Studies on the Biochemistry of *Penicillium charlesii*

INFLUENCE OF VARIOUS DICARBOXYLIC ACIDS ON GALACTOCARULOSE SYNTHESIS

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1. It has been shown that *Penicillium charlesii* continues to synthesize galactocarulose when L-malic acid, malonic acid, succinic acid, fumaric acid, maleic acid or oxaloglycollic acid is substituted for DL-tartaric acid in the Raulin-Thom nutrient medium. 2. The quantity of galactocarulose synthesized per g. of mycelia was markedly decreased by substitution of L-malic acid, malonic acid, succinic acid, fumaric acid or maleic acid for DL-tartaric acid. Substitution of oxaloglycollic acid for DL-tartaric acid did not depress the galactocarulose synthesized/g. of mycelia; however, the quantity of fungal mass formed was decreased approximately fivefold. 3. Based upon ^{14}C incorporation into galactocarulose, succinic acid, fumaric acid or malonic acid did not serve as direct precursors of galactose as did tartaric acid. Oxaloglycollic acid, L-malic acid and maleic acid were not tested. 4. The relative quantity of galactocarulose synthesized per g. of mycelia decreased as the concentration of diammonium dicarboxylate added to the growth medium was increased. Tartaric acid, oxaloglycollic acid, fumaric acid and malonic acid were tested. 5. The quantity of mycelia formed and the quantity of galactocarulose synthesized per g. of mycelia were greater when the growth medium contained L-tartrate than when it contained D-tartrate.

The Raulin-Thom medium (Birkinshaw & Raistrick, 1933a,b) is widely employed as a nutrient source for fungi. Despite the widespread use of this medium there exists significantly little recorded information which relates to biochemical function(s) of tartaric acid in the medium. However, numerous investigations have been recorded which define tartrate as a stimulant or depressant of some biochemical function, a product of some catabolic process or a substrate for an enzyme system.

Kun & Hernandez (1956) have shown that tartrate is oxidized in the presence of kidney mitochondria and NAD. The product of the reaction was identified as oxaloglycollate. In addition several species of bacteria and fungi metabolize the tartaric acids. *Pseudomonas putida* was shown to convert the DL-enantiomorphs of tartrate into oxaloacetate (la Riviere, 1956). Other strains of *Pseudomonas* have been shown to effect a similar dehydration of tartrate (Shilo, 1957; Rosenberger & Shilo, 1963). Dagley & Trudgill (1961, 1963)

have shown that *Pseudomonas* A decarboxylates L-tartrate to glycerate. *Aspergillus niger* strain 72-4 contains an enzyme system which converts tartrate into oxalate via a fluoride- and fluoroacetate-insensitive process (Cleland & Johnson, 1956).

The formation of tartrate by *Aspergillus niger* from glucose (Bernhauer & Bockl, 1932) and from 5-oxogluconate (Gokhokidze, 1949) has been reported. An enzyme system from *Aspergillus fumigatus* was shown to convert *trans*-1-epoxy-succinate into *meso*tartrate (Martin & Foster, 1955).

It has been previously demonstrated in this Laboratory (Gander, 1960) that *Penicillium charlesii* G. Smith utilizes tartrate as a source of carbon in the biosynthesis of a polygalactofuranoside, galactocarulose. This polysaccharide is unique in that it has been shown to be composed exclusively of β -D-galactofuranosyl units linked 1 $\rightarrow$ 5 (Haworth, Raistrick & Stacey, 1937). The evidence suggests that four carbon atoms of galactose are derived from tartrate and two carbon atoms are derived from glucose (Gander, 1960).

There are no reported experiments demonstrating the requirement, or lack of requirement, for tartrate in the biosynthesis of galactocarulose when NH_4^+ is the sole nitrogen source in the

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growth medium. However, it has been demonstrated that galactocarulose is formed when *P. charlesii* is grown on the Czapek-Dox medium which contains glucose as the only carbon source and nitrate as the nitrogen source (Clutterbuck, Haworth, Raistrick, Smith & Stacey, 1934).

