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# The Biosynthesis of Galactofuranosyl Residues in Galactocarolose

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1. Cell-free extracts of *Penicillium charlesii* G. Smith were used in a study of the biosynthesis of the galactofuranose polymer, galactocarolose. 2. UDP-glucose and UDP-galactopyranose were precursors of galactocarolose and it was shown that the galactofuranose residues in the polymer were formed from glucose without fission of the hexose carbon chain. 3. A new nucleotide, UDP-α-D-galactofuranose, was formed by the system and was a major product when polymer synthesis was inhibited by $F^-$ or $Zn^{2+}$; the nucleotide was isolated and its structure determined. 4. UDP-α-D-galactofuranose was efficiently utilized for polymer synthesis and shown to be formed from the pyranose nucleotides. 5. A route for the biosynthesis of galactocarolose, involving a novel ring contraction of the hexose residue while still attached to the nucleotide, is proposed.

Increasing numbers of oligo- and poly-saccharides from various micro-organisms have been shown to contain D-galactose in the furanose-ring form. Several of the type-specific substances of *Pneumococcus* spp. contain this unit (Roberts, Buchanan & Baddiley, 1963; Rao, Buchanan & Baddiley, 1966; Rao, Watson, Buchanan & Baddiley, 1969), and galactofuranosyl residues have been found in the O-antigenic portion of some lipopolysaccharides (Berst *et al.* 1969). Other micro-organisms known to produce polysaccharides containing such residues include *Mycoplasma mycoides* var. *mycoides* (Plackett & Buttery, 1964), *Gibberella fujikuroi* (Siddiqui & Adams, 1961), *Peltigera horizontalis* (Lindberg, Silvander & Wachmeister, 1964) and *Penicillium chrysogenum* (Myazaki & Yadomae, 1969). Galactocarolose, an extracellular β-D-(1→5)-linked polygalactofuranose produced by *Penicillium charlesii* G. Smith (Haworth, Raistrick & Stacey, 1937; Gorin & Spencer, 1959), was the first example of a polysaccharide composed of these units. Hitherto little was known about the biosynthesis of galactofuranosyl units, but, if, as is shown in the present study, they are transferred to polymer chains from nucleotide intermediates in the manner usual for glycoside biosynthesis, then their origin presents a particularly interesting problem, as the only nucleotide previously described containing galactose is UDP-galactopyranose. The process of formation of a nucleoside diphosphate galactofuranose is not immediately obvious.

The isolation of a phosphate-free nucleoside containing a galactofuranose unit from *Penicillium charlesii* G. Smith has been claimed (Maynard & Gander, 1966); it was also suggested that it might act as an intermediate in the biosynthesis of galactocarolose. This suggestion seems unlikely in view of the present knowledge of polysaccharide biosynthesis, and recent attempts to isolate such a nucleoside have been unsuccessful (D. S. Price, G. J. F. Chittenden & J. Baddiley, unpublished work). Investigations on the biosynthesis of galactocarolose described below, with a cell-free enzyme preparation from *P. charlesii*, have led to the isolation of a new nucleotide containing galactose in the furanose form, and this has been shown to be an intermediate for the introduction of these units into the polysaccharide; hexofuranose nucleotides were hitherto unknown. The structure of this intermediate has been established and the mechanism of the biosynthetic route is discussed. Some aspects of the work have been described briefly (Garcia Trejo, Chittenden, Buchanan & Baddiley, 1970).

## MATERIALS AND METHODS

Radioactive substrates were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-glucose, UDP-galactose and UDP-glucose dehydrogenase were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

*Analytical methods.* Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Radioactive material was located on chromatograms with a radiochromatogram scanner (Baird-Atomic Inc., Cambridge, Mass., U.S.A.); quantitative measurements were carried out with an integrator attached to the strip scanner or by cutting out appropriate areas of paper and examining in a Beckman LS-150 liquid-scintillation

counter with a scintillant of the following composition: toluene (A.R.) 2 litres; 1,5-diphenyloxazole, 8g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.2g. For water-soluble samples, the scintillant was mixed with Triton X-100 (2:1, v/v) (Patterson & Greene, 1965).

*Paper chromatography.* This was carried out on Whatman no. 1 or 3MM paper that had been washed with 2M-acetic acid, with the following descending solvent systems: *A*, M-ammonium acetate–ethanol, pH3.6 (5:2, v/v) (Paladini & Leloir, 1952); *B*, M-ammonium acetate–ethanol, pH7.5 (5:2, v/v) (Paladini & Leloir, 1952); *C*, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); *D*, propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (Baar & Bull, 1953); *E*, ethyl acetate–acetic acid–water (9:2:2, by vol.) (Gorin & Spencer, 1959); *F*, propan-1-ol–aq. $NH_3$ (sp.gr. 0.88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949). Compounds were detected with the alkaline $AgNO_3$ reagent (Trevelyan, Procter & Harrison, 1950) or aniline hydrogen phthalate (Partridge, 1949) for reducing sugars, and the molybdate reagent (Hanes & Isherwood, 1949) for phosphoric esters.

*Ultraviolet spectra.* These were determined on a Unicam SP. 800 spectrophotometer.

