METABOLISM OF d-RIBOSE-1-C¹⁴ AND C¹⁴-LABELED d-GLUCONATE IN AN ENZYME SYSTEM OF THE GENUS PROPIONIBACTERIUM

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ABSTRACT

STJERNHOLM, RUNE L. (Western Reserve University, Cleveland, Ohio) AND FRANK FLANDERS. Metabolism of d-ribose-1-C¹⁴ and C¹⁴-labeled d-gluconate in an enzyme system of the genus Propionibacterium. J. Bacteriol. 84:563-568. 1962.—Ribose-1-C¹⁴ and potassium gluconate labeled in different positions were incubated with cell-free extracts of Propionibacterium shermanii. The resulting propionate, acetate, and succinate were isolated and the C¹⁴ distribution determined by degradation. It is proposed that the extensive randomization observed is caused by the conversion of the labeled substrates to fructose-6-phosphate via the transketolase-transaldolase sequence followed by the Embden-Meyerhof pathway and that the triosephosphates produced by these metabolic routes are metabolized via pyruvate to succinate and propionate.

When carbohydrates are fermented by members of the genus Propionibacterium, propionic acid, carbon dioxide, and smaller amounts of acetic and succinic acids are produced. One of the difficulties in evaluating the pathways of the propionic acid fermentation from the hexose level has been that the C¹⁴ of specifically labeled glucose is randomized to every position of the propionate, and therefore the labeling patterns do not provide a clear picture of possible metabolic events. In resting-cell experiments with Propionibacterium shermanii, Wood, Stjernholm, and Leaver (1956) found that when glucose-1-C¹⁴ was the substrate there was an almost equal distribution of tracer into all carbons of the propionate, and with glucose-3,4-C¹⁴ there was appreciable activity in the alpha and beta carbons of the propionate. In addition, since the CO₂ from glucose-1-C¹⁴ had a higher specific activity than that from glucose-6-C¹⁴, it was evident that a second type of cleavage occurred. Various members of the genus Propionibacterium are capable of dissimilating sodium gluconate (Van Demark and Fukui, 1956), and several of the enzymes in the hexose monophosphate shunt have been identified in these organisms (Van Demark and Fukui, 1956); therefore, it seemed likely that the additional pathway of glucose dissimilation was a complete or partial pentose cycle.

In the present study, d-ribose-1-C¹⁴ and potassium-β-glucurate labeled in different positions were incubated with cell-free extracts of P. shermanii (52W). Propionic acid and acetic acids were isolated and used as indicators of metabolism. The C¹⁴ distribution in each of the carbons of these metabolites was determined by degradation. The labeling patterns in the acids indicate that the conversion of d-ribose and d-glucurate to propionic acid can be explained by the synthesis of fructose-6-phosphate via the transketolase-transaldolase sequence followed by the Embden-Meyerhof pathway and the transcarboxylation cycle (Swick and Wood, 1960).

MATERIALS AND METHODS

Cell-free extracts of P. shermanii (52W) were used, because Wood, Kuika, and Edson (1956) had shown that extracts of this strain are able to ferment glucose to propionic acid and remain active for several hours at 30C.

P. shermanii was maintained in lactate-grown stab cultures. Liquid cultures were grown for 3 days at 30C in a medium containing 0.5% glycerol, 0.5% Yeast Extract, 0.05% potassium phosphate buffer (pH 6.8), and 1 mg per liter of each of the following vitamins: calcium pantothenate, thiamine hydrochloride, and biotin. The cells were harvested by centrifugation at 6,000 X g for 10 min at 3 C, washed by suspension in distilled water, and recentrifuged; 1 liter of medium produced 3 to 4 g of cells (wet wt). A cell-free extract was prepared with the aid of a Nossal shaker (Nossal, 1953); 5 g of cells were suspended in 10 ml of 0.05 M potassium phosphate and 0.001
ml-cysteine at pH 6.8 and mixed with an equal volume of glass beads (Ballotini no. 13). The mixture was shaken for 15 sec and then cooled to 0 C. This process was repeated four times. The glass beads were removed by centrifugation at 13,000 x g for 15 min, and the remainder of the cell debris by centrifugation at 35,000 x g for 30 min. This preparation contained 13 to 20 mg of protein per ml (Gornall, Bardawill, and David, 1949) and was used without further treatment.

