HIGH YIELD SYNTHESIS OF ^{14}C LABELLED INTERMEDIATES OF THE L-TYPE PENTOSE PATHWAY: OCTULOSE MONO- AND BISPHOSPHATES, SEDOHEPTULOSE 1,7-BISPHOSPHATE AND D-ARABINOSE 5-PHOSPHATE

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SUMMARY

Methods for the enzymic synthesis, isolation, purification and analysis of the ^{14}C labelled intermediates that are characteristic of the L-type pentose phosphate pathway are described. These are D-glycero D-ido octulose 1,8-bisphosphate and 8-monophosphate; D-glycero D-altro octulose mono- and bisphosphates; sedoheptulose 1,7-bisphosphate and D-arabinose 5-phosphate. The procedures for the preparation of octulose- and sedoheptulose bisphosphates are based on the aldolase-catalyzed condensation of dihydroxyacetone phosphate with appropriately ^{14}C-labelled aldo pentose 5-phosphates and erythrose 4-phosphate respectively. D-Glycero D-ido octulose 8-phosphate and its altro epimer were prepared in transketolase catalyzed reactions involving β-hydroxypyruvate and appropriately ^{14}C-labelled aldohexose 6-phosphates. [U-^{14}C] D-arabinose 5-phosphate was synthesized by reacting [U-^{14}C] glucosamine 6-phosphate with ninhydrin. The authenticity of each of the ^{14}C-labelled sugar phosphates was confirmed using a variety of chromatographic methods, enzymatic analytic methods and NMR spectroscopy.

The ^{14}C labelled compounds are used in investigations involving the search for the identity of the pentose pathway in tissues in vitro, for the measurement of L-type pathway enzyme reactions and for testing the mechanistic predictions of the L-type pathway in vitro.

Key Words: Octulose mono- and bisphosphates; sedoheptulose 1,7-bisphosphate; arabinose 5-phosphate; allose 6-phosphate; erythrose 4-phosphate; β-hydroxypyruvate; glucosamine 6-phosphate; Liver-type pentose pathway reactions; aldolase; transketolase.

ABBREVIATIONS

PP, pentose pathway; P, -phosphate; D-g D-i Oct, D-glycero D-ido octulose; D-g D-a oct, D-glycero D-altro Octulose; Seh, sedoheptulose (altro-heptulose); Ara, arabinose; Rib, ribose; Ery, erythrose; Glc, glucose; Fru, fructose; Xlu, xylulose; Rub, ribulose; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; Butyl-PBD, 2-(4'-tert.-butylphenyl) -5-(4"-biphenylyl)-1,3,4-oxadiazole. All sugars and sugar phosphates mentioned in this paper have the D-configuration.

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INTRODUCTION

There are at least two forms of the pentose pathway of glucose metabolism in nature. They are called the F-type (for fat cell) and L-type (for liver cell) pathways (1). The F-type pathway has featured in textbooks of general biochemistry since 1956 and it was unequivocally shown by a mechanistic and quantitative study to be present in adipose tissue (2). The L-type pathway was first described in 1971 (3), its reaction sequence was demonstrated using liver enzymes in vitro (4) and it was quantitatively measured in rat hepatocytes, 22% (5); in hepatocytes of regenerating liver, 12% (6); in perfused rat heart, 80% (7) and in Morris hepatoma 5123TC cells, 30% (8). The L-type PP features reactions of D-g D-i Oct 1,8-P_2, D-g D-i oct 8-P, D-g D-a Oct 1,8-P_2, D-g D-a Oct 8-P, Seh 1,7-P_2 and Ara 5-P. These compounds all occur as intermediates during the conversion in vitro of ribose 5-P to hexose and triose phosphates by enzyme preparations of liver, heart, a variety of Morris hepatomas, chlorella and spinach chloroplasts. The L-type PP has no role for transaldolase (EC2.2.1.2) an enzyme of the F-type PP, but is dependent on all other enzymes of the F-PP and reactions catalyzed by aldolase (EC4.1.2.13) (9), a new phosphotransferase enzyme (10), and an enzyme which interconverts D-ribulose 5-P and Ara 5-P which may be the same as D-arabinose 5-phosphate 3'-ketol isomerase (EC5.3.1.3). These three enzyme activities are characteristic of the L-type PP and catalyze reactions involving octulose phosphates and Seh 1,7-P_2 and Ara 5-P. In 1978 the octulose phosphates, Seh 1,7-P_2 were isolated, characterized and measured in fresh rat liver for the first time (11).