This paper describes experiments designed to determine if tartaric acid is an obligatory requirement in galactocarulose synthesis and the relationship that exists between ammonium dicarboxylate concentration or ammonium carbonate concentration in the growth medium and the quantity of galactocarulose released into the growth medium.

MATERIALS AND METHODS

Maintenance and growth of cultures. Cultures of *Penicillium charlesii* G. Smith (A.T.C.C. strain 1887) were maintained on 2% agar plates fortified with the Czapek-Dox medium. Spores were transferred to 500 ml. wide-mouth Erlenmeyer flasks which contained 120–150 ml. of Raulin-Thom growth medium (Clutterbuck *et al.* 1934) modified by containing 4.0 g. of $(\text{NH}_4)_2\text{HPO}_4/1.5\text{l.}$ The growth media were autoclaved at 15 lb./in.² and 120° for 15 min. at the time of preparation of the media. Cultures were maintained at 23° throughout the growth period of approx. 28 days.

When the organism was grown on a ¹⁴C-labelled substrate the flasks were placed in 15 in. x 18 in. cylindrical jars which contained 200 ml. of 5N-NaOH in a separate beaker to trap the CO₂ evolved.

Pseudomonas saccharophila Doudoroff (A.T.C.C. 9114), obtained through the courtesy of Dr M. Doudoroff, were maintained and grown essentially according to the procedure of Wallenfels & Kurz (1962).

Oligosaccharide isolation and degradation. At the end of the experimental period the growth medium was separated from the mycelia by filtration and the medium was concentrated about 40-fold under reduced pressure at 20–30°. Either 6.7 vol. of absolute ethanol or 5 vol. of acetone was added to the solution and the mixture held at –4° for 12 hr. The polysaccharides precipitated as a result of this treatment were separated from the solvent by centrifuging. The residue containing the polymeric material was twice resuspended in aq. 85% (v/v) ethanol or aq. 80% (v/v) acetone and the centrifuging repeated. This process served to remove any free aldoses or lower oligosaccharides present in the preparation. The residue obtained was dissolved in a small volume of distilled water and stored at –15°.

The sample was treated with 0.1N-H₂SO₄ at 95° for 60 min., which quantitatively released galactose, but mannocarulose, a second oligosaccharide produced by *P. charlesii* under the conditions of growth defined previously, was not affected by this treatment. It has been previously shown (Clutterbuck *et al.* 1934) that 3N-H₂SO₄ is required to hydrolyse mannocarulose under similar conditions. Solid BaCO₃ was added to neutralize the cooled hydrolysate. The BaSO₄ was removed by centrifuging and the neutral solution treated with a mixed Dowex 1 (OH-form) and Dowex 50 (H⁺ form) resin. The deionized solution obtained after removal of the resin was concentrated to dryness under reduced pressure at 15–20° and the residue dissolved in a minimum of double-distilled water.

Carbohydrate separation and analyses. Galactose could be separated from mannocarulose and any other polymeric carbohydrates by paper chromatography. Chromatography was carried out on either Whatman no. 1 or Whatman no. 3MM paper with the following solvents: A, butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1957); B, the aqueous phase of ethyl acetate-pyridine-water (36:10:11.5, by vol.) (Ginsberg, 1960); C, butan-1-ol-acetic acid-water (52:13:35, by vol.) (Putman, 1957); D, methanol-formic acid-water (16:3:1, by vol.) (Bandurski & Axelrod, 1952). The chromatograms were developed at 22–24°. Reducing sugars were detected on the chromatograms with a solution containing 0.5M-AgNO₃ in aq. 0.05N-NH₃ soln. Non-reducing carbohydrates were sought with the aid of the periodate-benzidine reagent (Cifonelli & Smith, 1954).

Carbohydrate was eluted from the paper with water at 4°.

