Journal of Labelled Compounds — January-March — Vol. V, No. 1

Synthesis of glucose-3 and -4-^{14}C of high specific activity and radiochemical purity

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Received on 15th July 1968

SUMMARY

A method for the enzymatic synthesis of hexoses-3-^{14}C and hexoses-4-^{14}C is presented. The procedure is based on the synthesis of 3-phosphoglyceric acid-1-^{14}C (3PGA) by the action of the ribulose diphosphate carboxylase in the presence of ribulose-1,5 diphosphate and $^{14}CO_2$, followed by the reversal of the glycolytic reactions leading from glucose to 3PGA. For the synthesis of hexoses-3-^{14}C, dihydroxyacetonephosphate-1-^{14}C and cold glyceraldehyde-3-P are condensed by the aldolase reaction, while for the synthesis of hexoses-4-^{14}C cold dihydroxyacetonephosphate is condensed with glyceraldehyde-3-P-1-^{14}C. The problems and the results of such syntheses are discussed.

INTRODUCTION

The aim of the present research was the synthesis of hexoses specifically labelled with ^{14}C in the 3 and 4 positions, and having high radiochemical purity and specific activity. Though glucose-3,4-^{14}C is commercially available, both glucose-3-^{14}C and glucose-4-^{14}C are not. Glucose-3,4-^{14}C has been biologically synthesized *in vivo* in rat liver [1, 2], and *in vitro* by the use of purified enzymes [3].

We have synthesized glucose 3 and 4 ^{14}C by a procedure based on the possibility to reverse the glycolytic reactions between glucose and 3-phosphoglyceric acid (3PGA). The first step was therefore the synthesis of 3PGA-1-^{14}C by the ribulose-1,5 diphosphate (RuDP) carboxylase reaction :

$$\text{Ru DP} + CO_2 \longrightarrow 2\ \text{3PGA}$$

In this reaction one molecule of RuDP in the presence of $^{14}CO_2$ yields two molecules of 3PGA, one of which is labeled in the carbon one position [4]. The specific activity of the 3PGA thus synthesized is therefore one half of that

of the bicarbonate used. As $NaH^{14}CO_3$ of 50-52 mC/mmole specific activity is now commercially available, it is possible to synthesize 3PGA-1-^{14}C having a specific activity of 25 mC/mmole.

Glucose labeled either in the carbon 3 or in the carbon 4 can be obtained from 3PGA-1-^{14}C by the use of the following enzymes : phosphoglycerilkinase, phosphoglyceraldehyde dehydrogenase, triosephosphate isomerase, aldolase, acidic phosphatase, hexokinase, phosphohexose isomerase and phosphatase.

To synthesize glucose 3 or 4 ^{14}C having a maximal radiochemical purity (which essentially means with no cross contamination between the carbons 3 and 4) the main problem was that of the scarce purity of some of the enzymes commercially available. The presence of triosephosphate isomerase in many commercial enzymes was the most injurious contamination, because it caused the mixing between the two triosephosphates and therefore the cross contamination of the carbons 3 and 4 of fructose diphosphate (FDP).

The commercial phosphoglycerilkinase was the most strongly contaminated enzyme and though this contamination was of no significance for the synthesis of the hexoses-3-^{14}C, it raised serious problems in the synthesis of hexoses-4-^{14}C. It was therefore necessary for us to further purify this enzyme to eliminate the triosephosphate isomerase activity. Moreover we interrupted the series of the reactions in such a way that, at the moment of the condensation of the triosephosphates to give FDP, only aldolase was present.

In such a way from 3PGA-1-^{14}C we have obtained hexoses-3-^{14}C of the same specific activity as the starting 3PGA, and of high radiochemical purity. The yield of hexoses-3-^{14}C was about 30 % with respect to the 3PGA used.

The synthesized hexoses-4-^{14}C also showed a high radiochemical purity and specific activity. Their yield however was quite low. The reason was that trace amounts of triosephosphate isomerase still present even in the "purified" 3PGA kinase caused some of the glyceraldehyde-3-P to be transformed into dihydroxyacetonephosphate (DAP), which had then to be removed from the reaction mixture.

