The Formation of 1-Phosphoerythrulose-4-C^{14} by Homogenates of Swiss Chard Leaves[1]

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Received July 21, 1954

Introduction

Recently the presence of a soluble enzyme in rat liver was described (1) which catalyzed an aldol type condensation between dihydroxyacetone and formaldehyde to form erythrulose phosphate. The condensing enzyme was shown to differ from the classical aldolase of Meyerhof, Lohmann, and Schuster (2). In the extension of these studies using C^{14}-labeled formaldehyde, this reaction has also been demonstrated to occur in blendorized preparations of Swiss chard. The product has been further characterized as 1-phosphoerythrulose-4-C^{14} by oxidative degradation with periodate.

Materials and Methods

Fresh Swiss chard leaves were stripped from stems, weighed, and homogenized for 1–2 min. in a Waring blendor with sufficient distilled water at 0° to give a 30% preparation of leaf tissue. This suspension was filtered through a double layer of surgical gauze prior to use.

Radioactive formaldehyde was prepared by the magnesium reduction of formate-C^{14} (1, 3) or by the decomposition of glycine-2-C^{14} with ninhydrin (4). Carrier nonradioactive formaldehyde was prepared from paraformaldehyde by repeated distillation from 4 *N* H_2SO_4. The colorimetric determination of formaldehyde was carried out according to the chromotropic acid procedure of MacFadyen (5) scaled to a working volume of 4.0 ml. The specific activity of formaldehyde was determined by a conversion to the dimedon derivative after the addition of a known amount of carrier formaldehyde (1). The crystalline product, recrystallized to constant specific activity, was plated directly on weighed aluminum disks and counted with a thin end-window Geiger-Müller tube for a period sufficient to achieve a 5% statistical accuracy. Other radioactive materials were plated and

[1] This work was supported by a grant from the Alexander and Margaret Stewart Trust Fund and an institutional grant from the American Cancer Society.

handled in a similar manner. Glycolic acid determinations were made according to the method of Calkins (6). The Fiske and SubbaRow method (7) was used for the measurement of phosphorus. Authentic L-erythrulose was prepared by the fermentation of *meso*-erythritol with *Acetobacter suboxydans* (8), isolated as the *o*-nitrophenylhydrazone (m.p. 152°), and the free sugar regenerated with benzaldehyde (9).

In a typical experiment 20 ml. of the homogenate was incubated in 125-ml. Erlenmeyer flasks with 2.0 ml. of 0.01 *M* potassium iodoacetate for 10 min. at 37.5° under nitrogen with shaking and then combined with 10 ml. of 0.06 *M* fructose 1,6-diphosphate, 4.0 ml. of 0.01 *M* $MgCl_2$ and 2.0 ml. of formaldehyde-C^{14} solution containing 7.24 mg. with a specific activity of 175 counts/min./μg. formaldehyde unless otherwise specified. This reaction mixture, composed of constituents adjusted to pH 7.4, was incubated with constant shaking under nitrogen at 37.5° for 45 min. The reaction was stopped by the addition of 4.0 ml. of 100% (*w/v*) trichloroacetic acid (TCA), and the precipitated proteins were removed by filtration prior to analysis of the filtrate.

Results

The Isolation of Erythrulose Phosphate

The filtrates from four identical flasks after deproteinization with TCA were combined and analyzed for total formaldehyde disappearance. A total of 8.0 mg. (27 %) containing 1,400,000 counts/min. was metabolized. The filtrate was extracted ten times with an equal volume of ethyl ether to remove the major portion of the TCA. Evaporation of an aliquot of the extracted filtrate on a tared aluminum disk and counting for radioactivity revealed that in good agreement with formaldehyde disappearance, 1,440,000 counts/min. could be accounted for as nonvolatile, water-soluble metabolites. The solution was diluted 20 times with distilled water and passed through a freshly prepared column (2 × 16 cm.) of Dowex 1 (monochloroacetate form). The nonpolar or weakly acidic substances not retained on the column amounted to 573,000 counts/min.; this fraction (containing the free sugars) was set aside for subsequent study. Elution of the retained radioactivity with 15-ml. aliquots of 0.3 *M* monochloroacetic acid yielded a broad radioactive peak in samples 19–33 and accounted for 501,000 counts/min. The remainder of the counts were not eluted with this concentration of acid or identified.