Total sugar was determined by the phenol-H₂SO₄ method (Dubois, Gilles, Hamilton, Rebers & Smith, 1951) and the cysteine-H₂SO₄ procedure (Dische, Shettles & Osnos, 1949). Total hexose was determined by the latter method and by the procedure of Gurin & Hood (1939). Reducing sugar was determined by the Nelson (1945) procedure. Glucose was specifically assayed with β -D-glucose-oxygen oxidoreductase (EC 1.1.3.4) (glucose oxidase) or a combination of crystalline yeast ATP-D-glucose 6-phosphotransferase (EC 2.7.1.2) and D-glucose 6-phosphate-NADP oxidoreductase (EC 1.1.1.49), in which the assay was performed spectrophotometrically.

Galactose was specifically determined by use of the D-galactose-NAD oxidoreductase (EC 1.1.1.48) (D-galactose dehydrogenase) obtained from *Pseudomonas saccharophila* Doudoroff by the procedure of Su & Hassid (1962) and the chemical procedure described by Fisher, Hansen & Norton (1955). D-Galactose dehydrogenase was prepared according to the procedure of Doudoroff, Contopoulou & Burns (1957).

Estimation of the extent of galactocarulose synthesis. At the end of the experimental period the growth medium was separated from the fungal mats and the expressible fluid removed by pressing the mats between several layers of cheese-cloth and filter paper. This procedure was repeated several times until there was no moisture in the fresh absorbent filter paper. The weight obtained from these fungal mats is designated 'wet wt. of mycelia'. These fungal mats contained about 70% of water that could be removed by drying in an oven at 70° for 48 hr.

The growth medium was concentrated to a small volume and the concentrate applied as a strip to Whatman no. 3MM chromatography paper. The chromatograms were first developed through ascending chromatography with solvent D. After drying, the chromatograms were developed in the same direction with solvent A. This procedure moved the oligosaccharides, aldoses, ketoses and acidic compounds out from the point of application of the sample, leaving galactocarulose and mannocarulose behind. These were eluted from the paper with distilled water at 4°. Qualitative analyses revealed that only galactose and mannose were released when treated with 0.1N-H₂SO₄ or 3N-H₂SO₄ respectively as previously described.

The quantity of galactocarulose formed is given as μ moles of galactose released by 0.1N-H₂SO₄/g. wet wt. of fungal mats.

Assay of radioisotopic compounds. The specific activity

of ^{14}C -labelled malonate, succinate and fumarate was estimated by counting a known quantity of acid in a Packard model 314 Tri-Carb liquid scintillation counter. The sample was dissolved in 20 ml. of absolute ethanol-toluene (3:2, v/v). The solvent contained 2.0 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.2 g. of 2,5-diphenyloxazole/l. The liquid scintillation system gave approx. 40% efficiency with ^{14}C -toluene as a standard in each counting series. The observed counting rate was divided by the counting efficiency to obtain the rate at 100% efficiency. The values are reported on this corrected basis.

Galactose was isolated and estimated quantitatively by the procedures described in the previous section. A sample of the solution containing galactose was counted in the liquid scintillation counter and the quantity of radioactivity is expressed on the same basis as that for the dicarboxylic acids.

Chemicals and reagents. Succinic acid, fumaric acid and malonic acid as well as maleic anhydride were products of Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.). L-Malic acid, D-mannose, tris and D-galactose were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). DL-Tartaric acid was obtained from Malinkrodt Chemical Works (St Louis, Mo., U.S.A.). Oxaloglycollic acid, L-tartaric acid and D-tartaric acid were purchased from Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). $[2,3-^{14}\text{C}_2]$ Succinic acid, $[\text{U}-^{14}\text{C}]$ succinic acid, $[2,3-^{14}\text{C}_2]$ fumaric acid, $[1-^{14}\text{C}]$ malonic acid and DL- $[1,4-^{14}\text{C}_2]$ tartaric acid were obtained from the Nuclear-Chicago Corp. (Des Plaines, Ill., U.S.A.).