Experimental

Glycolytic enzymes, acidic phosphatase were purchased from the Biochemia s.r.l.; ATP, NADP, NAD, NADH, glyceraldehyde -3-P, DAP, RuDP from the Sigma Chemical Co., and $NaH^{14}CO_3$ was purchased from the Radiochemical Centre.

The RuDP carboxylase was isolated and purified from spinach leaves according to Racker [5] and further purified by gel filtration according to Trown [6].

The glycolytic intermediates were determined enzymatically by standard spectrophotometric methods.

Labeled compounds were localized on the chromatograms with a Vanguard

radiochromatoscanner, and the radioactivity of the purified compounds was determined in a Packard Tricarb liquid scintillation counter.

(*a*) *Synthesis of 3PGA-1-^{14}C.*

The reaction mixture, having a final volume of 8 ml was contained in a Warburg flask and consisted of 300 μmoles of Tris-Cl pH 8.2, 64 μmoles of $MgCl_2$, 40 μmoles of RuDP, 36 μmoles of $NaH^{14}CO_3$ having a specific activity of 40 mC/mmole. The last addition was the purified RuDP carboxylase. The side arm of the flask contained 0.8 ml of perchloric acid (PCA) 100 % w/v. The sealed flask was left for 5 hours at room temperature. At that time 0.5 ml of 5 N NaOH were added to the central well, and the PCA was added to the reaction mixture. The flask was left for about two hours in an ice bath with stirring.

The mixture was then centrifuged and the supernatant neutralized with KOH. After the precipitated perchlorate had been washed twice with water, the solution was decationized on a Dowex 50 (H^+ form) column. The solution contained 56.4 μmoles of 3PGA : the yield of 3PGA was about 70 % with respect to the RuDP used.

Aliquots of the solution were chromatogrammed on No. 1 Whatman paper with phenol-water (4 : 1), methanol-formic acid-water (80 : 15 : 5) or with butanol-acetic acid-water (100 : 28 : 70). Irrespective of the solvent system used, only one radioactive spot was obtained.

The specific activity of the 3PGA was 18.45 mC/mmole. About 74 % of the $NaH^{14}CO_3$ radioactivity initially added was found to be incorporated into 3PGA-1^{14}C (see Table 2).

TABLE 1. Degradation of the lactic acid obtained from the 3PGA-1-^{14}C synthesized by the RuDP carboxylase reaction

	cpm
Radioactivity recovered in the CO_2 after total oxidation	22.500 23.000
Radioactivity recovered in the CO_2 after partial oxidation	22.000 23.000

(*b*) *Radiochemical purity of the synthesized 3PGA-1-^{14}C.*

Though it has been demonstrated that in the reaction catalyzed by RuDP carboxylase, the carbon of the CO_2 is all localized in the position 1 of the 3PGA [3, 4], the following procedure was used to test the radiochemical purity of the 3PGA-1-^{14}C synthesized [7, 8].

An aliquot of the chromatographically purified 3PGA was enzymatically transformed into lactic acid. A part of it was partially oxidized with $KMnO_4$ at 75° C for 30 minutes (oxidation known to release as CO_2 only the carbon 1 of the lactic acid) while another part was completely oxidized with $AgNO_3$ and persulfate at 80° C for 12 hours [7, 8], a procedure known to completely oxidize the lactic acid. The released CO_2 was collected in KOH, precipitated as $BaCO_3$ and its radioactivity assayed. Table 1 shows the results obtained in this experiment. It can be seen that the radioactivity released from carbon 1 is the same as the total radioactivity of the compound. This confirms that the entire radioactivity of the 3PGA is localized in the carbon 1 of the molecule.

(*c*) *Synthesis of dihydroxyacetonephosphate-1-*^{14}C

In a 3ml final volume the reaction mixture contained : 150 μmoles of Tris-Cl pH 8.2, 4.5 μmoles of $MgCl_2$, 20 μmoles of NADH, 60 μmoles of ATP, 15 μmoles of 3PGA-1-^{14}C having a specific activity of 18.45 mC/mmole and the following enzymes : phosphoglyceril kinase 30 μg, glyceraldehyde-3-P dehydrogenase 300 μg, triosephosphate isomerase 20 μg. The reaction was followed spectrophotometrically at 340 mμ. After 3 hours the reaction was stopped by the addition of 10 % PCA. The solution was left for 3 hours in an ice bath, then centrifuged. Two mg of bovine albumine was then added to the supernatant and it was centrifuged again. The supernatant, neutralized with KOH, was then decationized on a Dowex 50 (H^+ form) and treated with Norite A in order to eliminate the nucleotides.