*Growth of organism.* A culture of *Penicillium charlesii* G. Smith (N.R.R.L. 1887) was purchased from the Commonwealth Mycological Institute, Kew, Surrey, U.K. The organism was grown in a medium with the following composition: D-glucose, 450g; L-tartaric acid, 24g; diammonium L-tartarate, 24g; diammonium hydrogen phosphate, 3.6g; $K_2CO_3$, 3.6g; $Mg_2CO_3$, 2.4g; $(NH_4)_2SO_4$, 1.5g; $ZnSO_4,7H_2O$, 0.42g; $FeSO_4,7H_2O$, 0.42g; water to 9 litres. Stock cultures of the organism were maintained on 2% nutrient agar fortified with this medium. Fresh slopes were inoculated with mature spores every 4–6 weeks. Such cultures were used to inoculate 1200ml Fernbach flasks, each containing 340ml of the medium, and then grown for 12–15 days at 25°C under stationary conditions. The culture medium was removed by decantation and the mycelium washed several times with ice-cold water.

*Preparation of cell-free extracts.* The mycelial pads were finely ground in a pestle and mortar that had been precooled to −10°C. For one standard pad (wet wt. about 20g), Ballotini no. 11 beads (7–10g) were added and the mass was ground vigorously for 2–4min at 0°C, whereupon 0.2M-potassium phosphate buffer, pH6.8 (12ml), was added and the grinding continued for a further 3min. A total of 20ml of buffer (1ml/g wet wt. of mycelium) was used in the grinding and in washing out the mortar. Beads and cell debris were removed by filtration through a precooled slot-holed funnel. After centrifugation at 650*g* for 20min at 0°C, the mixture yielded a light-brown opalescent supernatant solution that was decanted from the remaining debris. Such preparations contained 1–2mg of protein/ml and had pH6.5–6.8.

Storage overnight at −15°C resulted in 50% loss of activity and after 36h no activity was observed. Activity could not be maintained by the addition of dithioerythritol (Cleland, 1964). Preliminary attempts at purification were unsuccessful, and incubations were carried out with freshly prepared extracts termed 'enzyme preparation I'.

Freeze-drying of this solution gave a light-brown powder that was stored overnight at −15°C in the presence of acetone (A.R.) (25ml/pad). The solid was collected by centrifugation and then dried *in vacuo* at −10°C. This material was stored at −15°C, and at this temperature activity was retained for at least 5 weeks. For incubations, this material (40mg) was resuspended in water (0.95ml) to give a final protein content of 2–3mg/ml and was termed 'enzyme preparation II'.

## RESULTS

*Substrate requirements for polymer synthesis.* The substrate requirements for the biosynthesis of galactocarolose are given in Table 1. Polymer formation was indicated by the detection of a labelled product that did not migrate from the origin of paper chromatograms in solvent *A* or *C*. This method of observing the formation of polymer has been used previously in work on galactocarolose (Gander, 1960) and on the biosynthesis of peptidoglycans, teichoic acids and related polymers (Nathenson & Strominger, 1963; Baddiley, Blumsom & Douglas, 1968; Brooks & Baddiley, 1969). The amount of incorporation into the polymer is expressed as a percentage of the total radioactivity on the chromatograms.

It was found that [U-$^{14}$C]glucose, [U-$^{14}$C]glucose 1-phosphate, UDP-[U-$^{14}$C]glucopyranose and UDP-[U-$^{14}$C]galactopyranose were all suitable substrates for the production of the polymer, but [U-$^{14}$C]-galactose was not utilized. With glucose, the addition of UTP enhanced appreciably the extent of synthesis, but this was somewhat less effective with glucose 1-phosphate. The enzyme preparations used both UDP-[U-$^{14}$C]glucopyranose and UDP-[U-$^{14}$C]galactopyranose very efficiently for polymer synthesis, which suggested that both nucleoside diphosphate sugars are probably on the route from glucose to galactocarolose.

The cell-free biosynthesis of polysaccharides sometimes requires the presence of small oligosaccharides as acceptors for the units being added. However, in this case addition of unlabelled galactocarolose (Clutterbuck, Haworth, Raistrick, Smith & Stacey, 1934) had little effect on the extent of synthesis. Moreover, hydrolysis of the enzyme preparations with 1.5M-sulphuric acid for 90min at 100°C, followed by chromatography in solvent *D*, gave galactose among the hydrolysis products; this indicated that if an acceptor is required then there were sufficient amounts of it in the system.

The product was characterized by hydrolysis with 0.15M-sulphuric acid for 90min at 100°C to free galactose (Clutterbuck *et al.* 1934), and by its degradation to arabinose by the method of Gorin & Spencer (1959). Degradation products were identified by comparison with authentic samples by paper chromatography in solvent *D*. It has been shown that the polysaccharide is completely hydrolysed under these mild conditions (Clutterbuck *et al.* 1934); under the same conditions lactose remained

