An Enzymatic Method for the Preparation in High Yield of [5-^{14}C]- and [4,5,6-^{14}C] Glucose.

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Summary

[5-^{14}C]- and [4,5,6-^{14}C] glucose were prepared from [2-^{14}C]- and [U-^{14}C] glycerol and fructose 6-phosphate by reactions involving the enzymes glycerokinase, glycerol 3-phosphate dehydrogenase, triose phosphate isomerase, transaldolase, lactate dehydrogenase and phosphoglucose isomerase. The authenticity of each of the ^{14}C-labelled sugars was verified by chromatographic and enzymic procedures and the distribution of ^{14}C isotope confirmed by specific degradative procedures. These specifically labelled substrates are required for the quantitative estimation of the new pentose pathway reactions in liver, photosynthetic and some tumour tissues.

Key Words

[5-^{14}C] glucose, transaldolase exchange; quantitation; pentose cycle; liver; tumours.

Abbreviations

ATP, adenosine 5-triphosphate; NAD^+, nicotinamide adenine dinucleotide; NADH nicotinamide adenine dinucleotide, reduced form; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate.

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Introduction

Transaldolase is a group transferring enzyme that is found in many tissues and which has a broad specificity for its donor and acceptor substrates (1). Ljungdahl et al (2) used transaldolase and demonstrated the feasability of the formation in low yield, of [^{14}C]fructose 6-phosphate from [^{14}C]glycerol by the following series of reactions:

$$[2\text{-}^{14}C]\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} [2\text{-}^{14}C]\ \alpha\text{-phosphoglycerol} + \text{ADP} \quad (1)$$

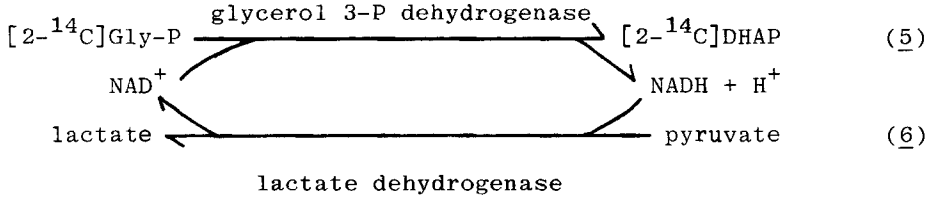
$$[2\text{-}^{14}C]\ \alpha\text{-phosphoglycerol} + NAD^{+} \xrightleftharpoons{\text{glycerol 3-P dehydrogenase}} [2\text{-}^{14}C]\text{DHAP} + \text{NADH} + H^{+} \quad (2)$$

$$[2\text{-}^{14}C]\text{DHAP} \xrightleftharpoons{\text{triosephosphate isomerase}} [2\text{-}^{14}C]\text{GAP} \quad (3)$$

$$[2\text{-}^{14}C]\text{GAP} + \text{F6P} \xrightleftharpoons{\text{transaldolase}} [5\text{-}^{14}C]\text{F6P} + \text{GAP} \quad (4)$$

Recent studies in this laboratory concerned with the reaction scheme of the non-oxidative pentose phosphate pathway in liver have led to the demonstration of a new reaction sequence for this pathway of glucose metabolism (3,4) and theoretical methods for measuring the quantitative contribution of this alternate form of the pentose phosphate pathway have been developed (5). It is mandatory for the application of these theoretical methods that [5-^{14}C]- and [4,5,6-^{14}C] glucose are used as substrates for liver and other gluconeogenic tissues. As neither of these ^{14}C-labelled substrates are commercially available, we have adapted the reaction sequence shown above (Reactions 1-4) to optimize the yield of [^{14}C]hexose 6-phosphate.

In terms of maximizing the yield of [^{14}C]fructose 6-phosphate formed by the reactions 1 to 4 above, two major limitations need to be considered. The transaldolase reaction (Reaction 4) is not a 'mass transfer' reaction, rather it is a reaction involving the exchange of carbon atoms 4,5 and 6 of fructose 6-phosphate with glyceraldehyde 3-phosphate. The reactants and products maintain the same concentration throughout the time course of the reaction sequence. The position of equilibrium thus sets a limit on the yield of ^{14}C isotope from [^{14}C]-glyceraldehyde 3-phosphate which can be incorporated into fructose 6-phosphate. This is so because at isotopic equilibrium only half of the available [^{14}C]glyceraldehyde 3-phosphate can be incorporated into the lower 3 carbons of fructose 6-phosphate. The second limitation involves the unfavourable equilibrium of reaction 2. The K_{eq} of this reaction, in the direction of dihydroxyacetone phosphate formation, is 10^{-12} (6). Thus an NAD^+ regenerating system has been incorporated in the incubation mixture which involves using pyruvate and lactate dehydrogenase to force the reaction in the direction of dihydroxyacetone phosphate formation, this is illustrated by Reactions 5 and 6.



The use of the NAD^+ regenerating system increased the yield of ^{14}C isotope incorporation into hexose 6-phosphate by a factor of 3.