All the operations described were found to be essential to eliminate any trace of triosephosphate isomerase : in fact after a simple precipitation with 7 % PCA a trace of that enzyme was still present in supernatant solution and mixed the two triosephosphates in the following step (synthesis of FDP).

The final solution contained 5.4 μmoles of DAP and ca. 0.2 μmoles of glyceraldehyde-3-P. The latter compound could be eliminated by incubating the solution in the presence of sodium arsenate, NAD and glyceraldehyde-3-P dehydrogenase. This step however was not essential because the glyceraldehyde-3-P-^{14}C would be largely diluted by the unlabeled compound added for the synthesis of FDP. An aliquot of the solution was chromatogrammed in phenol-water (4 : 1), and two radioactive spots appeared, one due to the unreacted 3PGA, and a second one due to DAP. The latter was eluted and the specific activity of the DAP resulted the same as that of the starting 3PGA (18.4 mC/mmole).

(*d*) *Synthesis of fructose-3-*^{14}C.

In a 3 ml final volume the reaction mixture contained : 60 μmoles of Tris-Cl pH 8.2, 5 μmoles of DAP-1-^{14}C, 15 μmoles of glyceraldehyde-3-P, and 200 μg of aldolase. After 20 minutes at 20° C the reaction was stopped as usual with 10 % PCA.

After neutralization with KOH the solution was adjusted to pH 5.4 with

acetic acid and treated for 12 hours with acidic phosphatase (1 mg/ml). At the end of this incubation the solution was chromatogrammed in a phenol-water solvent system (4 : 1). Three spots were localized : one (RF 0.29) corresponding to the glyceric acid, a second one (RF 0.58) corresponding to fructose, a third much smaller spot (RF 0.8) corresponding to dihydroxyacetone. The fructose was eluted from the paper and was shown to have a specific activity of 18.5 mC/mmole as one would theoretically expect.

(*e*) *Synthesis of glucose-3-^{14}C.*

4 μmoles of fructose-3^{14}C eluted from the chromatogram were treated with 20 μg of hexokinase and 20 μg of phosphoglucoisomerase, in the presence of 3 mM ATP, 2 mM $MgCl_2$ and 50 mM Tris-Cl pH 8.2 in a final volume of two ml. After two hours at room temperature the reaction was stopped by the addition of 0.2 ml of 10 % acetic acid. The solution was heated in a boiling bath for 10 minutes, then adjusted to pH 5.4 and treated with phosphatase as described above.

After the usual precipitation with 10 % PCA the products of the reactions were chromatogrammed with a phenol-water solvent system and two radioactive spots, corresponding to glucose and fructose (with RF 0.39 and 0.55 respectively) were localized.

Table 2 gives a summary of the synthesis of glucose-3-^{14}C.

TABLE 2. Synthesis of glucose-3-^{14}C

	μmoles	Radioactivity μC	Specific radioactivity mC/mmole	% of initial radioactivity
$NaH\text{-}^{14}CO_3$	36	1440	40	
RuDP	40			
3PGA-1-^{14}C	56.4	1070	18.45	74
DAP-1-^{14}C	20.2	365	18.4	25
Fructose-3-^{14}C	17	320	18.5	22
Glucose-3-^{14}C	13	246	18.5	17
Glyceric acid-1-^{14}C	25	460	18.5	32

(*f*) *Synthesis of glucose-4-^{14}C.*

In order to synthesize hexoses-4-^{14}C, glyceraldehyde-3-P-1-^{14}C and unlabeled DAP have to be condensed by the aldolase reaction. Glyceraldehyde-3-P-1-^{14}C can be obtained from 3PGA-1-^{14}C with 3PGA kinase and glyceraldehyde-3-P dehydrogenase in the presence of NADH, ATP, Mg^{++}. However the high contamination of the 3PGA kinase by triosephosphate isomerase makes this step very difficult. In fact when commercial yeast 3PGA kinase

(purchased either from Biochemia s.r.l. or from the Schuchardt G.m.b.H.) was used, practically all the glyceraldehyde-3-P synthesized was immediately converted to DAP. When the reaction was stopped, a 95/5 ratio DAP/ glyceraldehyde-3-P was found. Such a ratio could be expected from the equilibrium constant of the triosephosphate isomerase reaction. The yield of glyceraldehyde-3-P-1-^{14}C was therefore extremely low.