RESULTS

Influence of dicarboxylate on pH and glucose uptake. The possibility of there being an absolute requirement for tartaric acid in the biosynthesis of galactocarolose by *P. charlesii* was examined by substituting various dicarboxylic acids for tartrate in the Raulin-Thom medium followed by estimating the quantity of galactose released from the polysaccharide by hydrolysis in dilute acid.

The Raulin-Thom medium was prepared as previously described (Clutterbuck *et al.* 1934) except that an equimolar quantity of oxaloglycollic acid, maleic acid, succinic acid, fumaric acid, L-malic acid or malonic acid was substituted for tartrate. Because oxaloglycollic acid is destroyed by high temperatures it was added aseptically to the cooled Raulin-Thom salts solution. Experimental flasks were set up in triplicate and samples of the growth medium were removed at 24 hr. intervals after inoculation of the medium. The pH and total hexose content of each sample were determined.

Fig. 1 shows that when the growth medium contained DL-tartrate there was a rapid decrease in the total sugar concentration in the medium between the third and eleventh days. The pH of the medium also decreased rapidly between the third and sixth days. This was followed by a gradual increase in pH during the remainder of the

Fig. 1. Changes in pH and glucose in the growth medium when *P. charlesii* was grown on the Raulin-Thom medium containing glucose (277 $\mu\text{moles/ml.}$) and DL-tartrate (35.4 $\mu\text{moles/ml.}$) as the carbon sources. Samples of the growth medium were removed at the indicated interval and the pH (O) and glucose (Δ) content were measured as described in the text.

Fig. 2. Changes in pH and glucose in the growth medium when *P. charlesii* was grown on a modified Raulin-Thom medium containing fumaric acid (17.74 $\mu\text{moles/ml.}$) substituted for tartaric acid in the medium. In addition the medium contained 7 μmoles of ammonium carbonate/ml. as a source of nitrogen to replace a portion of that afforded by ammonium tartrate present in the Raulin-Thom medium. Experimental methods and symbols are the same as those used in Fig. 1 and are described in the text.

experimental period. Fig. 2 shows the relationship of pH and total sugar in the medium when fumarate was substituted for DL-tartrate. This Figure demonstrates that after a rapid initial uptake of glucose there is little carbohydrate removed from the medium between the fourth and fourteenth days and that this is followed by a rapid disappearance of carbohydrate from the growth medium. After an initial small decrease the pH gradually increased but not nearly as rapidly as in the flasks containing tartrate. The flasks containing malate-,

malonate- or succinate-modified Raulin-Thom media showed changes in pH and carbohydrate content of the medium similar to the fumarate system. Chromatography of the various samples taken at 24 hr. intervals revealed that glucose remained the major carbohydrate component of the growth medium during the first 2 weeks' incubation period.

Only limited growth of the organism occurred in flasks which contained oxaloglycollate and less than one-half of the glucose of the medium was removed from the growth medium during the experimental period. Mycelia produced under these conditions were devoid of the normal green matting of spores. The pH dropped from an initial value of about 3.0 to a final pH 2.3.

Galactocarulose synthesized. All flasks containing *P. charlesii* growing on the Raulin-Thom or a modified medium contained polysaccharide in the medium after 7 days. Hydrolysis of the polymer in dilute acid released galactose. The growth medium was analysed for polysaccharide and total mass of fungi expressed on a wet-weight basis at the end of the 28 days' experimental period.

Table 1 shows that the quantity of mycelia formed during the experimental period was greatly depressed when malonate, fumarate, succinate, maleate, malate or oxaloglycollate was substituted for tartrate. The Table also shows that the quantity of galactose formed per g. of mycelia was greatest when either tartrate or oxaloglycollate served as the dicarboxylate in the growth medium.

