*Biochem. J.* (1978) **175**, 909–912
*Printed in Great Britain*



# Preparative-Scale Enzymic Synthesis of D-[$^{14}$C]Ribulose 1,5-Bisphosphate

By GLENN D. KUEHN* and TZU-CHIN HSU
*Department of Chemistry, Box 3C, New Mexico State University, Las Cruces, NM* 88003 *U.S.A.*

(*Received* 13 *April* 1978)

A procedure is described to prepare uniformly labelled D-[$^{14}$C]ribulose 1,5-bisphosphate enzymically from uniformly labelled D-[$^{14}$C]glucose through the coupled reactions catalysed by hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) and 5-phosphoribulokinase (EC 2.7.1.19). All reagents utilized in the method are commercially available. The procedure is a reliable preparative-scale method for synthesizing the dibarium salt of D-[$^{14}$C]ribulose 1,5-bisphosphate with a specific radioactivity up to 7mCi/mmol and a purity near 90%. The final product was free of other $^{14}$C-labelled sugars, sugar phosphate esters, $P_i$ and nucleotides.

Ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) catalyses primary $CO_2$ fixation in all photosynthetic plants, photosynthetic bacteria and chemosynthetic bacteria (McFadden, 1973). Recent recognition (Andrews *et al.*, 1973) that this enzyme may also be the primary catalyst for light-dependent photorespiratory synthesis of phosphoglycollate by oxygenation of ribulose 1,5-bisphosphate has generated much interest (Jensen & Bahr, 1977) in its structure and mechanism of catalysis. We have reported on the feasibility of covalently labelling the active site of ribulose 1,5-bisphosphate carboxylase isolated from a bacterium, tobacco and spinach by the technique of paracatalytic modification (Hsu & Kuehn, 1978; Kuehn & Hsu, 1978). This technique for active-site labelling requires the natural substrate, ribulose 1,5-bisphosphate, and an external oxidant, hexacyanoferrate(III), to convert a carbanionic intermediate of the normal reaction mechanism into a reactive species capable of amino acid side-chain modification (Christen, 1977). An arginine side chain in the region of the ribulose 1,5-bisphosphate-binding site has been proposed as a possible locus for modification (Kuehn & Hsu, 1978). Radioactively labelled ribulose 1,5-bisphosphate is required to investigate this modified site, but it is not commercially available. An attempt by the authors to prepare [$^{14}$C]ribulose 1,5-bisphosphate enzymically by a published procedure (Wishnick & Lane, 1969) yielded a product extensively contaminated with 6-phospho[$^{14}$C]gluconate, a potent competitive inhibitor with respect to ribulose 1,5-bisphosphate of ribulose 1,5-bisphosphate carboxylase. Moreover, the failure of two commercial enzyme preparations to support enzymic synthesis of ribulose 1,5-bisphosphate by the same method prompted publication of the present paper.

This procedure describes the enzymic preparation of uniformly labelled D-[$^{14}$C]ribulose 1,5-bisphosphate from uniformly labelled D-[$^{14}$C]glucose with coupled reactions catalysed by four commercially available enzymes. It is a preparative-scale method yielding the dibarium salt of D-[$^{14}$C]ribulose 1,5-bisphosphate with a specific radioactivity of about 7mCi/mmol. This specific radioactivity is adequate for investigating ribulose 1,5-bisphosphate-binding-site modifications on ribulose 1,5-bisphosphate carboxylase.

