$C-substituted pyruvate or L-alanine. Fig. 1D shows

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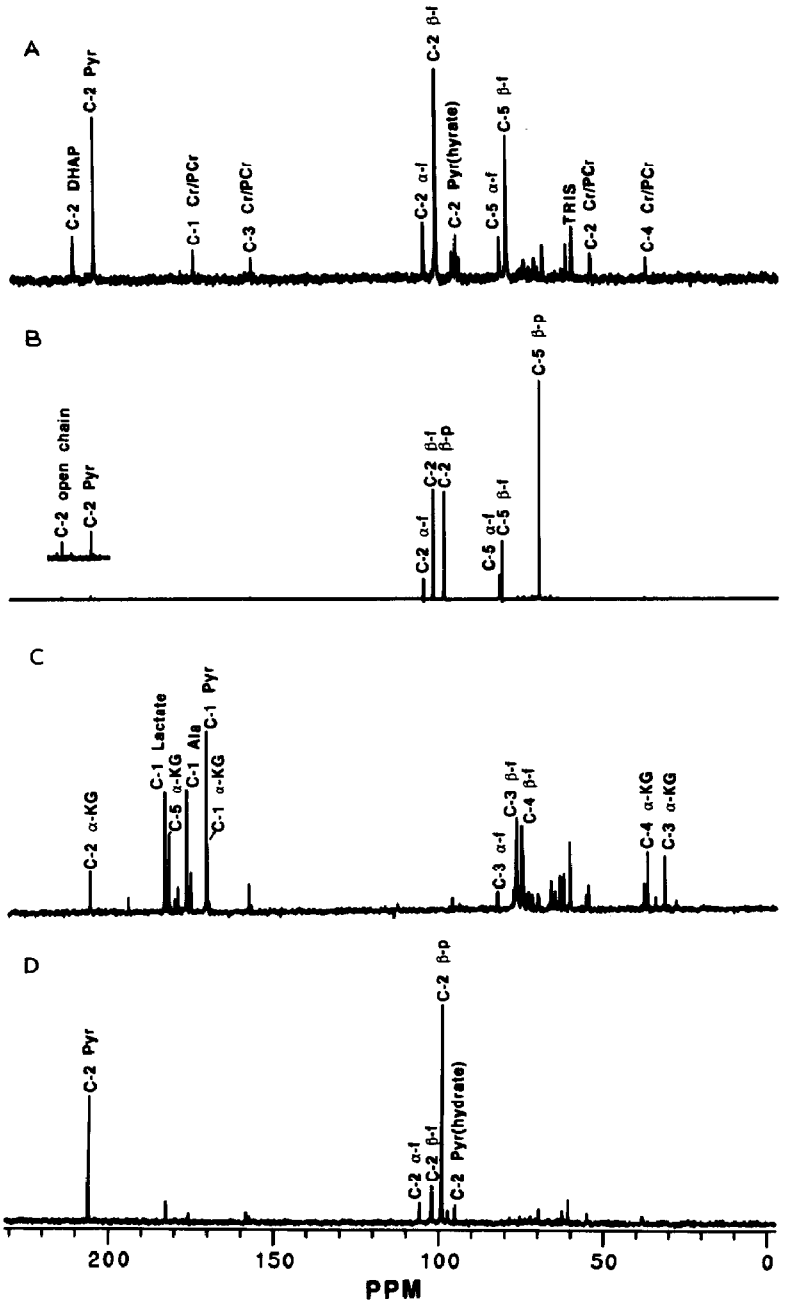
Fig. 1. Proton-decoupled $^{13}$C-n.m.r. spectra (125 MHz) of (A) the reaction mixture for the preparation of D-(2,5-$^{13}C_2$)fructose 1,6-bisphosphate from sodium (2-$^{13}$C)pyruvate. Resonances in the spectrum arising from $^{13}$C-substituted substrates may be assigned to the C-2 of pyruvate (Pyr) and its hydrate, the C-2 of DHAP, and the C-2 and C-5 of the furanose forms of D-(2,5-$^{13}C_2$)Fru-1,6-bisP (α-*f*, β-*f*). Resonances arising from unsubstituted material may be assigned to creatine and phosphocreatine (Cr/PCr), Tris buffer (TRIS),