Table 1. *Substrates used for polymer synthesis*

In all experiments enzyme preparation I (0.95ml) or enzyme preparation II (40mg in 0.95ml of water) was used and the volume was finally adjusted to 1ml with water. In Expts. 1–14 the mixture contained [U-$^{14}$C]-glucose (0.01ml; 1ml $\equiv 70\times10^5$ c.p.m.) and glucose (0.5$\mu$mol); in Expts. 15–21, [U-$^{14}$C]glucose 1-phosphate, potassium salt (0.01ml; 1ml $\equiv 67\times10^5$ c.p.m.), and $\alpha$-D-glucopyranose 1-phosphate, potassium salt (0.5$\mu$mol); in Expt. 23, UDP-[U-$^{14}$C]glucose (0.01ml; 1ml $\equiv 9.2\times10^6$ c.p.m.), and in Expt. 24 UDP-[U-$^{14}$C]galactose (0.01ml; 1ml $\equiv 13\times10^5$ c.p.m.), was taken. Where appropriate, nucleoside triphosphates (2$\mu$mol), $ZnSO_4,7H_2O$ (15$\mu$mol), $FeSO_4,7H_2O$ (15$\mu$mol), $MgCl_2$ (10$\mu$mol), NaF (5$\mu$mol) and galactocarolose (2mg) were added. Mixtures were incubated for 1h at 25°C, then heated for 2min at 100°C, concentrated to about 0.25ml *in vacuo* at 30°C, applied as bands to Whatman 3MM paper and chromatographed in ethanol–M-ammonium acetate, pH7.5 (Paladini & Leloir, 1952). The polymer remained at the origin. The amount of incorporation into the polymer is expressed as a percentage of the total radioactivity on the chromatograms.

| Expt. no. | Substrates | Polymer formed (% incorporation of labelled substrates) |
|---|---|---|
| 1 | [U-$^{14}$C]Glucose | 49.0 |
| 2 | [U-$^{14}$C]Glucose + UTP + ATP | 68.0 |
| 3 | [U-$^{14}$C]Glucose + UTP | 82.0 |
| 4 | [U-$^{14}$C]Glucose + ATP | 0.00 |
| 5 | [U-$^{14}$C]Glucose + UTP (enzyme boiled) | 0.00 |
| 6 | [U-$^{14}$C]Glucose + UTP + GTP | 80.0 |
| 7 | [U-$^{14}$C]Glucose + GTP | 56.0 |
| 8 | [U-$^{14}$C]Glucose + CTP | 45.0 |
| 9 | [U-$^{14}$C]Glucose + TTP + UTP | 2.4 |
| 10 | [U-$^{14}$C]Glucose + $Zn^{2+}$ + UTP | 8.0 |
| 11 | [U-$^{14}$C]Glucose + $Fe^{2+}$ + UTP | 59.5 |
| 12 | [U-$^{14}$C]Glucose + $Mg^{2+}$ + UTP | 64.0 |
| 13 | [U-$^{14}$C]Glucose + UTP + galactocarolose | 48.0 |
| 14 | [U-$^{14}$C]Glucose + $F^-$ + UTP | 14.0 |
| 15 | [U-$^{14}$C]Glucose 1-phosphate | 81.0 |
| 16 | [U-$^{14}$C]Glucose 1-phosphate + UTP | 89.0 |
| 17 | [U-$^{14}$C]Glucose 1-phosphate + UTP (enzyme boiled) | 0.00 |
| 18 | [U-$^{14}$C]Glucose 1-phosphate + $Zn^{2+}$ + UTP | 10.0 |
| 19 | [U-$^{14}$C]Glucose 1-phosphate + $Fe^{2+}$ + UTP | 61.0 |
| 20 | [U-$^{14}$C]Glucose 1-phosphate + $Mg^{2+}$ + UTP | 62.0 |
| 21 | [U-$^{14}$C]Glucose 1-phosphate + $F^-$ + UTP | 26.0 |
| 22 | [U-$^{14}$C]Galactose + UTP | 0.00 |
| 23 | UDP-[U-$^{14}$C]glucose | 91.0 |
| 24 | UDP-[U-$^{14}$C]galactose | 87.0 |
| 25 | UDP-[U-$^{14}$C]galactofuranose* | 76.0 |

* Initial radioactivity was not measured, owing to its instability.

essentially unchanged, and the ready hydrolysis of the polymer thus indicates the presence of furanosyl residues (Green, 1966).

Mannan, also known to be formed by the organism, was not detected in normal incubations. However, addition of GTP to the incubation mixtures yielded material that was not completely hydrolysed under the above conditions and which amounted to about 10% of the total labelled product. Hydrolysis of this with 1.5M-sulphuric acid for 90min at 100°C, followed by paper chromatography (solvent *D*), gave mannose.

*Properties of the enzyme system.* With glucose 1-phosphate as substrate the amount of polymer formed was proportional to the time of incubation (Fig. 1) during the first 20min; after that further synthesis did not occur. Enzyme preparations I and II were kept in the buffer for 25min at 25°C without the addition of substrate and were then treated with [U-$^{14}$C]glucose, [U-$^{14}$C]glucose 1-phosphate + UTP, UDP-[U-$^{14}$C]glucose or UDP-[U-$^{14}$C]galactose under the conditions described (Table 1). With glucose as the substrate there was no incorporation of label and with the glucose 1-phosphate a greatly diminished (32%) incorporation, whereas with the two nucleotides incorporation was still quite high (71 and 64% respectively). The results indicate that the point of inflexion in the curve (Fig. 1) did not necessarily represent the termination of activity of all enzymes in the