## Experimental

### *Materials*

The following special chemicals and enzymes were obtained from Sigma Chemical Co., St Louis, MO, U.S.A.: disodium ATP, monopotassium phosphoenolpyruvate, sodium $NADP^+$, dithiothreitol, bovine serum albumin, pyruvate kinase (EC 2.7.1.40) from rabbit muscle (type III), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) from yeast (type IV), hexokinase (EC 2.7.1.1) from yeast (type VI), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from baker's yeast (type XV), which was predialysed at 2°C for 2h in 50mM-Tris/HCl, pH7.6, just prior to use and phosphoribulokinase (EC 2.7.1.19) from spinach (type II), which was similarly predialysed as described above. Uniformly labelled D-[$^{14}$C]glucose with a specific radioactivity of 198 mCi/mmol was purchased from ICN Pharmaceuticals, Irvine, CA, U.S.A. Whatman DE-52 DEAE-cellulose came from Whatman Biochemicals, Kent, England. All other reagents used in the preparative procedure, including disodium EDTA, Tris buffer, $MgCl_2$, KCl, glucose,

* To whom reprint requests should be addressed.

NaOH, HCl, LiCl, anhydrous methanol, acetone, $Ba(OH)_2$, $Ba(C_2H_3O_2)_2$, water and absolute ethanol, were A.R. grade.

*Preparation of uniformly labelled [$^{14}C$]ribulose 1,5-bisphosphate*

The synthesis is based on the coupled reactions catalysed by four enzymes. The following components were added, in the order given, to a 50ml glass test tube at 2°C: Tris/HCl buffer, pH7.6 (400$\mu$mol); $MgCl_2$ (100$\mu$mol); KCl (20$\mu$mol); disodium ATP (35$\mu$mol); monopotassium phosphoenolpyruvate (265$\mu$mol); sodium $NADP^+$ (300$\mu$mol); dithiothreitol (10$\mu$mol); disodium EDTA (4$\mu$mol); D-glucose (115$\mu$mol). The pH was adjusted to 7.6 with 1M-NaOH. The following additional reagents were then added: bovine serum albumin (2mg); pyruvate kinase (215 units); glucose 6-phosphate dehydrogenase (325 units); 6-phosphogluconate dehydrogenase (55 units); phosphoribulokinase (45 units); 1mCi of uniformly labelled D-[$^{14}C$]glucose in 2ml of 20% (v/v) ethanol; and water to a final volume of 19ml. The pH was again adjusted to pH7.6 with 1M-NaOH. The reaction mixture was greenish-brown owing to the chloroplast preparation of phosphoribulokinase. Next, the reaction tube was warmed to 25°C and the synthesis was initiated by addition of 325 units of hexokinase dissolved in 1ml of 50mM-Tris/HCl, pH7.6. The reaction mixture was stirred continuously and the pH was monitored with a combination electrode. Throughout the synthesis, the pH was maintained at 7.6 by dropwise addition of 1M-NaOH. Approx. 1.4ml of 1M-NaOH was consumed in the reaction.

After about 45min there was no further pH change, indicating completion of the reaction. The content of the reaction tube was then poured into 6vol. of water at 2°C and was acidified to pH3.5 by adding 3M-HCl. The solution, which became turbid on acidification, was immediately applied to a DEAE-cellulose column (4cm internal diam. × 46cm; $Cl^-$ form) at 4°C. The column was equilibrated with 10mM-LiCl/1mM-HCl. Fractions of 17ml were collected automatically starting immediately after addition of the reaction mixture to the column. After addition of the reaction mixture, 450ml of 10mM-LiCl containing 1mM-HCl was passed through the column followed by a 4-litre LiCl linear gradient (0.01–0.2M-LiCl, containing 1mM-HCl). Fractions containing [$^{14}C$]ribulose 1,5-bisphosphate, which was detected by enzymic assay with purified ribulose 1,5-bisphosphate carboxylase (Racker, 1962; Kuehn & McFadden, 1969) and by radioactivity, were combined and concentrated under reduced pressure at 35°C. The resulting white solid was washed with 2 × 40ml of 20% (v/v) anhydrous methanol in acetone, then four times with cold acetone. The tetralithium salt of [$^{14}C$]ribulose 1,5-bisphosphate was next converted into the dibarium salt (Horecker *et al.*, 1958). The acetone-washed lithium salt was dissolved in 50ml of cold water and saturated $Ba(OH)_2$ solution was added dropwise to pH6.5. Dibarium [$^{14}C$]ribulose 1,5-bisphosphate partially precipitated during this adjustment. Then 2ml of 1M-barium acetate and 20ml of ethanol were added to ensure complete precipitation. The precipitate was collected by centrifugation at 12000*g* for 10min at 4°C, was washed with 80% ethanol, and was dried for approx. 1 week under reduced pressure over $P_2O_5$ at 4°C. The final product was stored over anhydrous $CaCl_2$ at −20°C.