The structures of the octulose phosphates and of Seh 1,7-P_2 were determined using ^{13}C-NMR spectroscopy (12). The intermediacy of octulose phosphates and Seh 1,7-P_2 in the pentose pathway was recently demonstrated using liver enzyme catalyzed reactions <u>in vitro</u> (13,14).

Investigations of the presence, enzymatic activity and mechanism of the L-type pathway reactions in extracts of animal tissue preparations, plants, microorganisms and neoplasms etc. has necessitated the development of methods for the synthesis and analysis of these radioisotopically labelled compounds and to that end the following procedures are described.

EXPERIMENTAL

Materials

All enzymes with the exception of yeast transketolase which was obtained from Sigma Chemical Co. St. Louis, Missouri USA, were purchased from Boehringer Mannheim Corp., FRG. All sugar phosphates and coenzymes were obtained from the Sigma Chemical Co. St Louis. Ion-exchange resins were from Bio-Rad, Richmond, California, USA. All other chemicals and solvents were of analytical grade and were purchased from either Ajax Chemicals, Sydney, Australia or from E. Merk, Darmstadt, FRG or from BDH, Poole, Dorset, UK. Radioactive substrates were obtained from either **Amersham International plc** Amersham, Bucks., UK or from New England Nuclear, Boston, Mass., USA.

Colorimetric reactions of the ketulose phosphates

The octulose mono- and bisphosphates and Seh 1,7-P_2 were measured using the unique absorption spectra of these compounds when they were reacted with the cysteine-sulphuric acid reagent (15)(16)(17). The spectra of the products of these reactions were recorded using a Gilford 2600 single beam spectrophotometer coupled with a Hewlett Packard 7225A plotter. The orcinol reaction for pentose phosphates (18) and the anthrone reactions for hexose phosphates (19) were carried out as described. Inorganic phosphate was measured by the method of (20).

Radioactivity measurements

Radioactivity measurements were made in an automatic liquid scintillation counter (Beckman LS350). Analysis of the radioactivity in the fractions collected during column chromatography was achieved by counting 0.01ml of a sample into 10.0ml of scintillation fluid (composed of 12.0g Butyl PBD, 1200ml Toluene and 800ml Methoxyethanol). All radioactivity measurements (except those concerned with establishing column profiles of chromatographically eluted substances) were conducted at least three times on duplicate samples and were corrected for background and quenching.

Synthesis of ^{14}C-Octulose bisphosphates

[4,5,6,7,8-^{14}C] D-*g* D-*i* Oct 1,8-P_2

Isotopically labelled D-*g* D-*i* Oct 1,8-P_2 was synthesized using DHAP and [U-^{14}C] Ara 5-P in the aldolase-catalyzed condensation reaction of equation (1).

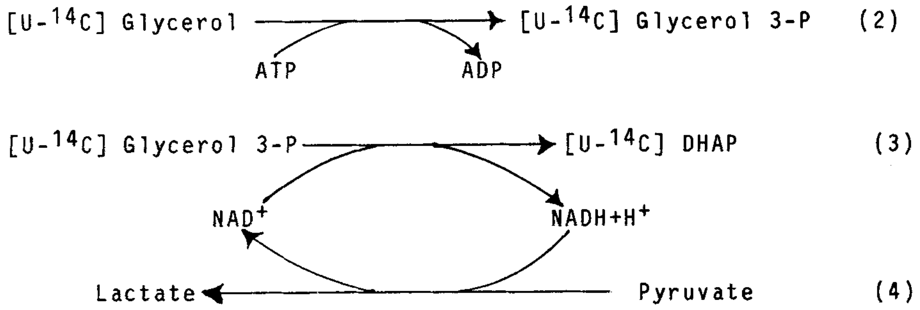
$$[U\text{-}^{14}C]\ \text{Ara 5-P} + \text{DHAP} \rightleftharpoons [4,5,6,7,8\text{-}^{14}C]\ \text{D-}\underline{g}\ \text{D-}\underline{i}\ \text{Oct } 1,8\text{-}P_2 \qquad (1)$$