Experimental Section

Enzymic Synthesis of [5-^{14}C]- and [4,5,6-^{14}C] Glucose 6-Phosphate

The reaction mixture (5.0ml) contained: 100μmole ATP; 200μmole NAD^+; 150μmole fructose 6-phosphate; 1mg glycerokinase (E.C.2.7.1.30; Boehringer Mannheim Corp.; from Candida mycoderma; 850U/mg); 1mg glycerol 3-phosphate dehydrogenase (E.C.1.1.1.8; Boehringer Mannheim Corp.; from rabbit muscle; 40U/mg); 100μg triose-phosphate isomerase (E.C.5.3.1.1; Boehringer Mannheim Corp.; from rabbit muscle; 5000 U/mg); 1.3 mg transaldolase (E.C.2.2.1.2; Boehringer Mannheim Corp; from yeast; 15U/mg); 160μg lactate dehydrogenase (E.C.1.1.1.27; Boehringer Mannheim Corp.; from rabbit muscle; 550 U/mg); 200μmole sodium pyruvate and 250μCi of [2-^{14}C]- or [U-^{14}C] glycerol (25mCi/m mol). All substrates, co-factors and enzymes were made up in 100 mM Tris/HCl, 20mM $MgCl_2$ pH 7.8. The reaction mixture was incubated for 2 hrs. at 25° before the addition of 140 μg phosphoglucose isomerase (E.C.5.3.1.9; Boehringer Mannheim Corp; from yeast; 350U/mg). The incubation was continued for one further hour and was then terminated by the addition of 5.0 ml of 0.6M $HClO_4$. The mixture was allowed to stand in ice for 30 min to complete the precipitation of protein. The protein was removed by centrifugation at 10,000 r.p.m. for 10 min. in a Sorval refrigerated centrifuge using the SS-34 rotor. The supernatant fluid was adjusted to pH 6.5 with KOH solution and allowed to stand at 0° for 30 min. The $KClO_4$ precipitate was removed by centrifugation as above and the reaction mixture placed on a column (1.5 x 30cm) of anion-exchange resin in the formate form (Dowex-1; x8; 200–400 mesh, Bio-Rad Laboratories, California, U.S.A.) followed

by 50 ml of water. The column was eluted with a linear gradient containing 500 ml of 2M HCOOH in a mixing bottle and 4M HCOOH in the reservoir at a flow rate of 1ml/min. Fractions (10 ml) were collected and the hexose 6-P peak identified using the anthrone reagent (7) and the appropriate fractions were collected (total volume 6 x 10ml). The solution of ^{14}C-labelled hexose 6-phosphates was concentrated under reduced pressure at 45^{o} using a Buchi rotary evaporator (Buchi Rotovaper R). The evaporated residue was then washed 4 times each with 100 ml of distilled water to remove the last traces of formic acid. The glucose 6-phosphate was separated from fructose 6-phosphate by descending paper chromatography using the GW3 solvent of Wood (8). The chromatography was run for 30 hrs and the glucose 6-phosphate and fructose 6 phosphate were located by scanning the paper for radioactivity using a Radiochromatogram Scanner (Packard Model 7201) (see Fig. 1). The glucose 6-phosphate was recovered from the paper by elution with water and concentrated to 5.0ml under reduced pressure using a Buchi rotary evaporator.

Dephosphorylation of glucose 6-phosphate and purification of glucose

The glucose 6-phosphate solution was made up to a volume of 10ml in a solution which was 100mM Tris/HCl, 10mM $MgCl_2$ pH 10.4 and dephosphorylated by the addition of 20mg of alkaline phosphatase (E.C.3.1.3.1; Sigma chemicals; from calf intestine; 2U/mg). The dephosphorylation incubation was maintained at 37^{o} for 3 hrs and was terminated by the addition of 10ml of 0.6M $HClO_4$ and the precipitated protein removed

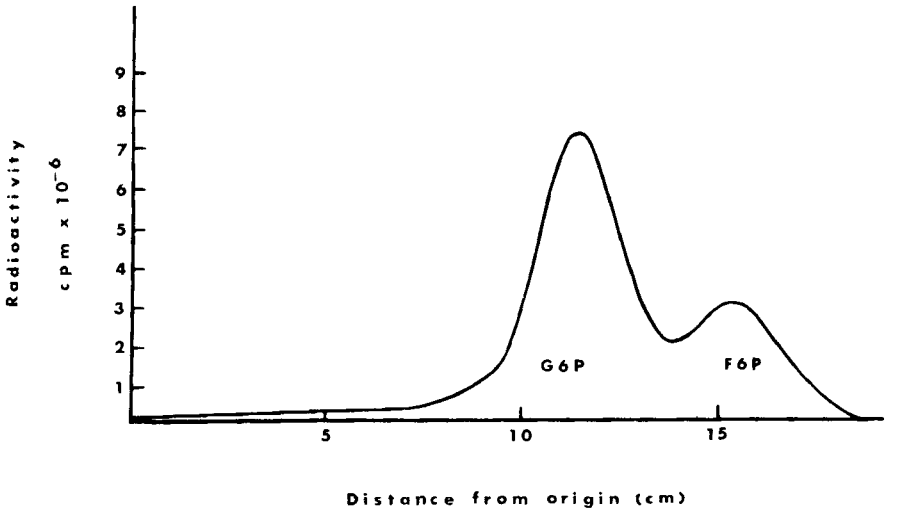


Fig. 1 The separation of glucose 6-P and fructose 6-P by paper chromatography.