*Paper chromatography and staining methods*

All paper-chromatographic separations utilized Whatman no. 1 paper in the solvent system 2-methylpropanoic acid/1M-$NH_3$/0.1M-EDTA (125:75:2, by vol.) (Tyszkiewicz, 1962). In this solvent system at 23°C the following relative migration distances, $R_{P_i}$, were observed for authentic commercial preparations: ribulose 1,5-bisphosphate, 0.41; 3-phosphoglycerate, 0.72; 6-phosphogluconate, 0.48. Developed paper chromatograms were stained with an ammonium molybdate reagent to detect sugar phosphate esters (Hanes & Isherwood, 1949) or with an $AgNO_3$ reagent to detect total sugars (Borders, 1972).

*Analyses*

6-Phospho[$^{14}C$]gluconate in column fractions was assayed spectrophotometrically at 30°C in a 1ml reaction mixture containing the following: $MgCl_2$ (0.5$\mu$mol); Tris/HCl, pH7.6 (90$\mu$mol); $NADP^+$ (0.2$\mu$mol); 6-phosphogluconate dehydrogenase (3 units).

$NADP^+$ in column fractions was similarly assayed in a reaction mixture containing the following: $MgCl_2$ (0.5$\mu$mol); Tris/HCl, pH7.6 (90$\mu$mol); 6-phosphogluconate (0.4$\mu$mol); 6-phosphogluconate dehydrogenase (3 units).

[$^{14}C$]Ribulose 1,5-bisphosphate was assayed spectrophotometrically (Racker, 1962).

Units of enzyme activity are expressed as $\mu$mol of product formed/min.

## Results and Discussion

The synthesis has been carried out three times, twice with relatively small quantities of D-[$^{14}C$]-glucose and once with 1mCi. The elution profile of $^{14}C$ radioactivity from the DEAE-cellulose chromatography step is shown in Fig. 1. [$^{14}C$]Ribulose 1,5-bisphosphate (fractions 192–212) began to be eluted from the column when the LiCl concentration reached 100mM. An earlier labelled component (fractions 135–170), which started to appear in the eluate at 71mM-LiCl, was found by separate enzymic analyses to contain at least 60% 6-phospho[$^{14}C$]-

Table 1. *Preparations of dibarium* D-[$^{14}$C]*ribulose* 1,5-*bisphosphate*

Preparative details are given in the text. The total dibarium D-[$^{14}$C]ribulose 1,5-bisphosphate (mol.wt. 580.74) isolated in preparation no. 1 was 71.9mg (124$\mu$mol). The purity of this preparation, determined by a coupled spectrophotometric–enzymic assay (Racker, 1962), was 91%. Thus the 'Total dibarium ribulose 1,5-bisphosphate isolated', second column, is expressed as the assayed amount of the salt. The values for total radioactivity in the third column were those provided by the commercial supplier of uniformly labelled D-[$^{14}$C]glucose and thus may be slightly uncertain.

| Preparation no. | Total dibarium ribulose 1,5-bisphosphate isolated ($\mu$mol) | Total radioactivity initially present (mCi) | Yield of ribulose 1,5-bisphosphate (%) | Fraction of total radioactivity in ribulose 1,5-bisphosphate (%) | Specific radioactivity of ribulose 1,5-bisphosphate (mCi/mmol) |
|---|---|---|---|---|---|
| 1 | 113 | 1 | 91 | 84.5 | 7.48 |
| 2 | 94 | 0.25 | 91 | 87.1 | 2.32 |
| 3 | 100 | 0.25 | 89 | 78.8 | 1.97 |

