AN ENZYMATIC METHOD FOR THE PREPARATION OF L-[1-^{14}C]GLYCEROL.

P.F. Blackmore, M.G. Clark and J.F. Williams.

School of Biochemistry,
The University of New South Wales,
Kensington. N.S.W. 2033
Australia.

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SUMMARY

Biologically labelled [1-^{14}C]glycerol (L-[1-^{14}C]glycerol) was prepared by a two step process. Step one involved the preparation of [1-^{14}C]glycerol 3-phosphate from $^{14}CO_2$ and D-ribulose 1,5-diphosphate using the enzymes, ribulose diphosphate carboxylase, phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase and L-α-glycerophosphate dehydrogenase. Step two involved the conversion of [1-^{14}C]glycerol 3-phosphate to [1-^{14}C]glycerol, by enzymatic dephosphorylation.

Based on the criteria of the constancy of the specific activity of glycerol and its derivatives, namely glycerol tribenzoate and glycerol p-nitrobenzoate, the compound was concluded to be pure. Degradation by methods which discriminate between DL-[1-^{14}C]glycerol, D-[1-^{14}C]glycerol and L-[1-^{14}C]glycerol indicated the compound to be L-[1-^{14}C]glycerol.

Abbreviations

ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; Tris, tris(hydroxymethyl)aminomethane.

INTRODUCTION

Studies by Schambye, Wood and Popjak[1] and Swick and Nakao[2] have shown that glycerol, although apparently a symmetrical molecule, may be metabolized asymmetrically, in confirmation of the hypothesis of Ogston[3]. Furthermore all commercially available [1-^{14}C]glycerol prepared by chemical methods has been found by other workers[4] and

us to be [1,3-^{14}C]glycerol (DL-[1-^{14}C]glycerol) when degraded by biological methods (Table 1). Our studies on carbohydrate metabolism in rabbit liver in vivo[5,6] have necessitated the use of biological [1-^{14}C]glycerol(L-[1-^{14}C]glycerol). As to our knowledge no L-[1-^{14}C]glycerol is commercially available we have developed a method for the preparation of this substance in high yield.

EXPERIMENTAL SECTION

The conversion of $^{14}CO_2$ to L-α-[1-^{14}C]glycerol 3-phosphate

The method used for the conversion of $^{14}CO_2$ to L-α-[1-^{14}C]-glycerol 3-phosphate was essentially that of Racker[7]. The reaction mixture was composed of 3μmoles $Na_2{}^{14}CO_3$, 165μCi (The Radiochemical Centre, Amersham, Bucks, England) dissolved in 0.08 ml of water; ribulose 1,5-diphosphate, sodium salt, 5μmoles; ATP, 600μmoles; NADH, 12μmoles; reduced glutathione, 20μmoles; $MgCl_2$, 50μmoles; triosephosphate isomerase, 25μg (0.8 units*); L-α-glycerophosphate dehydrogenase, 25 μg (1.2 units); glyceraldehyde 3-phosphate dehydrogenase, 1 mg (50 units); ribulose diphosphate carboxylase, 20 mg (0.5 units) and 1M-Tris-HCl buffer pH 7.4, 8 mmoles to give a total volume of 10.0 ml. Phosphoglycerate kinase, 25μg (50 units) was added to start the reaction. The production of L-α-[1-^{14}C]glycerol-phosphate was monitored spectrophotometrically by recording the decrease in absorbance at 340 nm due to the oxidation of NADH in a suitably diluted aliquot of the reaction mixture. When no further change in optical density at 340 nm was evident (usually 20 min.) the complete reaction mixture was heated at 100° C for 5 min. and the precipitate of protein removed by centrifugation at 5,000 g for 5 min.

Distilled deionized water, CO_2-free, was used for all preparations, and the average yield for this step was found to be 95 % based on NAD^+ production. A separate estimation of the yield based on the formation of acid stable radioactivity also indicated this to be approximately 95 %.

L-α-[1-^{14}C]glycerol 3-phosphate was isolated from the reaction mixture by anion-exchange column chromatography. The combined supernatant was adjusted to pH 8.0 with 0.1M NH_4OH and applied to a column (0.5 cm x 50 cm) of Dowex AG 1 x 4 resin in the borate form. The isolation of L-α-[1-^{14}C]glycerol 3-phosphate was achieved by elution using a linear gradient of 0.1M to 0.4 M ammonium tetraborate (178 ml of each). A total of 118 fractions of 3 ml each were collected at a constant flow rate of 0.65 ml/min. Samples of 0.01 ml were removed from every third fraction, dispersed in 5 ml of scintillant[8] and the radioactivity determined. The results in Fig. 1 show the column profile of radioactivity obtained for the isolation of L-α-[1-^{14}C]glycerol 3-phosphate together with a profile for authentic DL-α-glycerol phosphate obtained from a similar column prepared and eluted under identical

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* 1 unit of enzyme activity is defined as the amount of enzyme that converts a micromole of substrate to product in 1 minute under optimal conditions of assay at 30° C.

conditions. The fractions corresponding to the radioactive peak of L-α-[1-^{14}C]glycerol 3-phosphate were combined and the ammonium tetraborate removed by evaporations in vacuo with three successive volumes of methanol (each 350 ml).