After removal of most of the monochloroacetic acid by extraction five times with an equal volume of ether, the radioactive eluate was subjected to chromatography on a column (1.2 × 56 cm.) of Dowex 1 (formate form), using a constantly increasing concentration of formic acid for elution; the procedure of Busch *et al.* (10) was used with 200 ml.

of 1 *M* formic acid in the mixing chamber and 6 *M* formic acid in the reservoir. All of the radioactivity was eluted as a single band between tubes 50–60 with 9 ml./tube; analysis of the three peak samples revealed a molar ratio of incorporated C^{14} to organic phosphorus of 0.84. Accordingly, samples 50–57 were combined, extracted ten times with ether and subjected to another chromatographic purification using a similar column but with distilled water in the mixing chamber and 6 *N* formic acid in the reservoir. The molar ratio of C^{14} incorporated to organic phosphorus for a combined sample from tubes 50–52 was 0.90 and the sample was free of inorganic phosphorus. Comparative chromatograms on paper of the isolated product with a sample of erythrulose phosphate isolated previously from the liver enzyme system (1) demonstrated that both materials migrated identically in the ethanol–acetic–water (80:1:19) system (11).

Identification of Erythrulose

After hydrolysis with potato phosphatase (1), a sample of the free sugar containing 17,280 counts/min. was mixed with 10.8 mg. of bacterial erythrulose and refluxed for 10 min. with 15 mg. of *o*-nitrophenylhydrazine in methanol. After recrystallization, the *o*-nitrophenylhydrazine derivative (m.p. 150–151°) had a specific activity of 758 counts/min./mg. and accounted for 100% (17,300 counts/min.) of the radioactivity.

The isolation of *o*-nitrophenylhydrazone of plant erythrulose without the addition of carrier sugar was accomplished on a free sugar sample containing 1.72 mg. of erythrulose by radioactivity measurement. After repeated chromatography on Florisil columns (25 × 1 cm.), using alternately 10% methanol in $CHCl_3$ or 15% ethanol in $CHCl_3$ as the eluting solutions, and subsequent crystallization from heptane (Skellysolve)–ethanol mixture, 858 μg. of the derivative (measured by optical density of an ethanol solution) was isolated, m.p. 151°. The specific activity was 24,500 counts/min./μM as compared with 26,400 counts/min./μM for the precursor CH_2O which was used in this particular experiment.

From these data it was concluded that the carbon skeleton of the radioactive product, as with the liver enzyme system, was erythrulose. The optical configuration was not ascertained due to the small amount of pure derivative isolated.

The Periodate Oxidation of Erythrulose Phosphate

One-milliliter samples of plant erythrulose phosphate containing 1.68 μM of incorporated C^{14} and 1.87 μM organic phosphate (free of inorganic phosphate) were treated with 0.5 ml. of 0.3 M HIO_4 at room temperature for 1 hr., and then neutralized with KOH in a final volume of 5.0 ml. Following removal of the precipitated KIO_4 by centrifugation, the supernatant solution was passed through 3.0 × 0.8 cm. columns of Dowex 1 (chloride form), 100–200 mesh; after an initial 5.0-ml. water wash, the column was eluted with successive 5.0-ml. portions of dilute HCl of increasing concentrations: 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.10, 0.15, and 0.20 M HCl. In four trials 92–98 % of the organic phosphorus was recovered in the 0.04 and 0.05 M HCl fractions. HIO_4 and HIO_3 were eluted in the 0.02 and 0.03 M HCl fractions under these conditions.

Hydrolysis of the eluted organic phosphate fraction for 1 hr. with 1 N H_2SO_4 liberated only 1.25 % of the phosphorus in the inorganic form. This stability to acid hydrolysis excluded the possibility that the periodate oxidation product was glycolaldehyde phosphate since Fleury and Courtois had demonstrated that the latter was hydrolyzed to the extent of 50 % in 12 min. under these conditions (12).

These same authors found that phosphoglycolic acid was very stable to hydrolysis with 1 N H_2SO_4 at 100° (50 % hydrolyzed in 19.5 hr.), but much more labile to hydrolysis with 1 N acetic acid at 100° (50 % hydrolyzed in 6.5 hr.). Accordingly, a long hydrolysis with acetic acid was used for the characterization of the organic phosphate formed in the periodate oxidation of erythrulose phosphate.

Duplicate fractions of the eluate containing 96.2 μg. of organic P and free of inorganic P were evaporated to dryness in a vacuum desiccator over H_2SO_4 and KOH, and the residues were redissolved in 5.0 ml. of 1 N acetic acid. No inorganic P was released by this procedure, and a glycolic acid determination on an aliquot by the colorimetric method of Calkins (6) using 2.7 dihydroxynaphthalene revealed the presence of 20 μg. glycolic acid per sample. This small amount of glycolic acid could be accounted for by the slight hydrolysis of phosphoglycolic acid during the color development. The samples in 4.2 ml. of the 1 N acetic acid solution were subjected to hydrolysis under reflux at 95–100°C. for a period of 40 hr. After adjusting the hydrolysis volume back to 4.2 ml. with a small amount of distilled water, inorganic P and glycolic acid determinations were carried out on aliquots; 84.5 μg. inorganic P and

217 μg. glycolic acid were found. This increase in inorganic P corresponded to 88% hydrolysis of the organic phosphate present, and on a molar basis the ratio of glycolic acid to inorganic phosphate was 1.04; this was accepted as evidence that the difficultly hydrolyzable phosphate compound formed in the periodate oxidation media was phosphoglycolic acid.