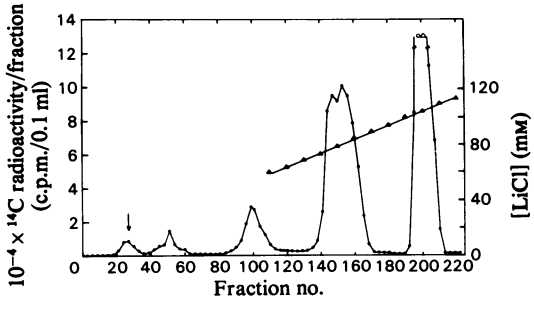


Fig. 1. *DEAE-cellulose chromatography of a reaction mixture after enzymic synthesis of* D-[$^{14}$C]*ribulose* 1,5-*bisphosphate from* 1 *mCi of* D-[$^{14}$C]*glucose*

$^{14}$C content (●) was determined by transferring 0.1ml from each fraction to 5ml of liquid-scintillation solvent (Kuehn, 1974) in a counting vial and counting in a Beckman LS-100c scintillation spectrometer. Each column fraction contained 17ml. The LiCl concentration gradient (▲) in column fractions was measured with a Radiometer CDM 2f conductivity meter. The 4-litre gradient was started at the arrow. Enzymic and paper-chromatographic analyses showed that pooled fractions 135–170 contained 6-phospho[$^{14}$C]gluconate and that fractions 192–212 contained [$^{14}$C]ribulose 1,5-bisphosphate. The $^{14}$C contents of fractions 198 (○) and 201 (△), which extended beyond the limits of the ordinate, were assayed to contain $2.19\times10^6$ and $0.691\times10^6$c.p.m./0.1ml respectively.

gluconate. It also contained about 13% unlabelled $NADP^+$. The labelled components in fractions with peak radioactivities in tubes 25, 51 and 99 were not identified.

The yields and purities of [$^{14}$C]ribulose 1,5-bisphosphate for three preparations are shown in Table 1. Preparation no. 1 summarizes the results of the procedure described above. The purity of the final dibarium salt in preparation no. 1 was 91% as determined by spectrophotometric assay (Racker, 1962). It had a specific radioactivity of 7.48mCi/mmol. No u.v.-absorbing material and no other sugar phosphates or sugars were found in 50$\mu$g (0.09$\mu$mol) of the salt analysed by paper chromatography. When 0.04$\mu$mol of synthesized [$^{14}$C]ribulose 1,5-bisphosphate was chromatographed on paper with authentic, commercial ribulose 1,5-bisphosphate, 89.3% of the total $^{14}$C radioactivity co-chromatographed with the sugar phosphate region that had an $R_{P_i}$ value of 0.41. Purified ribulose 1,5-bisphosphate carboxylase (0.1 unit) also converted 98.3% of the $^{14}$C radioactivity in 5.4$\mu$mol of synthesized [$^{14}$C]ribulose 1,5-bisphosphate into 3-phospho[$^{14}$C]glycerate in a 60min incubation at 30°C (Kuehn & McFadden, 1969).

The authors recommend caution in the choice of commercial suppliers for phosphoribulokinase. Two commercial preparations of this enzyme, purchased from suppliers different from that prescribed above, failed to synthesize [$^{14}$C]ribulose 1,5-bisphosphate under conditions described in the present paper.

The purity of [$^{14}$C]ribulose 1,5-bisphosphate prepared by this method is greater than that of unlabelled ribulose 1,5-bisphosphate currently available from major commercial vendors. All commercial preparations of ribulose 1,5-bisphosphate that we have tested contain a predominant contaminant with an $R_{P_i}$ of 0.09 that is detectable by paper chromatography as described in the present paper, and which stains like a sugar phosphate with ammonium molybdate reagent.

This work was supported by research grant PCM-7517261 from the U.S. National Science Foundation.

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