These results illustrate that the substitution of

various dicarboxylic acids for tartrate in the Raulin-Thom medium does not result in a cessation of galactocarulose formation by *P. charlesii*. However, growth of the organism was markedly decreased when other dicarboxylic acids were substituted for tartrate. The limited growth observed in these experiments may have reflected a condition of limiting nitrogen, since the acids were added in the free acid form along with the ammonium salts of the specified inorganic acids in the nutrient medium. This decreased the NH_4^+ concentration from 22.1 $\mu\text{moles/ml.}$ to 7.6 $\mu\text{moles/ml.}$

Experiments were performed to establish the relationship between the concentration of ammonium dicarboxylate added to the Raulin-Thom and modified Raulin-Thom growth media and the quantity of galactocarulose formed per g. of mycelia by *P. charlesii*. Table 2 shows this relationship. Larger quantities of mycelia were formed at higher concentrations of ammonium dicarboxylate than at lower concentrations of these salts. There appeared to be an inverse relationship between the quantity of galactocarulose formed and the quantity of ammonium dicarboxylate initially present in the medium. The highest ratio of galactocarulose synthesized per g. of mycelia resulted when the organism was grown in the presence of oxaloglycollate. The data of Fig. 3 show that most rapid uptake of glucose occurred in those flasks which contained highest concentrations of diammonium oxaloglycollate. Similar results were obtained with other dicarboxylic acids subjected to test in the growth media. These results suggest that in the

Table 1. *Production of mycelia and galactocarulose by Penicillium charlesii grown for 26 days in the presence of various dicarboxylic acids*

P. charlesii was grown on the Raulin-Thom medium or a modified Raulin-Thom medium in which various dicarboxylic acids were substituted for tartaric acid. The initial and final pH were measured. The wet weight of the mycelia is that weight obtained after expressing the liquid from the fungal mats into cheese-cloth and absorbent paper. This process was repeated until fresh absorbent paper was no longer moistened by the mats. The filtered nutrient medium was concentrated about 40-fold and the solution applied to Whatman no. 3MM chromatography paper. The chromatogram was developed first in solvent D followed by drying and further development in solvent A (see the Materials and Methods section). The materials at the line of application of the sample were eluted with water and the eluate was concentrated and subjected to hydrolysis in 0.3N- H_2SO_4 . The galactose released was estimated with galactose dehydrogenase. Galactocarulose synthesis is expressed as $\mu\text{moles of galactose/g. of mycelia on a wet-weight basis.}$

Dicarboxylate added	Mycelia formed (g.)	pH of medium		Galactose isolated (μmoles)	Galactocarulose synthesis ($\mu\text{moles/g. of mycelia}$)
		Initial	Final		
Tartrate	5.7	4.5	6.3	186.5	32.5
Malonate	2.6	3.6	4.3	49.5	19.0
Fumarate	2.3	3.4	4.4	31.5	13.7
Succinate	2.1	4.0	3.7	21.4	10.2
Maleate	1.8	3.2	3.6	9.0	5.0
Malate	1.6	3.3	3.7	11.5	7.8
Oxaloglycollate	1.1	3.0	2.3	36.7	33.3

Table 2. *Relationship between the concentration of ammonium dicarboxylate added to the growth medium and galactocarolose released by Penicillium charlesii*

Experimental conditions and methods are described in Table 1. To each flask was added 17.72 μ moles of the indicated dicarboxylic acid/ml. and in addition various quantities of diammonium dicarboxylate were added as shown in the Table.

Dicarboxylate	Concn. of ammonium dicarboxylate (μ moles/ml.)	Total mycelia synthesized (g.)	Galactocarolose synthesis (μ moles/g. of mycelia)
Tartrate	4.85	5.2	250.4
	14.56	15.0	92.1
	43.68	11.0	71.0
Oxaloglycollate	4.85	3.7	349.0
	14.56	5.6	225.0
	43.68	17.0	53.5
Fumarate	4.85	6.2	124.5
	14.56	15.7	40.1
	43.68	12.1	71.3
Malonate	4.85	6.1	192.9
	14.56	11.0	100.7
	43.68	11.5	48.2