The glucose 6 phosphate and fructose 6-phosphate were separated from one another after anion-exchange chromatography on Whatman 3mm chromatography paper in the GW_3 solvent described by Wood (8). The chromatography was run using the descending method for 30 hrs. The solvent composition was modified in that EDTA was omitted. The [^{14}C]glucose 6-phosphate and fructose 6-phosphate peaks shown above were located by scanning the paper for radioactivity using a Radiochromatogram Scanner (Packard Model 7201).

by centrifugation as above. The supernatant solution was de-ionized by passing through tandem columns (1.2 x 20 cm) of Dowex 50 (x4, H^+ form) and Dowex 1 (x8, acetate form). The columns were washed with 100 ml of water and the appropriate fractions were pooled and evaporated to near dryness in vacuo at 40° in a rotary film evaporator. The ^{14}C-labelled glucose solution was washed 4 times each with 100ml of distilled water to remove acetic acid and concentrated to a final volume of 2.0 ml. Enzymic analysis of the glucose solution indicated that it was free from fructose. The specific radioactivity of the glucose was approximately 1mCi/m mol and the yield was 60uCi.

Distribution of ^{14}C isotope in the carbon atoms of glucose

The glucose isolated by the procedures described above was biologically and chemically degraded carbon atom by carbon atom after the addition of 1m-mole of carrier D-glucose. The degradation procedures utilize Leuconostoc mesenteroides and appropriate chemical methods and have been described in detail elsewhere (9). An analytical control sample of glucose labelled with ^{14}C in positions 1,2 and 6 and of known isotopic composition was degraded simultaneously with the experimental samples.

The results of the degradations are shown in Table 1. When [2-^{14}C] glycerol was used, practically all of the ^{14}C was found in position 5 of glucose. The use of [U-^{14}C]-glycerol resulted in a nearly equal distribution of ^{14}C in carbon atoms 4,5 and 6 of glucose.

TABLE 1. Distribution of ^{14}C in the carbon atoms of glucose.

Carbon No.	Percentage of Radioactivity		
	[1,2,6-^{14}C]glucose	[5-^{14}C]glucose	[4,5,6-^{14}C]glucose
1	37.0	0	1.2
2	19.0	1.3	1.6
3	0	0.9	1.7
4	1.0	2.1	33.0
5	2.0	94.0	31.4
6	42.0	1.7	31.0
Recovery %	92	93	87

The distribution of ^{14}C isotope in the carbon atoms of glucose synthesized from [2-^{14}C] glycerol or [U-^{14}C] glycerol and fructose 6-phosphate was determined by biological and chemical degradation procedures (9). A standard [1,2,6-^{14}C]-glucose sample accompanied the degradations. The expected distribution of ^{14}C isotope in this standard glucose was C-1, 40%; C-2, 20%; C-3, 0%; C-4, 0%; C-5, 0%; C-6, 40%. The percentage recovery of label was determined by expressing the total label recovered (determined by the addition of the specific radioactivities of the six carbon atoms) as a fraction of the specific radioactivity obtained from the complete oxidation of the glucose by the method of Van Slyke and Folch (10).

Acknowledgements

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References

1. Clark,M.G., Williams,J.F., and Blackmore,P.F. -Cat. Rev.- Sci. Eng., 9:35 (1974).
2. Ljungdahl,L., Wood,H.G., Racker,E., and Couri,P. - J. Biol. Chem., 236: 1622 (1961).
3. Williams,J.F., Clark,M.G., and Blackmore,P.F. -Biochem. J. 176:241 (1978).
4. Williams,J.F., Blackmore,P.F. and Clark,M.G. -Biochem. J. 176: 257 (1978).
5. Longenecker,J.P., and Williams,J.F. manuscript submitted to the Biochemical Journal.
6. Bergmeyer,H.U. In Methods of Enzymatic Analysis, p. 468. Ed. By Bergmeyer,H.U., New York: Academic Press Inc. (1974).
7. Graham,D. and Smydzuk,J. -Anal. Biochem. 11: 246 (1965).
8. Wood,T. - J. Chromatog. 35: 352 (1968).
9. Williams,J.F., Rienits,K.G., Schofield,P.J. and Clark,M.G. -Biochem. J., 123: 923 (1971).
10. Van Slyke,D.D. and Folch,J. -J. Biol. Chem. 136: 509 (1940).