[U-^{14}C] Ara 5-P was synthesized as described below. Fru 1,6-P_2 was used as a source of triose phosphate in aldolase-mediated syntheses (21,22), but the yields were quite low unless triosephosphate isomerase (EC5.3.1.1) was included in the reaction mixture. This enzyme acted to maintain a low concentration of GAP, thus directing the condensation of DHAP with acceptor aldehydes such as pentose 5-phosphates. The reaction mixture in a final volume of 10.0ml and pH7.6 contained: Tris-HCl, 500μmol; [U-^{14}C] Ara 5-P, 800μmol (specific radioactivity 0.5Ci mol^{-1}); Fru 1,6-P_2, 25μmol (equivalent to 50μmol of triose-P); aldolase, 45U; and triosephosphate isomerase, 500U. The reaction mixture was incubated at 25° for 16-18h. The incubation was terminated by ultrafiltration to remove protein using an Amicon PM-10 membrane (molecular weight cut-off, 10,000). The filtrate was applied to a column (1.5 x 90cm) of anion-exchange resin in the formate form (AG-1 X 8; 200-400 mesh) followed by 150ml of deionized water.

Sugar phosphates were eluted from the column using a linear formate gradient (0.75ml min^{-1}) generated with the aid of an LKB microperspex peristaltic pump and consisting of 1.8L of H_2O in a mixing bottle and 1.8L of 4M HCOOH - M $HCOONH_4$. Fractions (7.5ml) were collected and each second fraction was tested using the appropriate colour reagents for sugar phosphates or monitored for radioactivity, Ara 5-P was located in the fractions using the orcinol reagent for pentoses and octulose bisphosphates using the cysteine-H_2SO_4 reagent. Fractions containing D-g D-i Oct 1,8-P_2 were pooled together and passed through a column (80ml) of Dowex 50W (H^+) resin (100-200 mesh) together with 1.5 bed-volumes of deionized water. The eluate was concentrated to 50ml at 40° under reduced pressure using a rotary evaporator (Buchi Rotovaper R), then continuously extracted with diethylether for 12-14h to remove formic acid, neutralized with NaOH to pH6.8, and lyophilized. The recoveries of sugar phosphates during the ether extraction were 75-95%, depending upon the sugar being processed. The yield of the Oct 1,8-P_2 was 60-65% (based on Fru 1,6-P_2) with a specific radioactivity of 0.35 Ci mol^{-1}. The product was free from Fru 1,6-P_2 as measured by specific enzymatic analysis (23). Analysis of the total and organic phosphorus of the D-g D-i Oct 1,8-P_2 showed that approx. 2% was not accounted for by the analysis on DHAP and Ara 5-P released when it was cleaved in the aldolase assay (15). The identity and purity of the product was further confirmed using mass spectrometry, gas liquid chromatography of the TMS derivatives of the dephosphorylated sugar (24) and natural abundance ^{13}C-NMR.

When Oct 1,8-P_2 labelled in the first three carbon atoms was required, ^{14}C-DHAP (either [U-^{14}C or 2-^{14}C]-labelled) were used as substrates. ^{14}C-Labelled DHAP was prepared *in situ* using

$[U\text{-}^{14}C$ or $2\text{-}^{14}C]$ glycerol according to the reactions of equations 2-5.



$$[U\text{-}^{14}C]\ \text{DHAP} + \text{Ara 5-P} \rightleftharpoons [1,2,3\text{-}^{14}C]\ \text{D-}\underline{g}\ \text{D-}\underline{i}\ \text{Oct 1,8-P}_2 \quad (5)$$

The enzymes catalysing the above reactions are glycerol kinase (EC2.7.1.30) (equation 2), glycerol 3-phosphate dehydrogenase (EC1.1.1.8) (equation 3), lactate dehydrogenase (EC1.1.1.27) (equation 4) and aldolase (equation 5). The glycerol 3-phosphate dehydrogenase-catalyzed reaction has an equilibrium constant of 10^{-12} in the direction of DHAP formation (25). Thus the coupling of lactate dehydrogenase (equation 3) via NAD^+ reduction-NADH oxidation was necessary to obtain substantial conversion of glycerol 3-P to DHAP. For this synthesis, the incubation mixture contained in a total volume of 20ml (pH7.6): $[U\text{-}^{14}C]$ glycerol, 7.8μmol (total of 250μCi, specific radioactivity 32Ci mol^{-1}); Ara 5-P, 180μmol; sodium pyruvate, 200μmol; ATP, 100μmol; NAD^+, 200μmol; glycerol kinase, 85U; glycerol 3-phosphate dehydrogenase, 40U; lactate dehydrogenase, 90U; aldolase, 12U; Tris-HCl, 2.0mmol; and $MgCl_2$, 400μmol. The mixture was incubated at 25° for 20h. $[1,2,3\text{-}^{14}C]$ D-g D-i Oct 1,8-P_2 was separated from the reactants as described above. The Oct 1,8-P_2 thus recovered represented 50% of the original ^{14}C-isotope added to the incubation mixture as $[U\text{-}^{14}C]$ glycerol.

