# Short Communications

## Evidence for the Incorporation of [$^{14}$C]Glyoxylic Acid into 2-Hydroxyglutaric Acid by *Aspergillus glaucus*

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Glyoxylic acid condenses enzymically with the CoA thio ester derivatives of various fatty acids, including acetate, propionate, butyrate and valerate (Rabin, Reeves, Wegener, Megraw & Ajl, 1965). The product of the condensation with propionyl-CoA in *Escherichia coli* is 2-hydroxyglutaric acid (Reeves & Ajl, 1962; Trop & Pinsky, 1966), which is further metabolized by cell-free extracts either to succinate or to pyruvate and acetate via citramalate (Reeves, Urbano & Ajl, 1965). The role of 2-hydroxyglutarate synthase (EC 4.1.3.9) in propionate metabolism, however, is not clear and investigations have been initiated to determine its physiological function. The present communication provides evidence for the incorporation *in vivo* of [$^{14}$C]glyoxylate into 2-hydroxyglutarate by whole mycelia of *Aspergillus glaucus* grown on propionate.

Richards & Lloyd (1966) found that *A. glaucus* grows on propionate–mineral salts medium in submerged culture at 37°, but that extracts of the mycelia lack both propionyl-CoA carboxylase (EC 6.4.1.3) and malonate semialdehyde dehydrogenase (acylating) (EC 1.2.1.18). Cell-free extracts, however, catalysed the disappearance of propionyl-CoA when incubated with glyoxylate and $Mg^{2+}$. Only mycelia grown on propionate or acetate possessed this activity, which was absent from glucose- or lactate-grown cells. In addition, 2-hydroxyglutarate was metabolized by extracts of propionate-grown mycelia to compounds that appeared to be chromatographically identical with lactate and acetate (Richards & Lloyd, 1966). These results suggested that *A. glaucus* grows on propionate by a mechanism involving the formation and dissimilation of 2-hydroxyglutaric acid. Further support for this suggestion is the observation that the other major pathways of propionate metabolism appear to be absent. Richards & Lloyd (1966) did not report, however, the formation of 2-hydroxyglutarate from either [1-$^{14}$C]- or [2-$^{14}$C]-propionate with intact mycelia.

For the present study, *A. glaucus* (kindly provided by Dr D. Lloyd) was grown in 500ml. of mineral salts medium (Reeves & Ajl, 1960) containing propionate (0·4%) for 4 days at 37° with continuous shaking. The mycelial growth was harvested by filtration on a Buchner funnel, washed with distilled water and resuspended in 40ml. of fresh growth medium. The mycelial mass was rendered to a finer suspension by a brief homogenization with the Potter–Elvehjem apparatus. The mycelial suspension then was incubated for 3hr. at 37° with shaking to ensure maintenance of metabolic activity.

After the incubation period, 2ml. portions of the mould suspension were distributed into test tubes and held in a water bath at 37°. To each tube was added 0·03ml. of a mixture of sodium [1-$^{14}$C]-glyoxylate (0·75$\mu$c, 0·11$\mu$mole) and sodium [2-$^{14}$C]glyoxylate (0·75$\mu$c, 0·16$\mu$mole). After incubation for either 30, 60 or 120sec., reactions were terminated by addition of 4ml. of 20% (w/v) KOH in 70% (v/v) methanol. The mycelial suspensions were then refluxed for 45min. in a boiling-water bath to liberate cytoplasmic constituents and deacylate CoA thio esters. The samples were centrifuged for 10min. at 3440*g* and the supernatants dried by flash evaporation at 80° to remove the methanol.

The following procedure was employed to desalt the samples and liberate the methanol-soluble products as free acids. The dried samples were dissolved in 2ml. of water, acetic acid was added dropwise to pH5–6 and a slurry of Dowex 50 ($H^+$ form) was added until pH2 was obtained. The resulting mixture was poured over a column bed of Dowex 50 ($H^+$ form) of approximately the same volume as the samples and washed with distilled water until the eluate was no longer radioactive. The eluates containing radioactivity were pooled, dried by flash evaporation at 40° and finally dissolved in 1·0ml. of water.

Labelled 2-hydroxyglutarate was determined after first converting it into the $\gamma$-lactone by the following method: 0·3ml. of the aqueous reaction mixture recovered from the Dowex column was made acidic with 2 drops of 2N-HCl and then boiled with 10ml. of benzene (redistilled) on a hot-plate until only 2ml. of benzene remained.