Fig. 3. Time-course for change in pH and glucose in the growth medium when *P. charlesii* was grown on a modified Raulin-Thom medium containing various quantities of diammonium oxaloglycollate substituted for DL-tartrate. Filled-in symbols represent pH values and the open symbols represent glucose values obtained at the indicated days. Diammonium oxaloglycollate concentrations of 4.85 μ moles/ml. (●), 14.56 μ moles/ml. (▲) and 43.6 μ moles/ml. (■) were initially added to the growth medium. (See Table 2 for the quantity of mycelia synthesized and the efficiency of galactocarolose synthesis.) Experimental methods are the same as those used in Fig. 1 and are described in the text.

previous experiment with oxaloglycollate either the NH_4^+ concentration or oxaloglycollate concentration was limiting growth of the organism.

The previous experiment did not distinguish the effect of the concentration of NH_4^+ from that of the dicarboxylic acid since they were both altered equally. An additional experiment was performed in which the NH_4^+ , in the form of $(\text{NH}_4)_2\text{CO}_3$, was

varied from 9.2 to 63 μ moles/ml. while the dicarboxylic acid concentration added to the medium was held constant at 22.6 μ moles/ml. Table 3 shows that there is a ratio of dicarboxylic acid to NH_4^+ concentrations which results in an optimum quantity of galactocarolose formed per g. of mycelia. This ratio, although not identical for all the dicarboxylic acids tested, is in the range 0.4–0.5 for tartrate, malonate and oxaloglycollate.

Influence of enantiomorphs of tartaric acid upon galactocarolose synthesis. The investigations described in this paper and by Gander (1960), in which the Raulin-Thom medium was used, contained DL-tartaric acid as the dicarboxylic acid. Experiments were conducted to determine if the organism would form galactocarolose when grown on either D-tartrate and D-glucose or L-tartrate and D-glucose. The experiments were carried out as previously described except that the pH and glucose content of the medium were not followed.

It was noted that the morphological characteristics of the organism grown on D-tartrate were very different from those grown on DL- or L-tartrate. In addition the growth medium did not change from the original pale yellow to a deep dark red-brown as is characteristic when *P. charlesii* is grown on DL-tartrate or L-tartrate. The fungal mat did not form green spores nor did it become compact, but instead grew in a large number of segregated colonies.

Table 4 shows that in the presence of L-tartrate the organism synthesizes more galactocarolose per g. of mycelia than in the presence of D- or DL-tartrate. However, both enantiomorphs allowed galactocarolose synthesis.

Incorporation of ^{14}C from ^{14}C -labelled dicarboxylic

Table 3. *Galactocarolose synthesis by Penicillium charlesii in the presence of various concentrations of ammonium salts*

Experimental conditions and methods are described in Table 1. Each flask contained 22.58 μ moles of dicarboxylate/ml., except those containing oxaloglycollate, which contained 14.56 μ moles/ml. The NH_4^+ concentration was varied as shown in the Table.

Dicarboxylate	Concn. of NH_4^+ (μ moles/ml.)	Total mycelia synthesized (g.)	Galactocarolose synthesis (μ moles/g. of mycelia)
Tartrate	9.23	5.2	55.2
	14.09	7.6	74.4
	23.82	8.3	92.2
	41.23	7.0	120.0
	44.80	8.2	71.7
Malonate	53.03	9.3	56.4
	9.23	5.3	51.2
	14.09	6.3	55.0
	23.82	7.6	74.2
	41.23	8.1	88.8
Fumarate	53.03	12.4	36.0
	9.37	6.5	29.4
	14.09	7.0	116.4
	23.82	7.2	102.3
	41.37	10.0	86.9
Oxaloglycollate	53.03	16.0	16.9
	19.9	4.6	94.6
	24.76	7.0	75.7
	33.52	6.4	85.9
	51.93	5.7	138.0
	62.70	7.6	58.3

Table 4. *Production of galactocarolose by Penicillium charlesii in the presence of D-, L- or DL-tartaric acid*

Experimental conditions and methods are described in Table 1.