$[^{14}C]$ D-g D-a Oct 1,8-P_2 was synthesized in essentially the same manner as the *ido*-epimer, except that Rib 5-P replaced Ara 5-P (see equation 6):

[U-^{14}C] Rib 5-P + DHAP $\rightleftharpoons$ [4,5,6,7,8-^{14}C] D-*g* D-*a* Oct 1,8-P_2 (6)

[U-^{14}C] Rib 5-P was first prepared from [U-^{14}C] glucose (250µCi, specific radioactivity 230Ci mol^{-1}) as described by (4). The yield of the reaction was 95% (the specific radioactivity of the product varied between 0.52 and 0.65Ci mol^{-1}). The incubation mixture for D-*g* D-*a* Oct 1,8-P_2 synthesis contained in a final volume of 20.0 ml (pH7.6): [U-^{14}C] Rib 5-P, 2mmol; Fru 1,6-P_2, 75µmol; aldolase, 45U; triose phosphate isomerase, 500U; and Tris-HCl, 1mmol. The reaction was carried out at 25° for 3h by which time 90% conversion of the reactants to D-*g* D-*a* Oct 1,8-P_2 had occurred as indicated by the spectra of the cysteine-H_2SO_4 reaction product. Rib 5-P was previously shown to be a better substrate for rabbit muscle aldolase than Ara 5-P (15), hence the synthesis of the *altro*- epimer only required a short incubation period (3h). After deproteinization using the ultrafiltration procedure, the mixture was eluted from a formate anion exchange column (1.5 x 90cm), using a formate linear gradient as described above. The further processing, purification and analysis of the compound was the same as that described for the *ido*-epimer. The yield of the D-*g* D-*a* Oct 1,8-P_2 was 80%-90% based on Fru 1,6-P_2. The product had a specific radioactivity of approximately 0.54Ci mol^{-1}.

Synthesis of ^{14}C-Labelled Octulose Monophosphates: [8-^{14}C] D-*g* D-*i* Oct 8-P

Isotopically labelled D-*g* D-*i* Oct 8-P and its *altro* epimer were synthesized via transketolase (EC2.2.1.1) catalyzed reactions which involved coupling carbons 2 and 3 of β-hydroxypyruvate with the appropriately ^{14}C labelled aldohexose 6-phosphates (see eqn. 7 for the synthesis of [8-^{14}C] D-*g* D-*i* Oct 8-P).

[6-^{14}C] Glc 6-P + β-hydroxypyruvate $\rightarrow$ HCO_3^- + [8-^{14}C] D-*g* D-*i* Oct 8-P (7)

The [6-^{14}C] glc 6-P was prepared from [6-^{14}C] glucose (80μmol, total ^{14}C radioactivity 50μCi, specific radioactivity 5.9 Ci mol^{-1}), ATP (400μ mol) and hexokinase (35U) in the presence of 0.4M TEA-HCl buffer containing 20mM $MgCl_2$. [6-^{14}C]Glc 6-P (95-97% yield) with specific radioactivity 0.62Ci mol^{-1} was separated from [6-^{14}C] glucose by formate anion exchange chromatography as described above.

The incubation mixture for the synthesis of [8-^{14}C] D-g D-i Oct 8-P contained in a total volume of 15ml at pH7.6: [6-^{14}C]Glc 6-P, 80μmol; β-hydroxypyruvate, 240μmol; transketolase (from yeast), 20U; TEA-HCl (pH7.4), 1.2mmol; $MgCl_2$, 150μmol and thiamine pyrophosphate, 1.5μmol. The reaction was carried out at 30° for 24h.