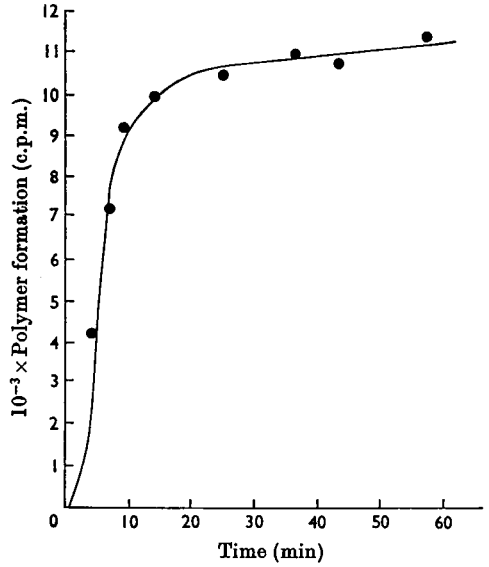



Fig. 1. Rate of polymer synthesis. The incubation mixtures contained enzyme preparation I (0.95ml), [U-$^{14}$C]-glucose 1-phosphate, potassium salt (0.01ml; 1ml≡12× $10^5$ c.p.m.), glucose 1-phosphate, potassium salt (0.5$\mu$mol), and UTP (2$\mu$mol); the volume was finally adjusted to 1ml with water. The mixture was incubated for the times indicated and the reaction was terminated by immersion in water at 100°C for 2min; the products were determined as described in the text.

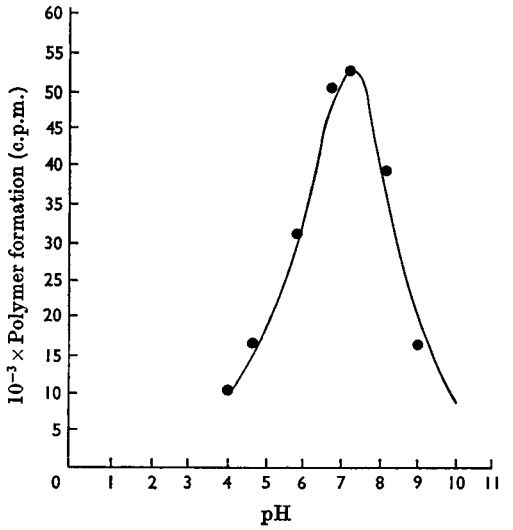



Fig. 2. Effect of pH on polymer synthesis. The incubation mixtures contained [U-$^{14}$C]glucose 1-phosphate solution (0.01ml; 1ml≡67×$10^5$c.p.m.), UTP (2$\mu$mol), enzyme preparation II (40mg) in buffer solution as indicated below, adjusted to a total volume of 1ml: pH4.5–5.8, 0.05M-succinic acid–NaOH; pH6.0–7.6, 0.2M-$Na_2HPO_4$–$NaH_2PO_4$ pH7.6–8.3, 0.1M-tris–HCl; pH9.0–10.0, 0.05M-glycine–NaOH. Reaction mixtures were incubated at 25°C for 1h, treated as described in the text, and the $^{14}$C at the origin of the chromatogram was measured directly in the scintillation counter.

preparation. The effect of varying the pH of the incubation mixtures is shown in Fig. 2; the curve showed a fairly sharp optimum at pH7.2.

The enzyme preparations I and II were obviously crude and further investigation of properties, i.e. the effect of enzyme and substrate concentration on reaction rates, was not attempted. Dialysis of enzyme preparation I for 2h at 0°C against 0.05M-potassium phosphate buffer, pH6.8, resulted in loss of activity with all substrates. The activity could not be restored by the addition of boiled enzyme, yeast extract, UTP, ATP (1mg/ml), NAD (0.5mg/ml), NADH (1mg/ml), $Mg^{2+}$, $Fe^{2+}$, $Zn^{2+}$ or $Mn^{2+}$ (final concentration 5mM). The additions and cofactors were added individually or in a variety of combinations. Dissolution of enzyme preparation II in aq. 1% Triton X-100 followed by the addition of saturated ammonium sulphate, to 40 and 66% saturation respectively, precipitated proteinous material but much activity was lost.

*Isolation of UDP-α-D-galactofuranose.* Addition of $Zn^{2+}$ or $F^-$ ions to the incubation mixtures (Table 1) strongly inhibited polymer synthesis and a new product was formed. This was isolated from the mixture in Expt. 18 and purified by paper chromatography (solvent *A*). It has an absorption maximum at 265nm and mobility ($R_{adenosine}$ 0.49) slightly higher than UDP-galactopyranose ($R_{adenosine}$ 0.44). It was readily hydrolysed in dilute acids, about 50% of it decomposing to galactose when chromatographed in solvent *B* at pH3.8; under the same conditions UDP-glucose and UDP-galactose were unaffected. Significant decomposition occurred when the nucleotide was recovered by extraction from papers with water (pH5.5–5.8) that had not been previously neutralized.

Hydrolysis of the nucleotide in 0.1M-hydrochloric acid for 10min at 100°C gave galactose, $P_i$ and UMP, identified by chromatographic comparison with authentic samples; the ultraviolet-absorption spectrum of the UMP was identical with that of an authentic sample. Oxidation with 0.08M-sodium metaperiodate for 30min at 0°C gave [$^{14}$C]formaldehyde, characterized as its dimedone derivative. The radioactivity of this derivative (4.3×$10^3$c.p.m.) represented about 12.5% of the total radioactivity of the nucleotide (3.35×$10^4$c.p.m.) (calculated value for one carbon atom: 5.6×$10^3$c.p.m.).