In an attempt to account for the radioactivity of the erythrulose phosphate molecule, determinations of the amount and specific activity of the formaldehyde derived by periodate oxidation were carried out. For this purpose duplicate samples following oxidation with periodate and neutralization with KOH were passed through 5.0 × 0.5 columns of IR-X resin in the hydrochloride form. The columns were washed with sufficient water to give 10 ml. of eluate. This eluate, as well as an appropriate blank from a column treated with the periodate reagents only, was analyzed for total formaldehyde.

The radioactivity of the erythrulose phosphate was completely accounted for as formaldehyde released by the periodate oxidation procedure. In duplicate experiments 58.7 and 56.5 μg. CH_2O were formed, corresponding to an average of 114% of theory based on micromoles of C^{14} incorporated or 102% based on micromoles of organic phosphate in the sample. The specific activity of the derived CH_2O was 171 counts/min./μg. as compared with 175 counts/min. for the CH_2O of the original reaction mixture.

With these findings it has been demonstrated that the radioactive CH_2O was incorporated enzymatically into erythrulose phosphate as a terminal primary alcohol group of a glycol chain and that the phosphate was attached to the sugar so as to yield nonradioactive phosphoglycolic acid as the product of periodate oxidation. In the 4-carbon sugar, erythrulose monophosphate, these observations are compatible only with the structure, 1-phosphoerythrulose-4-C^{14}.

Discussion

Recent reports (13) have demonstrated that free erythrulose is an intermediate in the reversible breakdown and synthesis of ribulose phosphate and sedoheptulose phosphate. The present reaction affords one pathway leading to this intermediate. In accordance with this possibility it has been observed that a fresh rat liver homogenate system which was rapidly synthesizing erythrulose phosphate also incorporated as much as 10% of the metabolized formaldehyde into ribose (14). It

remains to be demonstrated whether this reaction proceeds through the monophosphate, diphosphate, or the free erythrulose sugar in the homogenate system.

Similarly, this reaction in plant tissue could provide the 4-carbon compound suggested by Benson *et al.* (15) as a precursor of the 2-carbon acceptor for CO_2 in photosynthesis. In two preliminary experiments with fortified Swiss chard homogenates, radioactivity accumulated in the lactic acid and pyruvic acid fractions to the extent of 12 % of the metabolized formaldehyde.[2] Further work is necessary to define the pathway of this conversion.

In other experiments it has also been found that erythrulose phosphate can be reversibly cleaved to yield formaldehyde in a rat liver enzyme system.[3] This may indicate another source of 1-carbon intermediates; however, this appears to require the phosphorylated form of the sugar. The relative significance of this pathway and its relationship to pentose breakdown remain to be determined.

Summary

1. Blendorates of Swiss chard leaves which are fortified with fructose 1,6-diphosphate and Mg^{++} incorporate radioactive formaldehyde into the 4-carbon sugar, 1-phosphoerythrulose-4-C^{14}.

2. The chromatographic isolation and the characterization of this sugar by periodate oxidation are described.

3. The implication of this pathway in pentose and photosynthesis is discussed.

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[2] Fourteen milliliters of a freshly prepared 20% blendorate of Swiss chard leaves in 1.15% KCl was incubated in a reaction medium containing 0.005 *M* $MgCl_2$, 0.006 *M* hexose 1,6-diphosphate, 0.003 *M* CH_2O-C^{14}, 0.005 *M* nicotinamide, DPN (600 μg.), TPN (600 μg.), 0.001 *M* ATP, 0.01 *M* KH_2PO_4–K_2HPO_4 buffer at pH 7.4 and 0.021 *M* $NaHCO_3$ in a final volume of 40 ml. The incubation was carried out in an atmosphere of 5% CO_2–95% N_2 at 37.5° in a 250-ml. Erlenmeyer flask with constant shaking and the illumination of a 150-w. light bulb. After 30 min. 43 μ*M* of CH_2O-C^{14} had been metabolized. Carrier isolations of the *p*-bromophenacyl ester of lactic acid and the 2,4-dinitrophenylhydrazone of pyruvic acid accounted for 11.6 and 0.74%, respectively, of the metabolized CH_2O. A repeat experiment gave similar results.

[3] Unpublished data.

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