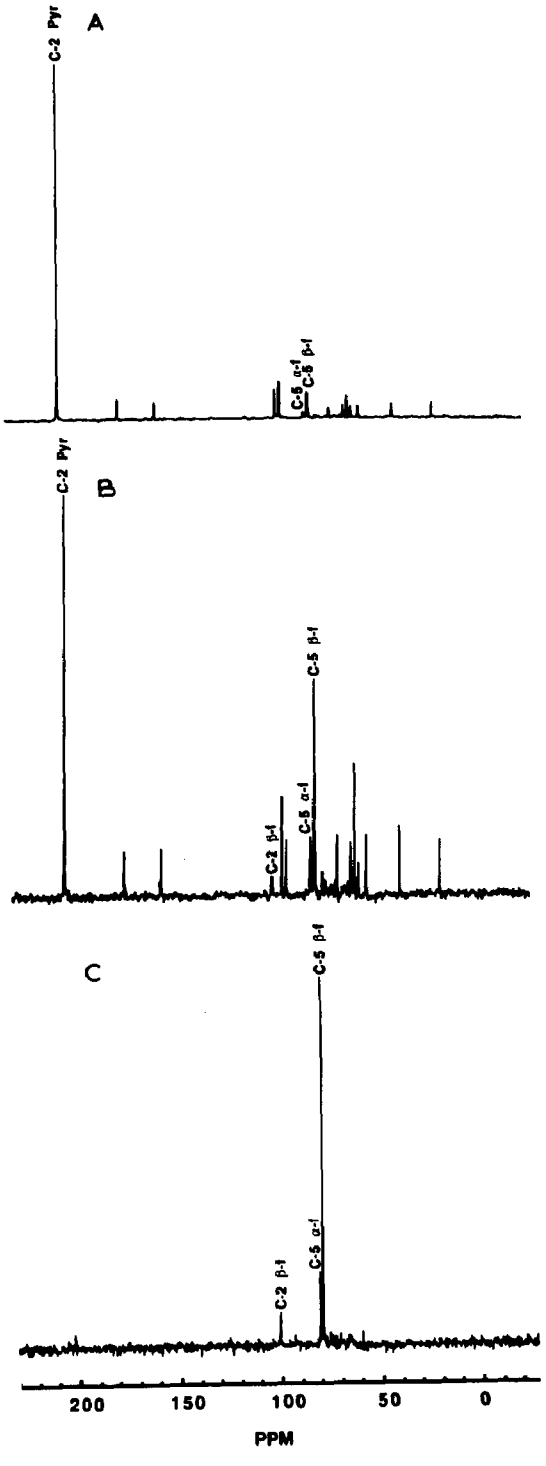

D-glucose and D-gluconate (unsubstituted peaks between about 60 and 80 p.p.m. and near the pyruvate hydrate resonance at about 93 and 97 p.p.m.) (B) Spectrum of chromatographically purified D-(2,5-$^{13}C_2$) fructose. Resonances arising from $^{13}C$-substituted carbons may be assigned to the *β*-pyranose (*β-p*) and the furanose (*α-f*, *β-f*) forms of the product. (2-$^{13}C$)Pyruvate is present as a contaminant. (C) Reaction for the preparation of D-(3,4-$^{13}C_2$)Fru-1,6-bisP from L-(1-$^{13}C$)alanine. Resonances from nonsubstituted carbons not appearing in spectrum A may be assigned to the carbons of α-ketoglutarate (α-KG). (D) Reaction mixture for the preparation of D-(2-$^{13}C$)fructose 1-phosphate from (2-$^{13}C$)pyruvate and D-glyceraldehyde.