Treatment of a sample of the nucleotide (3.42×$10^3$c.p.m.) with 0.2M-potassium hydroxide for 2h at 20°C, followed by neutralization and paper

chromatography (solvent *A*), gave a phosphate ($R_F$ 0.60) ($2.93 \times 10^3$ c.p.m.) together with UMP. This product, with 0.1M-sulphuric acid for 10min at 100°C, gave D-galactose 2-phosphate, $R_{Gal}$ 0.36 (solvent *F*) and galactose. The 2-phosphate ($2.2 \times 10^3$ c.p.m.) was indistinguishable from an authentic sample (Chittenden, Roberts, Buchanan & Baddiley, 1968) and represented about 80% of the radioactivity of the cyclic phosphate.

The nucleotide was capable of acting as a donor of galactofuranose units in the biosynthesis of galactocarolose. It was isolated by preparative paper chromatography at pH 7.5, eluted with water at pH 7.0, treated with Dowex 50 ($NH_4^+$ form) ion-exchange resin, and the solution was then concentrated to small volume by freeze-drying. Treatment of the product with cell-free extract (Table 1) resulted in 76% polysaccharide formation; the remaining radioactivity was located in the area corresponding to galactose, characterized further by paper chromatography.

*Incorporation of specifically labelled glucose into galactocarolose.* The incorporation of [1-$^{14}$C]-, [6-$^{14}$C]- and [U-$^{14}$C]-glucose into the polymer was examined and the results are shown in Table 2. Not only were C-1 and C-6 of glucose equally incorporated into the polymer, but they were incorporated to the same degree as were those in the uniformly labelled substrate.

Samples of galactocarolase were synthesized from [1-$^{14}$C]glucose and [6-$^{14}$C]glucose, hydrolysed with 0.15M-sulphuric acid for 90min at 100°C, and the galactose produced was isolated by elution from paper chromatograms (solvent *D*). The sugar was dissolved in water (150ml) and a sample (10ml) of this solution was dispersed on chromatography paper and the radioactivity counted. A known amount was then isotopically diluted with unlabelled galactose and then oxidized to potassium galactonate with iodine in methanolic potassium hydroxide solution at 40°C (Moore & Link, 1940). This was oxidized further to carbon dioxide (from C-1) and formaldehyde (from C-6) with periodate, by using the method described in Table 3. The carbon dioxide from the [6-$^{14}$C]glucose experiment had negligible radioactivity, whereas the formaldehyde accounted for nearly all of the $^{14}$C in the product. The isotope distribution was reversed in the experiments with [1-$^{14}$C]glucose.

*Evidence for the presence of UDP-galactose 4-epimerase in the cell-free extracts.* The efficient utilization of UDP-glucose and UDP-galactose (Table 1) suggested the presence of a 4-epimerase in the enzyme preparations. Suspension of enzyme preparation II in water at 0°C, followed by centrifugation at 20000*g* for 30min at −5°C, gave a pale-grey sediment. This was washed five times with 0.15M-sodium pyrophosphate buffer, pH 7.5, and suspended in 0.2M-potassium phosphate buffer, pH 6.8, to a final concentration of 500mg/ml. Incubation of samples (0.25ml) of this preparation with [U-$^{14}$C]glucose and UTP, [U-$^{14}$C]glucose 1-phosphate and UTP, UDP-[U-$^{14}$C]glucose or UDP-[U-$^{14}$C]galactose, under the usual conditions, did not yield polymer. With the exception of glucose, which was recovered unchanged, the substrates yielded a mixture of UDP-glucose and UDP-galactose, identified by comparison with authentic samples by paper chromatography in solvent *A*. The two nucleotides were present in an approximate ratio of 7:3. The results confirmed the presence of the 4-epimerase in the extract. They also demonstrated the participation of a pyrophosphorylase, since [U-$^{14}$C]glucose 1-phosphate was converted into the same mixture of nucleotides as were the preformed ones. Incubation of the unwashed pellet with the substrates resulted in the synthesis of galactocarolose, although in the experiment with [U-$^{14}$C]-glucose only 17% was converted into polymer.

Table 2. *Incorporation of* [1-$^{14}$C]*glucose,* [6-$^{14}$C]*glucose and* [U-$^{14}$C] *glucose into galactocarolose*

The labelled substrates (0.01ml; 10$\mu$Ci/ml) were incubated with enzyme preparation II (40mg) in water (0.95ml) together with UTP (0.005ml; 10$\mu$mol/ml) and the volume was adjusted to 1ml with water under the conditions given in Table 1. The radioactive base-line areas of the chromatograms were cut out and their radioactivities counted in toluene scintillant (10ml).

| Substrate | $10^{-3} \times$Initial radioactivity (c.p.m.) | $10^{-3} \times$Radioactivity of polymer (c.p.m.) |
|---|---|---|
| [U-$^{14}$C]Glucose | 90.6 | 59.9 |
| [1-$^{14}$C]Glucose | 92.2 | 59.4 |
| [6-$^{14}$C]Glucose | 91.8 | 56.3 |

## DISCUSSION

When the mycelium of *Penicillium charlesii* was ground in the cold with a phosphate buffer a crude unstable preparation was obtained, which synthesized galactocarolose from glucose, glucose 1-phosphate, UDP-glucose or UDP-galactopyranose (Table 1); the freeze-dried extract that had been washed with acetone was stable on storage but, like the buffer extract itself, was unstable in solution. The instability was most marked with the substrates glucose and glucose 1-phosphate, whereas it was less noticeable with the nucleoside diphosphate sugars. Both UDP-glucose and UDP-galactopyranose were highly effective substrates for polymer synthesis and consequently are to be regarded as natural precursors of galactocarolose. This is supported by the observation that the addition of UTP markedly enhanced the utilization of