Dicarboxylic acid	Mycelia formed (g.)	Analysis of carbohydrate fraction identical with galactose		
		Total sugar isolated (μ moles)	Fraction as galactose	Galactocarolose synthesis (μ moles/g. of mycelia)
DL-Tartrate	9.6	693	1.01	72.2
L-Tartrate	9.2	1134	0.95	123.2
D-Tartrate	7.1	777	0.96	109.4

acids into galactose. The previous experiments indicate that tartrate is a better precursor of the galactofuranose moieties of galactocarolose than malic acid, malonic acid, fumaric acid, maleic acid or succinic acid. However, the observation that galactocarolose was synthesized by the organism when any one of these acids was substituted for tartrate made it necessary to determine whether any of these acids would serve as precursors of the carbon skeleton of the galactofuranose moiety.

Twenty-five or 50 μ C of ^{14}C -labelled dicarboxylic acid was added to the modified Raulin-Thom medium containing 32 μ moles of unlabelled di-

carboxylic acid/ml. The labelled dicarboxylic acid was added to the medium either at the time of inoculation or after 16 days, when most of the original dicarboxylic acid would have been taken into the organism from the medium. The cultures were grown on 25 ml. of medium in 250 ml. culture flasks. Results of the analysis of the various fractions after 28 days' incubation are shown in Table 5. In all six media the incorporation of ^{14}C into the galactose moieties of galactocarolose was less than 200 counts/min./ μ mole of galactose. The quantity of galactocarolose in the medium was extremely low, ranging from 16 to 84 μ moles of

Table 5. Incorporation of ^{14}C -labelled dicarboxylic acids by *Penicillium charlesii*

P. charlesii was grown in a modified Raulin-Thom medium in which the indicated ^{14}C -labelled dicarboxylic acid was substituted for tartrate. Labelled substrate was either added initially or after 16 days as indicated. The CO_2 respired was trapped in 5N-NaOH . The specific activity of ^{14}C -labelled malonate, succinate and fumarate was estimated by counting a known quantity of acid in a Packard Tri-Carb liquid scintillation counter. The sample was dissolved in 20 ml. of the scintillation solvent described in the Materials and Methods section. The observed counting rate was divided by the counting efficiency (usually about 40%) to obtain the rate at 100% efficiency. A standard of ^{14}C toluene was included in each counting series to estimate the counting efficiency. Values are reported on this corrected basis. Galactose was isolated and was estimated quantitatively by the procedures described in the Materials and Methods section. A portion of the solution containing galactose was counted in the liquid scintillation system and the quantity of radioactivity is expressed on the same basis as that for the dicarboxylic acids.

^{14}C Dicarboxylic acid added	$10^{-4} \times$ Initial sp. activity of dicarboxylic acid (counts/min./ μmole)	Age of culture when ^{14}C added (days)	Sp. activity of galactose (counts/min./ μmole)
[1- ^{14}C]Malonate	1.65	16	102
[2- ^{14}C]Malonate	1.65	0	185
[1,4- $^{14}\text{C}_2$]Succinate	0.82	0	28
[2,3- $^{14}\text{C}_2$]Succinate	0.82	0	36
[U- ^{14}C]Succinate	1.65	16	—
[2,3- $^{14}\text{C}_2$]Fumarate	0.82	0	—

galactose/flask. This was probably a result of the large mass of fungal mat relative to the volume of nutrient medium. However, the fact that there was at least a 100-fold dilution in specific activity argues against the direct conversion into galactose of any of the dicarboxylic acids tested. Under similar conditions ^{14}C from [1,4- $^{14}\text{C}_2$]tartrate has been shown to be incorporated into galactocarolose (Gander, 1960) with only a twofold dilution. This experiment provides evidence that malonate, succinate and fumarate do not serve as effective precursors of the galactose moiety of galactocarolose.

