and 7 at 40° and the pH optimum for the glucose-oxidizing activity was at 5.6 at 30°. The activity was measured manometrically in a Warburg apparatus at 30°. The reaction mixture contained 0.2 ml of enzyme solution, 1.2 ml 0.1 *M* sodium acetate buffer, pH 5.6, and 0.2 ml of catalase solution (prepared by the method of KEILIN AND HARTREE[2]) in the main compartment, 0.2 ml 20 % glucose in the side arm, and 0.2 ml 20 % KOH in the center well. Under these conditions, the turnover number (moles O_2 consumed/mole enzyme/min) was 17,000 (the Qo_2 (μl O_2 uptake/mg protein/h) = 148,364 at 30°). The enzyme solution at pH 5.6 could be stored for 2 weeks at 5° without any loss of the activity. The flavin-adenine dinucleotide could be removed from the protein at below pH 2.8 at 0° in the presence of 80 % satd. ammonium sulfate. The activity was regained by the addition of FAD, in proportion to the amount of added FAD. Reactivation was complete on addition of an equal amount of FAD to that liberated. FMN had no effect on the reactivation. The activity was completely inhibited by 10^{-3} *M* *p*-chloromercuribenzoate and partially by 10^{-3} *M* aldehyde reagent such as dimedon, phenylhydrazine, N_2H_4, $NaHSO_3$ and NH_2OH.

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The formation of γ-hydroxy-γ-methylglutamic acid from a common impurity in pyruvic acid

Incubation of a recently purchased preparation of sodium [2-^{14}C]pyruvate with cell-free extracts of *Clostridium propionicum* resulted in the formation of a radioactive dicarboxylic amino acid. Several milligrammes of this product were isolated and identified as γ-hydroxy-γ-methylglutamic acid.

Methods for the cultivation of *C. propionicum* are described elsewhere[1]. Cell-free extracts were prepared by crushing the cells in a Hughes press[2], followed by addition of 2–3 vol. 0.05 *M* potassium phosphate buffer, pH 7.4, and centrifugation at 20,000 × *g* in order to remove whole cells and cell-debris.

The amino acid produced on incubation of [2-^{14}C]pyruvate with the extract was isolated by the following procedure. The incubation mixture was treated with 0.1 vol. 10 % $HClO_4$ and centrifuged to remove protein. The supernatant solution was then

passed over Dowex 50-H^+ and the material eluted by 1 N NH_4OH was collected and evaporated. An aqueous solution of this material was then put on a 24 × 1.1 cm column of Dowex 1-acetate, X8, 100–200 mesh. The column was washed with 50 ml water and gradient elution was performed by dropping 0.5 N acetic acid into 200 ml water with stirring. At 265 ml a radioactive material appeared in the eluate. At 330 ml the solution in the dropping funnel was changed to 6 N acetic acid and the remainder of the radioactive material was eluted. These fractions were pooled since the radioactive amino acid they contained appeared to be identical on the basis of paper chromatography.

γ-Hydroxy-γ-methylglutamic acid was synthesized chemically by the method of GROBBELAAR *et al.*[3]. First the dimer of pyruvic acid, γ-hydroxy-γ-methyl-α-oxoglutaric acid was prepared by the method of DE JONG[4] and the dinitrophenylhydrazone derivative was isolated and crystallized, m.p. 173°–177° (uncorr.). (Found: C, 40.34; H, 3.47; N, 15.37. Calc. for $C_{12}H_{12}O_9N_4$: C, 40.45; H, 3.40; N, 15.73.) γ-hydroxy-γ-methylglutamic acid was produced by catalytic reduction of this compound[5]. It co-chromatographed on paper with the radioactive enzymic product in four solvent systems.*

Since the amount of radioactive γ-hydroxy-γ-methylglutamic acid formed on incubation of [2-^{14}C]pyruvate with the extract varied with different lots of commercial [2-^{14}C]pyruvate, it appeared likely that the amino acid was derived from a contaminant. Paper chromatography of the dinitrophenylhydrazone of the [2-^{14}C]pyruvate used in these experiments, in the solvent system n-butanol–ethanol–0.5 N ammonia (70:10:20) revealed that 80–90 % of the radioactivity migrated with an R_F of 0.20, while only 10–20 % migrated with R_F's of 0.44 and 0.64. The latter two spots are ascribed to the syn and anti isomers of pyruvic acid dinitrophenylhydrazone. The lower spot often appears in chromatograms of pyruvic acid dinitrophenylhydrazone[6].