To overcome this problem we have attempted to purify further the 3PGA kinase. Many authors however [9, 10] have reported that it is very difficult to get rid of the isomerase activity during the purification of the kinase. Rao and Oesper [9] and Gosselin-Rey [10] reported that their preparations of purified 3PGA kinase still maintained some triosephosphate isomerase activity. By filtration on Sephadex G 100 (the isomerase activity was eluted slightly before the kinase) and recycling two or three times, it was possible for us to get a kinase preparation where the contaminating activity was 15 to 25 fold lower then initially. Even with our purified preparation however a certain amount of glyceraldehyde-3-P was still converted to DAP, thus reducing the yield of glyceraldehyde-3-P-1-^{14}C.

The 3PGA-1-^{14}C was incubated in the presence of $MgCl_2$, ATP, Tris, NADH, "purified" 3PGA kinase, glyceraldehyde-3-P dehydrogenase for 40'. The reaction was then stopped as described for the synthesis of DAP. After filtration on Dowex 50 and treatment with Norite A, NADH and glycerol-1-P dehydrogenase were added to the solution. The reaction was followed spectrophotometrically and, after all the DAP had been converted into glycerol-1-P, it was stopped as usual by addition of PCA. The volume of the solution was reduced by liophilisation. Cold DAP and aldolase were added to it and the solution incubated for 20 minutes. The following procedures were the same as those used in the synthesis of glucose-3-^{14}C.

(*g*) *Determination of the radiochemical purity of the glucose-3-^{14}C and 4-^{14}C.*

The radiochemical purity of the glucose-3^{14}C and 4^{14}C was determined by means of the enzymatic degradation already described [11].

Since 97 % of the radioactivity was localized in the carbon 3 of glucose-3-^{14}C, its radiochemical purity was of about 97 %.

The degradation of glucose-4-^{14}C showed it to have a radiochemical purity of 99 %.

Conclusions.

A method for the enzymatic synthesis of hexoses-3-^{14}C of high radiochemical purity and specific activity has been presented. The whole procedure is based on the possibility to reverse the glycolytic reactions leading from glucose to 3PGA. 3PGA-1-^{14}C was initially obtained by means of the reaction catalyzed by the RuDP carboxylase in the presence of RuDP and $^{14}CO_2$.

DAP-1-^{14}C, synthesized from 3PGA-1-^{14}C, was condensed with cold glyceraldehyde-3-P to give FDP-3-^{14}C and from this compound glucose and fructose-3-^{14}C were formed.

It is clear that for the synthesis of hexoses-3-^{14}C it is very important to interrupt the series of the reactions after the synthesis of DAP-1-^{14}C. One also must be certain that triosephosphate isomerase activity is absent from the reaction mixture where FDP is synthesized from the two triosephosphates. The commercially available aldolase is pure enough for this step. By our method glucose-3-^{14}C has been obtained with 18.4 mC/mmole specific activity and with a yield of about 30-35 % with respect to the 3PGA used.

More problems have been raised during the synthesis of hexoses-4-^{14}C. The key step consists here in the condensation of cold DAP with glyceraldehyde-3-P-1-^{14}C. Because of the persistent contamination of the 3PGA kinase with triosephosphate isomerase, the yield of glyceraldehyde-3-P-1-^{14}C is low, a great amount of it being lost as DAP. This has therefore to be eliminated from the reaction mixture before the aldolase reaction. The yield of hexoses-4-^{14}C is much lower than that of hexoses-3-^{14}C. In both cases however the radiochemical purity and specific activity of the synthesized compounds are very high.

The present research has been carried out under the Euratom contract No. 083-64-11 RIS-I.

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