All radioactive sugars were obtained from the National Bureau of Standards, Washington, D.C. Crystalline potassium D-gluconate-1-C14, -2-C14, and -6-C14 were prepared according to the method of Moore and Link (1940) from the correspondingly labeled glucose. The unlabeled substrates, glucose-6-phosphate (G-6-P), 6-phosphogluconate (6-PG), gluconic acid, di- and trichophosphopyridine nucleotides (DPN, TPN), and adenosine triphosphate (ATP), were commercial preparations. Dehydrogenase activity was determined spectrophotometrically by the reduction of DPN and TPN at 340 mp according to Adler et al. (1939). Fermentations were carried out in conventional Warburg vessels. To isolate the products of the fermentation, the acidified incubation mixture was centrifuged and the protein precipitate washed with water. The combined supernatants and washings were extracted continuously with ether for 48 hr, and the metabolic acids thus obtained were separated on a Celite column (Swim and Krampitz, 1954), purified, and degraded as previously described (Wood et al., 1955). The C14 was counted as CO2 in a proportional counter (Bernstein and Ballentine, 1950) after combustion with chromic acid (Van Slyke and Foleh, 1940).

RESULTS AND DISCUSSION

The presence of gluconokinase and two TPN-dependent dehydrogenases of the hexose monophosphate shunt is shown in Fig. 1. Both G-6-P and 6-PG reduced TPN. This was also true for gluconic acid in the presence of ATP. When DPN was substituted for TPN, no reduction

**TABLE 1. Recovery and specific activity of metabolic acids from fermentations of D-ribose and D-gluconate by Propionibacterium shermanii**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Propionate</th>
<th>Count</th>
<th>Acetate</th>
<th>Count</th>
<th>Succinate</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribose-1-C14</td>
<td>345</td>
<td>354</td>
<td>49.6</td>
<td>183</td>
<td>31.7</td>
<td>192</td>
</tr>
<tr>
<td>Gluconate-2-C14</td>
<td>146</td>
<td>627</td>
<td>20.2</td>
<td>170</td>
<td>4.5</td>
<td>850</td>
</tr>
<tr>
<td>Gluconate-6-C14</td>
<td>98</td>
<td>181</td>
<td>16.5</td>
<td>424</td>
<td>24.8</td>
<td>480</td>
</tr>
<tr>
<td>Gluconate-1-C14</td>
<td>99</td>
<td>130</td>
<td>11.6</td>
<td>190</td>
<td>1.0</td>
<td>310</td>
</tr>
</tbody>
</table>

* All fermentations were in 125-ml flasks under N2 at 30 C for 24 hr. Each flask contained 1,000 amoles of labeled substrate, 200 amoles of fructose-1,6-diphosphate sodium salt, and 5,000 amoles of potassium phosphate (pH 6.8). The bacterial extract contained about 200 mg of protein. Total volume was 30 ml.
† Expressed as count per min per amole.
‡ Expressed as amoles.

**FIG. 1. TPN reduction by 6-phosphogluconic acid (6-PG), glucose-6-PO4, (G-6-P), and gluconate with ATP.** The cuvettes contained: 20 amoles of the substrate indicated, tris(hydroxymethyl)aminomethane buffer (pH 7.4; 400 amoles), MgCl2 (20 amoles), cysteine (10 amoles), and TPN (0.8 amole). The extract (3 mg of protein) was a dialyzed ammonium sulfate (80%) precipitate. Water was added to make a total volume of 3 ml. Temperature was 28 C.
occurred, and little reduction occurred in the absence of added substrate.