DISCUSSION

The experiments described in this paper were designed to elucidate the metabolism of the tartrate contained in Raulin-Thom medium by *P. charlesii* and to determine if it could be replaced by malonic acid, fumaric acid, succinic acid, L-malic acid, maleic acid or oxaloglycollic acid. The choices of dicarboxylic acids to be substituted for tartaric acid were based on compounds that (a) bear a structural resemblance to tartrate, (b) are metabolites in biological systems or (c) act as metabolic inhibitors.

The results obtained show that the organism retained the ability to synthesize galactocarolose when tartrate was replaced by all other dicarboxylic acids tested. However, the quantity of galactocarolose synthesized per g. of mycelia was greatly reduced with all substitutions except when oxaloglycollic acid replaced tartaric acid in the medium. However, the organism did not grow as rapidly in

the medium containing oxaloglycollic acid and D-glucose as it did on DL-tartrate and D-glucose. The uptake of D-glucose was decreased in the flasks containing oxaloglycollic acid.

Although galactocarolose synthesis continued when tartaric acid was replaced by the other dicarboxylic acids tested, no evidence was obtained to suggest that the carbon skeletons of succinic acid, malonic acid or fumaric acid were effective precursors of the galactose moiety of galactocarolose. The influence of NH_4^+ concentration and dicarboxylic acid concentration upon the quantity of galactocarolose formed may be an indirect one. These experiments do not elucidate the role of tartaric acid in the biosynthesis of the galactose moiety of galactocarolose. The data do suggest that this organism does not contain a means of converting the Krebs cycle acids into precursors of the galactofuranose units.

Evidence for the incorporation of ^{14}C from DL-[1,4- $^{14}\text{C}_2$]tartaric acid into galactocarolose was presented by Gander (1960) and later Bentley, Bhate & Kiel (1962) observed incorporation of ^{14}C from DL-[1,4- $^{14}\text{C}_2$]tartaric acid into the polysaccharide although they did not determine which hexoses were labelled. Foster (1949) had suggested carolic acid and carlosic acid, two tetrionic acids found to accumulate in the growth medium, as possible precursors of the galactofuranose units. Bentley *et al.* (1962) observed that tartaric acid was not an effective precursor of carolic acid or carlosic acid while succinate, malonate and acetate were excellent precursors. Therefore it appears unlikely that these tetrionic acids are precursors of

galactocarulose. In addition, these investigators observed that ^{14}C from succinate, malonate and acetate became incorporated into the polysaccharides (presumably galactocarulose and manno-carulose) precipitated. They did not determine whether the ^{14}C was incorporated into the galactose moiety or some other component precipitated with the polymer. Recent investigations in this Laboratory have shown that a pigmented material remains bound to the polysaccharide through various alcohol and acetone precipitations but that it may be removed by chromatography of the growth medium on DEAE-Sephadex (Preston & Gander, 1965). Therefore it is not currently possible to determine whether the data of Bentley and co-workers, showing the incorporation of ^{14}C from succinate, malonate and acetate into polysaccharide, represents the actual incorporation of these acids into the galactofuranose units or whether it may represent incorporation into other components being precipitated with galactocarulose.

The relative quantity of galactocarulose in the medium at the end of the experimental period does not appear to be a simple function of final pH of the medium. In the series of experiments in which the diammonium dicarboxylate concentrations were varied, the greatest quantities of galactocarulose per g. of mycelia were observed at the lowest final pH values. In the series of experiments in which diammonium carbonate was varied the largest quantity of galactocarulose formed per g. of mycelia was observed at the highest final pH values, results opposite of those obtained by altering the diammonium dicarboxylate concentration. These data argue against the possibility that the pH of the medium is a major factor in determining the quantity of galactocarulose to be released by the organism.

Experiments with small volumes of nutrient medium and large surface areas, on which the organism grew, invariably led to extremely low yields of galactocarulose. Cells grown under these conditions were not subjected to the anaerobic conditions that resulted when organisms were grown under conditions where a relatively large portion of the cellular mass was submerged in the nutrient medium. This suggests that the polymer may result from a reductive pathway of tartrate metabolism in cells metabolizing in a partially anaerobic environment.

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