the $^{13}C$-n.m.r. spectrum of a reaction mixture made up in a manner similar to that shown in Fig. 1A, except that a two-fold excess of D-glyceraldehyde was initially added to the reaction mixture. The only resonances arising from $^{13}C$-substituted substrates appearing in the spectrum can be readily assigned to the C-2 of (2-$^{13}C$)pyruvate and its hydrate (26% by peak integration) and the C-2 of the $\beta$-pyranose (99.1 p.p.m.; 57%) and $\alpha$-(105.6 p.p.m.; 4%) and $\beta$-furanose (102.0 p.p.m.; 13%) forms of D-fructose 1-phosphate. The absence of a significant equilibrium concentration of DHAP, as evidenced by the absence of a C-2 DHAP resonance as appears in Fig. 1A at 212.2 p.p.m., can be accounted for by the large molar excess of D-glyceraldehyde. Assuming a free-energy change at least as large as the aldolase-catalyzed reaction using D-glyceraldehyde 3-phosphate as a second substrate[37], an equilibrium concentration of only 4$\mu$M is calculated, making the detection of the DHAP C-2 resonance unlikely within the signal-to-noise limitations shown in the spectrum.

In principle, D-fructose 1,6-bisphosphate $^{13}C$-substituted at one of C-4, C-5, or C-6 may be prepared by adding excess unsubstituted DHAP and eliminating TPI from the Scheme 2 reaction mixture. In practice, sufficient TPI activity is present in commercial preparations of some enzymes added to the reaction mixture that $^{13}C$-substituted GAP is converted to substituted DHAP at a rate greater than the competing aldol condensation with unsubstituted DHAP. The product D-Fru-1,6-bisP is then symmetrically substituted with an abundance of $^{13}C$ diluted in proportion to the excess of unsubstituted DHAP initially added. The most significant TPI activity was found to occur in commercial preparations of creatine phosphokinase and yeast enolase, with TPI activity in the latter being nearly 2.5 times the enolase activity. Accordingly, both enzymes were treated with chloroacetol phosphate, an inhibitor previously found to act specifically and irreversible on TPI[20,21]. Following chloroacetol phosphate treatment, it was assessed that TPI activity in creatine phosphokinase and enolase was reduced 80-and 10 000-fold, respectively, while CPK and enolase activities were unaffected.

Figs. 2A and 2B show $^{13}C$-n.m.r. spectra of a modified Scheme 2 reaction mixture containing one equiv. of unsubstituted DHAP and chloroacetol phosphate-treated enolase and phosphocreatine kinase. As was the case for the reaction mixture in Fig. 1A, (2-$^{13}C$)pyruvate was used as the $^{13}C$-substituted substrate. Far from equilibrium (Fig. 2A), $^{13}C$-substituted carbons give rise to resonances which can be assigned to the C-2 of pyruvate and its hydrate and the C-5 of the furanose anomers of D-Fru-1,6-bisP. As equilibrium is approached, the rate of condensation between unsubstituted DHAP and $^{13}C$-substituted D-GAP slows and isomerization of D-GAP to DHAP becomes a competing reaction. This latter step takes place in spite of chloroacetol phosphate inhibition of TPI in the enolase and creatine phosphokinase preparations, as evidenced by small

Fig. 2. Proton-decoupled $^{13}C$-n.m.r. spectra (50 MHz) of (A) the reaction mixture used in the preparation of D-(5-$^{13}C$)Fru-1,6-bisP from (2-$^{13}C$)pyruvate and DHAP. The reaction mixture had been allowed to react for 14 h at room temperature. (B) Reaction mixture after 19 h. (C) Product isolated from the reaction mixture following a 19-h incubation. About 6% of the product was estimated to be D-(2,5-$^{13}C_2$)Fru-1,6-bisP and 95% D-(5-$^{13}C$)Fru-1,6-bisP.


A
C-2 Pyr
C-5 α-1
C-5 β-1
B
C-2 Pyr
C-2 β-1
C-5 α-1
C-5 β-1
C
C-2 β-1
C-5 α-1
C-5 β-1
200
150
100
50
0
PPM

C-2 resonances of the two furanose forms of D-Fru-1,6-bisP appearing in the reaction mixture as equilibrium is approached (Fig. 2B). With the appearance of the C-2 resonance, the reaction was stopped, and the product D-Fru-1,6-bisP was purified in the usual manner (55% overall yield from (2-$^{13}$c) pyruvate). $^{13}$C-N.m.r. peak integration showed the isolated material to be approximately 95% D-(5-$^{13}$C)Fru-1,6-bisP and 5% D-(2,5-$^{13}C_2$)Fru-1,6-bisP (Fig. 2C).

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