The fermentations of ribose-1-C\textsuperscript{14} and gluconate-C\textsuperscript{14} by undialyzed extracts were carried out in the presence of fructose-1,6-diphosphate to maintain a supply of ATP. The yields of products and their specific activities are recorded in Table 1. No attempt was made to calculate recovery of C\textsuperscript{14}, oxidation-reduction balance, or carbon recovery. There was a wide variation in fermentation products in the four incubations. The C\textsuperscript{14}-distribution patterns in the products are expressed in percentages of the specific activity of each compound to circumvent these variations. With regard to the yield of products, it was noted that there was an increased production of succinate compared with that normally found with resting or proliferating cells fermenting either glucose or pentose. A similar observation was made by Wood et al. (1956) and is probably due to a net CO\textsubscript{2} fixation via the phosphoenolpyruvic carboxytransphosphorylase reaction (Siu, Wood, and Stjernholm, 1961).

A possible pathway for conversion of pentose to propionate is shown in Fig. 2. It is proposed that ribose is phosphorylated to ribose-5-phosphate, that an isomerase converts this compound to ribulose-5-phosphate, and that an epimerase would then form xylulose-5-phosphate, thus yielding the compounds which can serve as donors and acceptors in transketolase reactions. This is a reasonable assumption, since it has been shown by Volk (1959) that P. pentosaceum contains a d-ribokinase phosphoribulokinase and ribulose-5-phosphate-3-epimerase. For the purpose of illustration, the carbon atoms of the pentoses have been numbered. It is assumed that two molecules of pentose-5-phosphate
react in a transketolase reaction to yield one molecule of sedoheptulose-7-phosphate and one molecule of triosephosphate (reaction 1). These two molecules react with each other in a transaldolase reaction yielding one molecule of fructose-6-phosphate and one molecule of erythrose-4-phosphate (reaction 2). A third molecule of pentose-phosphate reacts with the erythrose-4-phosphate in a transketolase reaction, giving fructose-6-phosphate and triosephosphate (reaction 3). The fructose-6-phosphate molecules are then assumed to be converted via Embden-Meyerhof reactions to four triosephosphates (reaction 4). Thus, three pentoses will yield five trioses, which by further reactions in the Embden-Meyerhof scheme are converted to five pyruvate molecules. Figure 2 also shows where the carbon atoms of the original pentose will be located in the pyruvate. By inspection of the five trioses produced during these enzymatic conversions, it is observed that three trioses are derived from C-5, C-4, and C-3 of ribose which will become the β-carbon, α-carbon, and the carboxyl group, respectively, of the pyruvate. In addition, two other trioses are formed from the two top carbons of the pentose, having the distribution 1:2:1 and 1:2:2. Thus, it is seen that ribose-1-C\textsuperscript{14} would contribute three labeled carbon atoms to the pyruvate, two of which would be located in the methyl group and one in the carboxyl group. On a percentage basis, 66.6\% of the total radioactivity should be located in the methyl group and 33.3\% in the carboxyl group. If the pyruvate is further metabolized via succinate to propionate mediated by transcarboxylase, as outlined by Swick and Wood (1960), then the resulting propionate should be equally labeled in all three carbon atoms. Any acetate formed from the pyruvate should be labeled exclusively in its methyl group. Table 2 shows that ribose-1-C\textsuperscript{14} gave propionate labeled equally in all positions, which is in accordance with the postulated reaction sequence. In addition, acetate acid carried 91\% of its total C\textsuperscript{14} in the methyl group.