Table 3. *Distribution of* $^{14}C$ *in galactocarolose synthesized from* [1-$^{14}C$]*glucose and* [6-$^{14}C$]*glucose*

Samples containing the labelled potassium galactonate, derived from galactocarolose by acid hydrolysis and oxidation with $I_2$ (see the text), were dissolved in 0.5M-sodium phosphate buffer, pH 5.8, previously boiled to remove $CO_2$, and oxidized with 0.3M-$NaIO_4$ for 12h at room temperature with rigorous exclusion of $CO_2$ in this and subsequent stages. The $CO_2$ evolved was collected in M-NaOH and precipitated as $BaCO_3$ by the addition of 2% (w/v) $BaCl_2$. The $BaCO_3$ was centrifuged, washed with methanol and ether and dried to constant weight (yield 55%, based on galactose); radioactivity was measured by scintillation counting in the presence of Cab-O-Sil. Formaldehyde was obtained from the oxidation mixture by destruction of unreduced reagent and removal of formic acid (Reeves, 1941), followed by precipitation as the dimedone derivative.

| Substrate | $10^{-4}\times$Sp. radioactivity of galactose (c.p.m./mol) | $10^{-4}\times$Sp. radioactivity of $BaCO_3$ (c.p.m./mol) | Incorporation into C-1 (%) | $10^{-4}\times$Sp. radioactivity of formaldehyde dimedone derivative (c.p.m./mol) | Incorporation into C-6 (%) |
|---|---|---|---|---|---|
| [1-$^{14}C$]Glucose | 67800 | 61500 | 90.6 | — | |
| [6-$^{14}C$]Glucose | 55000 | 0.023 | 0.0001 | 57000 | 106 |

glucose; the effect was less noticeable with glucose 1-phosphate, perhaps because of the inability to control relative concentrations of the various nucleotide components of the mixture in the crude multi-enzyme preparation. ATP and TTP were powerfully inhibitory, whereas CTP was almost without effect. GTP caused the synthesis of small amounts of another polymer, in addition to galactocarolose, which yielded mannose on acid hydrolysis. This was consistent with the observation that the organism produces a mannan (Clutterbuck *et al.* 1934), and that mannose is usually incorporated into polysaccharides through GDP-mannose.

The enzyme preparations showed no particular metal ion requirements and high concentrations of $Zn^{2+}$ or $F^-$ were strongly inhibitory for polymer synthesis. The optimum pH of the system was about 7.2, and the addition of galactocarolose had little effect on the rate and extent of incorporation of label from the various substrates into polymer. However, a possible requirement for primer could not be excluded, as hydrolysis of the enzyme preparation revealed the presence of material containing galactose residues.

In studies with glucose and glucose 1-phosphate, polymer synthesis occurred smoothly during the first 20min (Fig. 1) but decreased sharply after that. It is probable that the point of inflexion represents loss of activity of enzymes responsible for the early stages of biosynthesis, as both UDP-glucose and UDP-galactopyranose were still effective substrates after 20min. Attempts to fractionate the enzyme system were mainly unsuccessful, probably because of the lability of some of the component enzymes in buffer solutions even at 0°C. This prevented useful kinetic studies and restricted work to establishing the nature of the intermediates in the biosynthetic route.

Table 1 shows that, although the system will synthesize polymer from glucose or glucose 1-phosphate, the synthesis is markedly enhanced by the addition of UTP, and the nucleotides UDP-glucose and UDP-galactopyranose are highly effective substrates. The enzyme preparations presumably contain small amounts of UTP and other nucleotides, which convert glucose or glucose 1-phosphate into UDP-glucose and UDP-galactopyranose. The inability of the system to utilize galactose is consistent with the observation (Gander, 1960) that whole cells of this organism are unable to utilize galactose.

Although $Zn^{2+}$ or $F^-$ powerfully inhibited the synthesis of polymer, the substrates glucose, glucose 1-phosphate, UDP-glucose and UDP-galactopyranose were all converted in the presence of these ions into a new nucleotide. This product was exceptionally labile towards acid and alkali; it was partly destroyed by paper chromatography at pH 3.8. On gentle acid hydrolysis it gave galactose, $P_i$ and UMP, whereas with alkali it gave UMP and a cyclic phosphate of galactose. Further hydrolysis of the cyclic phosphate with dilute acid gave galactose, $P_i$ and an 80% yield of galactose 2-phosphate. These reactions indicate that the cyclic phosphate was a galactose 1:2-phosphate. Glycosyl 1:2-cyclic phosphates are known to yield about 75% of the corresponding 2-phosphate on gentle acid hydrolysis, the other products being the free sugar and $P_i$ arising from the hydrolysis of the intermediate sugar 1-phosphate (Paladini & Leloir, 1952; Remy, Remy & Buchanan, 1955; Piras, 1963). It is also known that careful alkaline hydrolysis of sugar 1-pyrophosphates and nucleoside diphosphate sugars possessing a vicinal *cis*-hydroxyl group adjacent to the pyrophosphate group gives sugar 1:2-cyclic phosphates. It follows that the new nucleotide is a UDP-galactose differing from UDP-galactopyranose in its very slightly greater chromatographic mobility and its markedly greater susceptibility to hydrolysis. These

properties are in agreement with a galactofuranose 1-pyrophosphate structure in the nucleotide. Furanoside 1-phosphates usually hydrolyse in acid 200–400 times faster than do the corresponding pyranosides (Bunton & Humeres, 1969), and furanosides generally have higher $R_F$ values than the pyranosides.