The radioactivity of authentic 2-hydroxy[5-$^{14}$C]-glutaric acid was recovered almost completely by this procedure and the product possessed chromatographic properties identical with those of authentic 2-hydroxyglutaric acid lactone (kindly supplied by Dr I. I. Salamon). Little radioactivity chromatographing with 2-hydroxyglutaric acid remained in these benzene solutions, indicating almost quantitative conversion into the lactone by this method.

Incorporation of [$^{14}$C]glyoxylate into 2-hydroxyglutaric acid and other carboxylic acids was determined by two-dimensional thin-layer chromatography. Gelman ITLC (type SG) chromatography media (20cm. × 20cm.) (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) were spotted with either aqueous reaction mixture (10$\mu$l. containing 8000–10000 counts/min.) or benzene-soluble lactonization product (100$\mu$l. containing 1000–2000 counts/min.). A standard 2-hydroxyglutaric acid lactone (5$\mu$l.) was spotted over the labelled material. The media were developed with, first, light petroleum (b.p. 30–60°)–anhydrous diethyl ether–formic acid (28:12:1, by vol.) and, in the second dimension, chloroform–methanol–formic acid (80:1:1, by vol.). The solvent fronts were allowed to ascend 15cm., and the media dried in a hood for 10min. after each development and finally sprayed with 0·04% bromophenol blue (ethanolic) to locate the spots. Areas to be measured for radioactivity were cut from the medium and placed in 10ml. of 0·04% 2,5 - bis - (5 - *tert.* - butylbenzoxazol - 2 -yl)thiophen (Packard Instrument Co., Downers Grove, Ill., U.S.A.) made up in toluene–ethanol (2:1, v/v). These were counted in a Packard Tri-Carb liquid-scintillation spectrometer.

Table 1. *Incorporation of* [$^{14}$C]*glyoxylate into various carboxylic acids by whole mycelia of* Aspergillus glaucus

Reaction mixtures and methods of assay are described in the text. Yields are expressed as counts/min. per $10^6$ counts/min. in the total reaction mixture eluted from the Dowex 50 ($H^+$ form) column. Each recovery is corrected for background emission.

| Compound | Radioactivity recovered (counts/min.) At 30sec. | At 60sec. | At 120sec. |
|---|---|---|---|
| Malate | 266400 | 273000 | 223000 |
| Fumarate | 12400 | 16300 | 11800 |
| Succinate | 14200 | 16100 | 10600 |
| 2-Oxoglutarate | Nil | Nil | Nil |
| Glycollate | 36200 | 40000 | 37500 |
| Citramalate | 2500 | 870 | 1100 |
| 2-Hydroxyglutarate* | 3190 | 1860 | 1340 |

* Recovered as the lactone derivative (see the text).

Results of the pulse-labelling of *A. glaucus* with [$^{14}$C]glyoxylate are presented in Table 1. It is apparent that [$^{14}$C]glyoxylate is immediately and extensively incorporated into malate. This is not unexpected, since propionate-grown *Aspergillus* possesses an active malate synthase (Richards & Lloyd, 1966). Significant amounts of labelled succinate and fumarate are also recovered early and may, in part, be derived from malate by the reductive functioning of the tricarboxylic acid cycle. In addition, considerable radioactive glycollate is recovered and reflects the presence of an active glyoxylate reductase in propionate-grown *Aspergillus*.

Of particular interest in the present study is the recovery of significant amounts of labelled 2-hydroxyglutarate. This compound is formed within 30sec. and the radioactivity incorporated into it diminishes with time. If 2-hydroxyglutarate is dissimilated by *Aspergillus* as it is by *E. coli*, then its early metabolism may yield significant proportions of the labelled succinate and citramalate shown in Table 1. Table 1 reveals that incorporation of [$^{14}$C]glyoxylate into all the intermediates studied is essentially complete by the end of 30sec. It should be noted that incorporation of radioactivity into 2-hydroxyglutarate was not obtained when boiled mycelia were employed in pulse-labelling experiments otherwise identical with those described above.

The present study cannot fully assess the importance of 2-hydroxyglutarate in the growth of *A. glaucus* on propionate. Experiments in which sodium [$^{14}$C]propionate was used for pulse-labelling will allow a more critical evaluation of the importance of the pathway. The present study does, however, conclusively demonstrate the ability of *Aspergillus* to form the first intermediate in the 2-hydroxyglutarate pathway (Rabin *et al.* 1965).

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