If gluconate is oxidatively decarboxylated, then gluconate-2-C\textsuperscript{14} will give ribulose-5-phosphate labeled in C-1, and the expected isotope distribution should coincide with that obtained from ribose-1-C\textsuperscript{14}. It is noted that a fair agreement exists between the two substrates, again indicating that the transketolase-transaldolase reactions are responsible for part of the randomization obtained in the end products. The lower activity in the carboxyl groups of propionate and succinate in the gluconate-2-C\textsuperscript{14} incubation is probably best explained by assuming fixation of unlabeled CO\textsubscript{2} partly derived from C-1 of the gluconate. With gluconate-6-C\textsuperscript{14} as the labeled substrate, β-labeled pyruvate should result, which, after passage through the transcarboxylation cycle, would yield equal labeling in C-3 and C-2 of the propionate, with no activity in the carboxyl group. The methyl group of acetic acid should be labeled exclusively. The observed values are in agreement with the predicted values. When gluconate-1-C\textsuperscript{14} is the substrate, C\textsuperscript{14} should enter the end products only by CO\textsubscript{2} fixation, e.g., via phosphoenolpyruvic carboxytransphosphorylase (Siu et al., 1961). In this experiment, the carboxyl group of propionate contained all the radioactivity; the acetic acid was virtually unlabeled. The succinate showed an identical C\textsuperscript{14} pattern in comparison with the propionate, indicating the precursor-product relationship between the two compounds.

The C\textsuperscript{14} distributions obtained when glucose-1-C\textsuperscript{14} and glucose-6-C\textsuperscript{14} were fermented are recorded in Table 3. The purpose of these incubations was to show that a crude cell-free extract of Propionibacterium shermanii will metabolize glucose in essentially the same manner as a resting-cell suspension of bacteria. The values obtained by Wood et al. (1955) with resting cells have been included for comparison. Wood and Katz (1958) discussed how randomization of C\textsuperscript{14} within the top three carbons of the hexose monophosphates may occur.

### Table 2. Distribution of C\textsuperscript{14} in the products from fermentations of p-ribose and p-gluconate by Propionibacterium shermanii*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Propionate</th>
<th>Acetate</th>
<th>Propionate from succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-3</td>
<td>C-2</td>
<td>C-1</td>
</tr>
<tr>
<td>Ribose-1-C\textsuperscript{14}</td>
<td>35</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Gluconate-2-C\textsuperscript{14}</td>
<td>38</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>Gluconate-6-C\textsuperscript{14}</td>
<td>49</td>
<td>49</td>
<td>2</td>
</tr>
<tr>
<td>Gluconate-1-C\textsuperscript{14}</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* For conditions of these fermentations see Table 1. The recoveries of C\textsuperscript{14} from the carbon-by-carbon degradations were 96 to 102\% of the values obtained from total oxidations.
by exchange via transaldolase-transketolase reactions together with phosphoribulosylamine and ribulose-5-phosphate-3-epimerase reactions. Thus, it is possible for glucose-1-C\textsuperscript{14} to yield fructose-6-PO\textsubscript{4} labeled in C-1 and C-3 positions (see Wood and Katz, 1958, Fig. 2). Further metabolism of this hexose monophosphate would bring about extensive randomization into all carbon atoms of propionate, similar to the results obtained with ribose-1-C\textsuperscript{14} or glucose-2-C\textsuperscript{14}. The most notable discrepancy between the resting cells and the extract is in the fermentation of glucose-6-C\textsuperscript{14}. With resting cells, a larger proportion of the C\textsuperscript{14} is situated in the carboxyl group of the propionate. If glucose-6-C\textsuperscript{14} yields \( \beta \)-labeled pyruvate, which is further metabolized by recycling in the Krebs cycle, these intermediates will become labeled in the carboxyl groups. Since the extract is almost free of particles, this route of the fermentation pathway may be absent. The C\textsuperscript{14}-distribution pattern observed with differently labeled glucose can now be better understood, since a complete hexose monophosphate shunt exists in the propionic acid bacteria, allowing extensive randomization to occur. The propionic acid fermentation may therefore occur via reactions in the Embden-Meyerhof pathway and the pentose cycle. The resulting trioses are then metabolized to pyruvate, which may be decarboxylated to acetaldehyde, or, alternatively, may be transformed, with additional randomization, via oxaloacetate and succinate to propionate.

Rappoport and Barker (1954) fermented L-arabinose-1-C\textsuperscript{14} with growing cells of \( P. \) pentosaceum. The isotope distribution in the metabolic acids is in accord with the results reported here and can be explained by the same sequence of reactions.

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LITERATURE CITED