It was then necessary to adopt the following strategy to remove any unreacted Glc 6-P from the 24h reaction mixture before proceeding with the ion-exchange chromatographic separation of octulose monophosphate. The reaction mixture was placed in a boiling water bath for 5 min, cooled, ultra-filtered and the filtrate incubated at 30° for 2h with a 2-mol excess of ATP over the amount of unreacted Glc 6-P, 20U of glucosephosphate isomerase (EC5.3.1.9) and 10U of 6-phosphofructokinase (EC2.7.1.11). Protein was removed by ultrafiltration and [8-^{14}C] D-g D-i Oct 8-P was isolated, free from other contaminants, by formate ion-exchange chromatography. The yield of [8-^{14}C] D-g D-i Oct 8-P (specific radioactivity 0.4Ci mol^{-1}) was 60% based on the ^{14}C radioactivity initially applied as [6-^{14}C]Glc 6-P. The remaining ^{14}C was recovered in Fru 1,6-P_2 (20%) and in dephosphorylated products (20%). Spinach and rat liver transketolase were also used for the synthesis of [8-^{14}C] D-g D-i Oct 8-P and afforded good yields and required shorter reaction periods (26). However the preparation of these enzymes was time consuming and significant losses of transketolase activity using enzyme from these sources, was experienced upon prolonged (1 month) storage. An alternative method for the preparation of octulose monophosphates involved selective acid hydrolysis of the octulose 1,8-bisphosphates (27). However, the method was capricious and the yields were frequently low (20%-40%).

[8-^{14}C] D-g D-a Oct 8-P was synthesized by reacting [6-^{14}C] allose 6-P (100µmol) and β-hydroxypyruvate (300µmol) with transketolase (20U), as described for the synthesis of the *ido*-epimer, except that the incubation only required 3h. The deproteinized mixture was eluted from a column of formate anion exchange resin, using a linear gradient of formic acid-ammonium formate as described for the synthesis of octulose bisphosphates, to give D-*g* D-*a* Oct 8-P in 80% yield. In contrast to the synthesis kinetics of D-*g* D-*i* Oct 8-P, the rate of D-*g* D-*a* Oct 8-P formation was rapid, presumably because allose 6-P is a better analogue of Rib 5-P (the most active aldose 5-P for transketolase) than Glc 6-P. This method was efficient, in terms of both yield and purity, to that involving the acid hydrolysis of D-*g* D-*a* Oct 1,8-P_2.

[6-^{14}C] Allose 6-P was synthesized (28) from [5-^{14}C]Rib 5-P, which in turn was prepared from [6-^{14}C] glucose (29). Condensation of the [5-^{14}C]Rib 5-P with cyanide yielded a mixture of epimeric hexonitrile phosphates which were catalytically hydrogenated without prior purification. The resulting mixture of allose and altrose 6-phosphates were separated on a column (2.6 x 100 cm) of DEAE-Sephadex A-25, using a 0.05 to 0.4M linear gradient (3.5L) of ammonium tetraborate (pH8.4). The individual sugar phosphates were then passed through a column (2.6 x 100 cm) of Sephadex G-10 in order to remove residual salts. The anthrone reaction (19) indicated a 20% yield of pure allose 6-P (30).

Enzymatic analysis of octulose phosphates

(a) Octulose bisphosphates were determined by measuring the DHAP liberated by the aldolase catalyzed reactions of equations (1 and 6). The reaction mixture at 30° and in a total volume of 1.0ml contained: 100μmol TEA-HCl buffer (pH7.6), 0.16μmol NADH, 1.7U glycerol 3-phosphate dehydrogenase, 1.8U rabbit muscle aldolase, 0.04-0.1μmol Oct 1,8-P_2. 9 Units of aldolase were used for the assays of the _ido_-epimer, to ensure rapid completion of the reaction.

(b) Octulose 8-phosphates were assayed by measuring the amount of Fru 6-P and Glc 6-P formed in a transketolase-mediated reaction with Ery 4-P as the aldehyde acceptor (equation 8). The reaction mixture in a total volume of 1.0ml and at 30° contained: 80μmol TEA-HCl buffer (pH7.6), 0.3μmol $NADP^+$, 0.3μmol Ery 4-P, 10μmol $MgCl_2$, 0.1μmol thiamine pyrophosphate, 0.5U yeast transketolase, 1.7U glucosephosphate isomerase, 1.4U glucose 6-phosphate dehydrogenase (EC1.1.1.49) and 0.02-0.05μmol D-_g_ D-_i_ Oct 8-P.

$$\text{D-}\underline{g}\text{ D-}\underline{i}\text{ Oct 8-P} + \text{Ery 4-P} \rightleftharpoons \text{Glc 6-P} + \text{Fru 6-P} \qquad (8)$$