Conversion of L-α-[1-^{14}C]glycerol 3-phosphate to L-[1-^{14}C]glycerol

The residual powder, free of ammonium tetraborate and containing approximately 5umoles of L-α-[1-^{14}C]glycerol 3-phosphate was dissolved in 3 ml of phosphatase reagent. (1 mg Sigma Type I plant acid phosphatase per ml of 10mM $MgCl_2$-0.1M sodium acetate-acetic acid buffer pH 4.8). Dephosphorylation was allowed to proceed at 37° C for 3 hr after which time the reaction was stopped by heating at 100° C for 5 min. The supernatant was passed through a mixed-bed resin (2 g) composed of equal parts of Dowex 50W (H^+ form) and Dowex I (HCO^-_3 form). The resin was washed with 10 ml H_2O and the combined eluate concentrated to approximately 0.2 ml employing care to prevent loss of the product by drying[9]. The concentrate (approx. 0.2 ml) was applied as a 20 cm wide band to Whatman 3 MM paper. By using authentic glycerol as a reference the L-[1-^{14}C]glycerol was isolated from possible contaminants by ascending chromatography in ethyl acetate-pyridine-water (12:5:4, by vol). Glycerol was located by spraying the reference with alkaline $AgNO_3$[10] and scanning a 2.5 cm strip of the chromatogram for radioactivity using a Nuclear Chicago Actigraph III. The [1-^{14}C] glycerol was eluted and concentrated to 5 ml, assayed[11], and the specific radioactivity determined.

Exactly 1umole of the prepared [1-^{14}C]glycerol was added to 500μ moles of unlabelled glycerol and the tribenzoate prepared[12]. After recrystallization from alcohol the m.p. was determined (75-76°C; uncorrected; published value 76°C). Identical amounts of the prepared radioactive glycerol and unlabelled glycerol were used for the preparation of the p-nitrobenzoate derivative[12] (m.p. 186-187° C; uncorrected; published value 188° C). The specific radioactivity of each derivative was determined by collecting approximately 20 mg onto a preweighed filter disc and counting in a Gas Flow Counter. All counts were corrected to infinite thinness. The specific radioactivities of the [^{14}C]glycerol tribenzoate and [^{14}C]glycerol p-nitrotribenzoate were 4.8 x 10^4 and 4.6 x 10^4 c.p.m./umole, respectively. Approximately 1umole of the prepared [^{14}C]glycerol was diluted with 250umoles of unlabelled glycerol and degraded by the method of Genovese et al.[4] (Table 1).

The average yield of product L-[1-^{14}C]glycerol, obtained by the method described was 65 %. The prepared aqueous solution of L-[1-^{14}C] glycerol (specific radioactivity 27.5 mCi/mmole and 0.011 mCi/ml) was stored as a liquid at -40° C with no detectable decomposition after 6 months.

TABLE I.

The percentage distribution of ^{14}C in various preparations of [^{14}C]glycerol

Source	% distribution of ^{14}C in the carbon atoms of glycerol			% recovery of label
	C-1	C-2	C-3⁂	
Commercial preparation I of [1-^{14}C]glycerol	50.0	0.7	49.3	101.2
Commercial preparation II of [1-^{14}C]glycerol	48.5	0	51.5	107.1
Commercial preparation of [2-^{14}C]glycerol	2.1	95.9	2.0	97.9
Prepared L-[1-^{14}C]glycerol	99.7	0.2	0.1	99.4

Unlabelled glycerol (250μmoles) was added to 2.0×10^5 d.p.m. of each of the commercial preparations of [^{14}C]glycerol, and to 1.2×10^5 d.p.m. of the prepared [1-^{14}C]glycerol. Samples of [^{14}C]glycerol were degraded to CO_2 by the step-wise procedure of Genovese *et al.*[4]. The distribution of ^{14}C in each carbon atom of glycerol is expressed as a percentage of the total and the percentage recovery of label was determined by the fraction

$$\frac{\text{sum of individual carbon atom specific radio-activities}}{\text{specific radioactivity of glycerol}}$$

⁂ C-3 is the carbon atom which is adjacent to the phosphate group in L-α-glycerol 3-phosphate formed by biological phosphorylation.

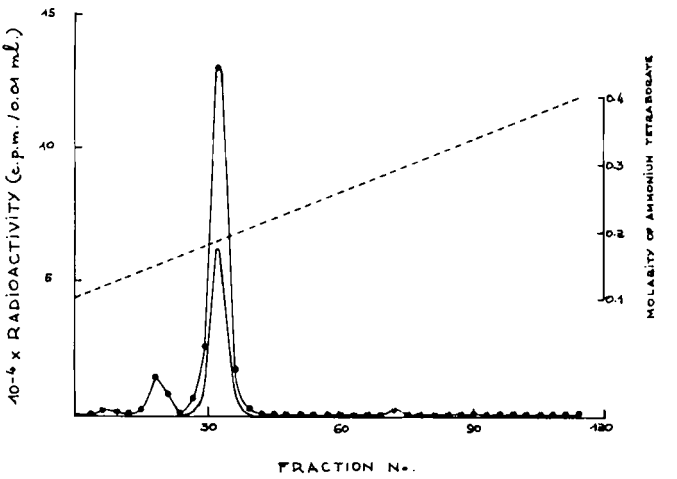

Fig. 1. The isolation of L-α-glycerol 3-phosphate by anion-exchange chromatography.

A column (0.5 cm x 50 cm) of Dowex 1 x 4 resin in the borate form was used. L-α-glycerol 3-phosphate was eluted with a linear gradient of 0.1 - 0.4 M-ammonium tetraborate, and fractions of 3.0 ml each were collected.

———, Total phosphate profile for the isolation of authentic DL-α-glycerol phosphate obtained by the analysis of every third fraction[13].

Radioactivity profile for the isolation of [1-^{14}C]glycerol 3-phosphate (0.01 ml sample). Under the conditions employed the fractions containing [1-^{14}C]glycerol 3-phosphate were free of ATP, ADP, NADH, NAD^+ and unreacted ribulose 1,5-diphosphate.

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