The synthetic dinitrophenylhydrazone of γ-hydroxy-γ-methyl-α-oxoglutaric acid was compared with the dinitrophenylhydrazone of the contaminant by paper chromatography in two solvent systems: n-butanol–ethanol–water (70:10:20), in which the R_F of both was 0.42, and in n-butanol–ethanol–0.5 N ammonia (70:10:20), in which the R_F of both was 0.19. Both dinitrophenylhydrazones were subjected to catalytic reduction[5] and the resulting amino acids were chromatographed on paper in 80 % phenol. The R_F of both was 0.16.

The contaminant in [2-^{14}C]pyruvic acid is probably γ-hydroxy-γ-methyl-α-oxoglutaric acid on the basis of this evidence. DE JONG observed the formation of this compound from pyruvic acid at room temperature[4].

In view of these results the enzymic formation of γ-hydroxy-γ-methylglutamic acid is thought to occur by transamination. The reactions are:

$$\underset{\text{Pyruvic acid}}{2\ CH_3\cdot{}^{14}CO\cdot COOH} \xrightarrow[\text{condensation}]{\text{Non-enzymic}} \underset{\gamma\text{-hydroxy-}\gamma\text{-methyl-}\alpha\text{-oxoglutaric acid}}{HOOC\cdot{}^{14}C(OH)(CH_3)\cdot CH_2\cdot{}^{14}CO\cdot COOH}$$

$$\downarrow \text{ Transaminase}$$

$$\underset{\gamma\text{-hydroxy-}\gamma\text{-methylglutamic acid}}{HOOC\cdot{}^{14}C(OH)(CH_3)\cdot CH_2\cdot{}^{14}CHNH_2\cdot COOH}$$

* R_F values of the enzymic and synthetic products were: 0.17 with 80 % phenol, 0.19 with n-butanol–acetic acid–water (63:27:10), 0.53 with methanol–water–pyridine (80:20:4) and 0.60 with isopropanol–pyridine–water (1:1:1).

Glutamate was found to be an efficient amino donor in the enzymic step. An alanine-glutamate transaminase has been demonstrated in extracts of this organism[1] and the reaction reported here is probably a non-specific transamination. When purified radioactive pyruvate was used there was no evidence of condensation catalyzed by these extracts. Other aqueous solutions of radioactive pyruvate have been found to be contaminated with γ-hydroxy-γ-methyl-α-oxoglutaric acid and the proportion of contaminant increases with time, even at —20°.

γ-Hydroxy-γ-methylglutamic acid has been found to constitute a large part of the free amino acid pool of the fern *Adiantum pedatum*[3]. It has also been isolated from other plants[7]. LINKO AND VIRTANEN have reported on the synthesis of labelled γ-hydroxy-γ-methylglutamic acid from ^{14}C-labelled pyruvate in whole plants and suggest that this and related amino acids are synthesized from pyruvate by an aldol condensation followed by transamination[8]. FOWDEN AND WEBB[9] have performed similar experiments in a study of the synthesis of γ-methyleneglutamic acid by groundnut plants.

From the results reported here, it is clear that pyruvate should be carefully checked for contamination with γ-hydroxy-γ-methyl-α-oxoglutaric acid in studies concerned with the biosynthesis of possible condensation products of pyruvate. The solvent system, ether–acetic acid–water (13:3:1) provides a simple separation on paper of pyruvic acid, R_F 0.5–0.6, from γ-hydroxy-γ-methyl-α-oxoglutaric acid, R_F 0.1–0.2.

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