Synthesis of ^{14}C Sedoheptulose 1,7-P_2

[1,2,3-^{14}C] Seh 1,7-P_2 was prepared from [U-^{14}C] DHAP and Ery 4-P in the aldolase-catalyzed reaction of equation 9.

$$[\text{U-}^{14}\text{C}]\text{DHAP} + \text{Ery 4-P} \rightleftharpoons [1,2,3\text{-}^{14}\text{C}]\text{Seh 1,7-P}_2 \qquad (9)$$

When [4,5,6,7-^{14}C] Seh 1,7-P_2 was required, [U-^{14}C]Ery 4-P was used, which in turn was prepared from [U-^{14}C]Glc 6-P by the method of (31). The reaction mixture for [1,2,3-^{14}C]Seh 1,7-P_2 synthesis was contained in a total volume of 15.0ml at 25° and consisted of: [U-^{14}C]DHAP (prepared from [U-^{14}C] glycerol _in situ_ as described for the synthesis of [1,2,3-^{14}C] D-_g_ D-_i_ Oct 1,8-P_2), 40μmol; Ery 4-P, 40μmol; TEA-HCl, 2.0mmol, pH7.6; aldolase, 20U. The incubation was carried out for 1h and the reaction stopped by ultrafiltration as described above. The deproteinised mixture was loaded onto a formate anion exchange column (1.5 x 90cm) and Seh 1,7-P_2 was eluted using a linear gradient of formic acid-ammonium formate as described for the isolation of octulose phosphates. The yield of Seh 1,7-P_2 was greater than 90% and the product was free from sugar monophosphates. When ^{14}C-Ery 4-P was used, then unlabelled DHAP was replaced by Fru 1,6-P_2 (as the triose-P source). Alternatively, Seh 1,7-P_2 was prepared by phosphorylation of Seh 7-P with [1-^{32}P]ATP, catalyzed by rabbit muscle 6-phosphofructokinase followed by isolation and purification of the product from other reactants by formate anion-exchange chromatography. The synthesis of [1-^{32}P]Seh 1,7-P_2 from Ery 4-P and [1-^{32}P]Fru 1,6-P_2 was described by (32).

Synthesis of [U-^{14}C] arabinose 5-P

[U-^{14}C]Ara 5-P (0.5Ci mol^{-1}) was prepared from [U-^{14}C] glucosamine by the following variant of the method of (33). [U-^{14}C]glucosamine 6-P was first prepared from [U-^{14}C]glucosamine as follows: the reaction mixture contained in a total volume of 2.0ml at 30°: [U-^{14}C]glucosamine-HCl, 0.21μmol (50μCi, specific radioactivity 239Ci mol^{-1}); glycylglycine-KOH buffer (pH7.4), 0.25mmol; ATP, 2.0μmol; $MgCl_2$, 4.0μmol; and hexokinase (EC2.7.1.1), 10U. After 30 min the entire reaction mixture was applied to 3MM Whatman chromatography paper (40 x 40cm) and chromatographed in solvent GW_3 (34). The [U-^{14}C]glucosamine 6-P was located on the chromatogram by scanning the paper for radioactivity using a radiochromatogram scanner (Packard Model 7201) and 99% of the ^{14}C was found to be coincident with authentic glucosamine 6-P. The [U-^{14}C]glucosamine 6-P, following elution from the paper, was added to 100μmol of unlabelled glucosamine 6-P in a total volume of 30.0ml together with ninhydrin (500μmol), citrate buffer, pH4.7 (0.75mmol), and heated at 100° for 40 min. The reaction mixture was cooled to 0°, filtered through a Whatman No 1 filter and then applied to a formate anion exchange column (1.5 x 90cm). [U-^{14}C]Ara 5-P was isolated using a linear gradient of 4M formic acid-1M ammonium formate (4L) as described above. The yield of the final product was 80%-90% based on the original reactants. A small peak (less than 1%) of the total orcinol positive material was consistently observed before the Ara 5-P peak and was identified as Rib 5-P. Care was exercised to keep Rib 5-P from contaminating the Ara 5-P product. Bulk preparations of [U-^{14}C]Ara 5-P, isolated using larger volumes on ion-exchange resin and eluting solvent, was shown to be free of Rib 5-P, Xlu 5-P and Rub 5-P by specific enzymatic analysis (35), by NMR spectroscopy and by gas liquid chromatographic analysis of the dephosphorylated derivatized product (24).

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