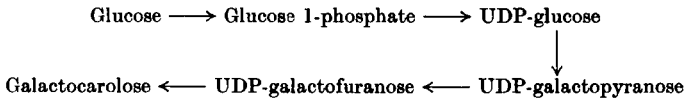
Confirmation of the presence of a galactofuranosyl residue in the nucleotide was obtained by oxidation with periodate. One-sixth of the carbon of the galactose residue was converted into formaldehyde (measured as [$^{14}$C]formaldehyde) during 30min at 0°C, with 0.08M-periodate. The ready formation of the cyclic phosphate from the nucleotide indicates that the galactofuranosyl residue possesses the $\alpha$-configuration at the anomeric centre. The close steric proximity between the hydroxyl group at C-2 of galactofuranose and the pyrophosphate group at the $\alpha$-1-position would ensure ease of cyclization, whereas a $\beta$-configuration on the almost flat furanose ring would not permit cyclization even under vigorous conditions. The above considerations establish the structure UDP-$\alpha$-D-galactofuranose (I) for the new nucleotide; no previous examples of nucleotides containing hexofuranose residues have been described.

UDP-galactofuranose was capable of acting as a substrate for galactocarolose synthesis without further additions. In the experiments in Table 1 it was utilized to about 76%, and spontaneous decomposition to galactose accounted for most of the unused nucleotide. From experiments on the metabolic requirements of whole cells (Gander, 1960; Jordan & Gander, 1966) it was suggested that the galactofuranose units in galactocarolose might arise from the condensation of $C_2$ and $C_4$ precursors. Although the nature of the precursors established in the present work makes this suggestion unlikely, the possibility was examined by using specifically labelled glucose as substrate in the cell-free system. From the results with [1-$^{14}$C]- [6-$^{14}$C]- and [U-$^{14}$C]-glucose (Table 2) it is seen that C-1 and C-6 were equally incorporated into polymer, and label from [U-$^{14}$C]glucose appeared in corresponding amount. Moreover, hydrolysis of the product from [1-$^{14}$C]-glucose to galactose, and then selective degradation of this to determine the $^{14}$C content at C-1, showed that the C-1 of glucose was converted entirely into C-1 of galactose (Table 3). Similarly, with [6-$^{14}$C]-glucose no $^{14}$C appeared in C-1 of galactose. It was also shown that C-6 of glucose was converted entirely into C-6 of galactose in the polymer. Thus it is concluded that glucose is converted into galactocarolose by a direct route involving glucose 1-phosphate, UDP-glucose, UDP-galactopyranose and UDP-galactofuranose, without interchange or rearrangement of the six-carbon-atom chain of the hexoses.

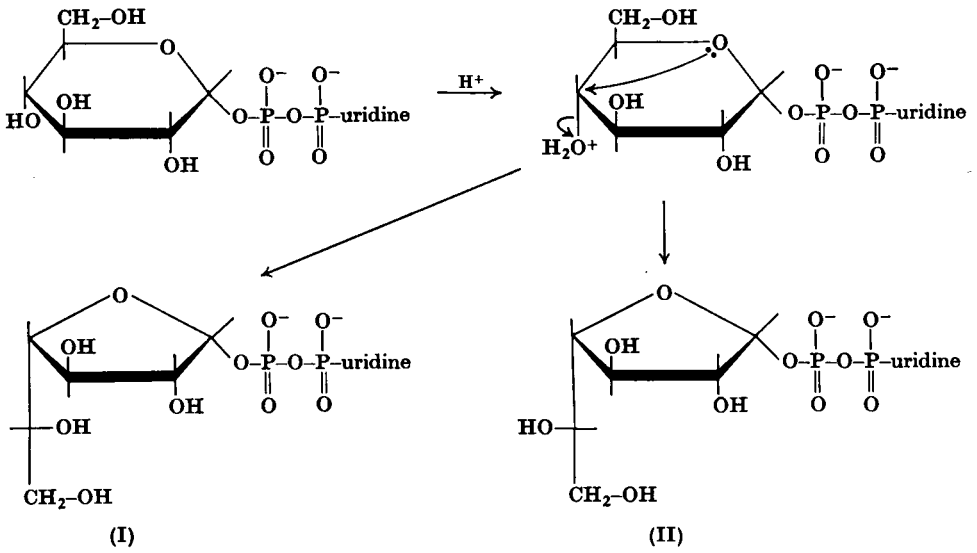
The equally efficient utilization of UDP-glucose and UDP-galactopyranose suggested the presence of UDP-galactose 4-epimerase in the crude enzyme preparations. This was confirmed by using a well-washed particulate component of the acetone-washed enzyme preparation. When the particulate component was incubated with glucose 1-phosphate and UTP, UDP-glucose or UDP-galactopyranose, no polymer was formed, but the products in each case were UDP-glucose and UDP-galactopyranose in the ratio 7:3, in close agreement with the expected equilibrium value for the 4-epimerase; glucose was not utilized by the preparation, and if the washing step was omitted galactocarolose was synthesized. These results establish the presence of the 4-epimerase and suggest the presence of UDP-glucose pyrophosphorylase. By using the quantitative procedure of Maxwell, Kurahashi & Kalckar (1962) it was shown that the preparation contained about 500 units of epimerase/mg of protein.

The utilization of the various substrates, and the occurrence of a nucleotide possessing a galactofuranose residue, indicate the route shown in Scheme 1 for the biosynthesis of galactocarolose from glucose in *P. charlesii*.

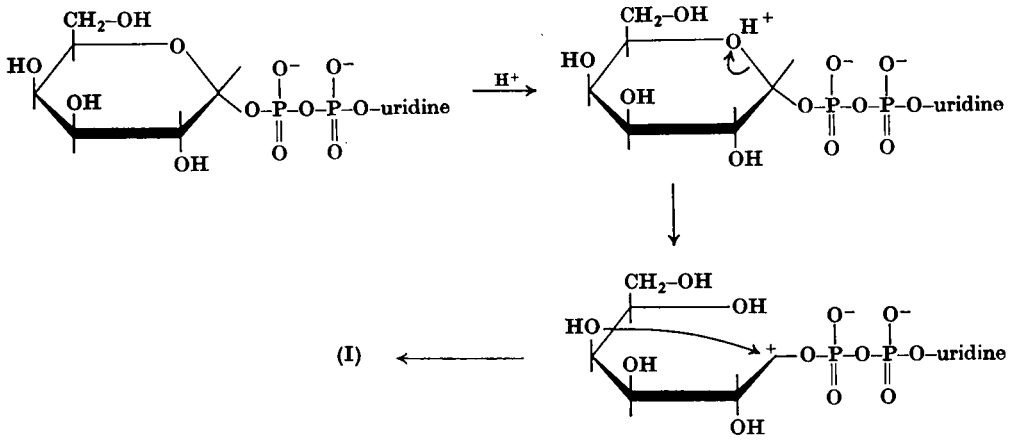
Galactose cannot itself be a precursor, as it is not utilized by either the whole cells or the cell-free extracts. It is likely that both UDP-glucose and UDP-galactopyranose are involved, as both are equally efficient precursors. Moreover it is not obvious what other purpose the 4-epimerase might have if UDP-galactopyranose were not on the direct route. Nevertheless, the present investigation does not exclude a possible direct conversion of UDP-glucose into UDP-galactofuranose, and a mechanism for such a transformation is discussed below. Sarvas & Nikaido (1969) have reported that, in a cell-free system from a T1 form of *Salmonella*, galactofuranose residues of lipopolysaccharide are synthesized from UDP-galactopyranose. The mutant used in the study was 'blocked in galactose metabolism', but it is not clear whether it lacked galactose 4-epimerase. If this is indeed the case,




Scheme 1. Biosynthesis of galactocarolose from glucose.




Scheme 2. Possible mechanism for the transformation of UDP-glucose into UDP-galactofuranose.




Scheme 3. Possible mechanism for the transformation of UDP-galactopyranose into UDP-galactofuranose.

and if it is reasonable to assume similar routes for the two organisms, then the most probable route for furanose ring synthesis must proceed directly from UDP-galactopyranose to UDP-galactofuranose.

The possible mechanism of the transformation of UDP-glucose or UDP-galactopyranose into UDP-galactofuranose is of particular interest, as in either case a ring contraction must have occurred while the linkage between the sugar and the pyrophosphate remained intact. Such ring contractions have not been encountered before in enzymic reactions of carbohydrates, and analogies must be sought in the general chemistry of monosaccharides. In fact, it is possible to propose mechanisms for the direct transformation of either of the pyranose nucleotides into UDP-galactofuranose. UDP-glucose could give the furanoside by the route outlined in Scheme 2; protonation of the hydroxyl group at the 4-position would be followed by attack on C-4 by the lone pair of electrons on the ring oxygen atom. The fission of the C–O bond at the 5-position would be accompanied by attack at that position by $OH^-$. Although this attack could lead to either the D-galacto (I) or L-altro (II) structure in the final product, enzymic control would be assumed to specify the former. This ring contraction resembles a reported case of chemically induced contraction of a 4-substituted hexopyranoside (Austin, Buchanan & Large, 1967).

A mechanism for the direct transformation of

UDP-galactopyranose into UDP-galactofuranose is given in Scheme 3. This mechanism proposes the preferential attack of a proton on the ring oxygen of the pyranose nucleotide. This resembles the first step in one of the possible mechanisms for the acid-catalysed hydrolysis of glycosides (Bunton, Lewis, Llewellyn & Vernon, 1955). However, although it is now believed that, for oxygen glycosides, preferential attack occurs at the glycosidic rather than the ring oxygen atom (Banks, Meinwald, Rhind-Tutt, Sheft & Vernon, 1961; Overend, Rees & Sequira, 1962), this may not be the case with the sugar pyrophosphate structures, where the pyrophosphate group would be expected to decrease markedly the basicity of the glycosidic oxygen. Thus with UDP-galactopyranose preferential protonation of ring oxygen would be expected. A comparable example is the action of protons on glycosides of thiols (Clayton, Hughes & Saeed, 1967; Hughes & Robson, 1966), where the decreased basicity of sulphur compared with oxygen is believed to cause preferential attack at the ring oxygen atom and subsequent ring contractions and anomerizations.

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