The Biosynthesis of C₅₅ Polyprenols by a Cell-Free Preparation of *Lactobacillus plantarum*

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A cell-free supernatant of lysates of *Lactobacillus plantarum* catalyses the synthesis of lipids from [2-¹⁴C]mevalonate. Of the added mevalonate, 7.5% is incorporated into lipids, which were fractionated into five components. About 4% of the radioactivity in these lipids co-chromatographs with compounds shown by mass spectrometry, n.m.r. and i.r. spectroscopy to be C₅₅ polyprenols, and about 2% co-chromatographs with a hexamer. The rest of the radioactivity is in more complex fractions. Analysis by mass spectrometry, n.m.r. and i.r. spectroscopy shows that the major C₅₅ polyprenol is undecaprenol, accompanied by an isomer containing one reduced isoprene unit. A Kuhn–Roth degradation of [¹⁴C]polyprenols indicates that the supernatant catalyses synthesis of these compounds *de novo*.

Polyprenol pyrophosphates are carriers of polysaccharides and peptides that serve as intermediates in the synthesis of peptidoglycans of *Micrococcus lysodeikticus* (Higashi *et al.*, 1967) as well as the *Salmonella* O-antigen (Wright *et al.*, 1967). Although C₅₅ polyprenols have been isolated from whole cells of lactobacilli (Thorne & Kodicek, 1966; Gough *et al.*, 1970), the natural form of polyprenols, their function and mechanism of synthesis in lactobacilli are hitherto unknown.

The ability of a cell-free preparation of *Lactobacillus plantarum* to incorporate mevalonate into unsaponifiable lipids was reported by Durr & Habbal (1970). The purification of polyprenols that are synthesized by such a preparation and the identification of C₅₅ polyprenols are presented below.

Materials and Methods

Preparation of the lysate

Cells of *L. plantarum* were grown and lysed with lysozyme and cysteine as described by Habbal & Durr (1969). Spermine tetrahydrochloride (0.25 mM) was included in the lytic mixture as described by Durr & Habbal (1970). The lysate was centrifuged at 4500g for 25 min. The supernatant was examined by phase-contrast microscopy and Gram staining and was found to be free of cells. The 4500g pellet did not incorporate DL-[2-¹⁴C]mevalonate into unsaponifiable lipids. Apparently unbroken but lysozyme-treated cells lost the ability to metabolize mevalonate.

Reaction mixture

The reaction mixture contained 4 μmol of DL-[2-¹⁴C]mevalonate (0.36 × 10⁶ c.p.m.), 2 μmol of L-

glutamate, 80 μmol of D-glucose, 320 μmol of K₂HPO₄, 4 μmol of MgSO₄ and 4 ml of supernatant (23 mg of protein/ml). The final volume was 4.7 ml, and the final pH was 7.4. The incubation time was 2 h at 30°C. Several incubation mixtures, containing a total of 880 μmol of DL-[2-¹⁴C]mevalonate (9 × 10⁴ c.p.m./μmol), were pooled. The reaction was stopped by immersing the flasks in boiling water for 3 min.

Extraction of mevalonate metabolites

The suspension was exhaustively extracted (four times) with an equal volume of butan-1-ol–pyridinium acetate prepared as described by Anderson *et al.* (1967). The turbid organic layer was clarified by centrifugation at 10000g for 5 min, and washed twice with half its volume of cold water. The organic solvent was evaporated *in vacuo*, and the residue was subjected to chromatography.

Chromatographic methods

DEAE-cellulose acetate. The residue was dissolved in a small volume of chloroform–methanol (7:1, v/v) and subjected to chromatography on a DEAE-cellulose acetate column (2.5 cm × 50 cm) by using the nine solvent systems described by Anderson *et al.* (1967). The chloroform–methanol (7:1, v/v) fraction, the first to be eluted, was evaporated *in vacuo*, and the residue was refluxed for 60 min with 200 ml of KOH (10%, w/v) in methanol–water (7:3, v/v), and then extracted with diethyl ether–light petroleum (b.p. 40–60°C) (1:1, v/v). The solvent was removed, and the residue was chromatographed on deactivated alumina.

Deactivated alumina. The residue was chromatographed on a column (1 cm × 15 cm) of deactivated alumina by consecutive elution with 100 ml of *n*-heptane and with 190 and 80 ml of *n*-heptane containing 5 and 20% (v/v) benzene respectively. Only the 20%-(v/v)-benzene eluate contained a radioactive fraction. The solvent was evaporated *in vacuo*.

Sephadex LH-20. The residue was applied to a column (2.5 cm × 30 cm) of Sephadex LH-20 prepared in chloroform. Elution with chloroform resolved the applied material into two fractions (I and II).

Treatment with charcoal. Eluates containing fraction I were pooled, stirred with 2 g of treated charcoal and filtered through a column (1 cm × 5 cm) of the same adsorbent. The solvent was evaporated *in vacuo*. Fraction II was similarly treated with charcoal.

Silicic acid chromatography. The column (1 cm × 12 cm) of silicic acid was developed with 15 ml of each of 1 and 4% (v/v) diethyl ether in light petroleum (b.p. 40–60°C). The next eluent, 30 ml of 8% (v/v) diethyl ether in light petroleum, contained fraction I. No other radioactive fractions appeared on further elution with more polar solvents.

Fraction II was similarly chromatographed. The only radioactive fraction was recovered in 25% (v/v) diethyl ether in light petroleum.

Thin-layer chromatography. This was performed on silica gel G plates by using three different solvents: (1) Di-isobutyl ketone–acetic acid–water (80:50:7, by vol.) (Marinetti *et al.*, 1957); (2) 10% (v/v) ethyl acetate in *n*-heptane (Thorne & Kodicek, 1966); (3) chloroform (Gough *et al.*, 1970). Radioactive spots were identified by radioautography by using the Medical Kodak X-ray film Gafstar, by keeping it in contact with the plate for one week. Spots were also detected by using iodine vapour.

Spectroscopy

Mass-spectroscopic analysis of fractions I and II was kindly performed by Dr. D. Rosenthal, Research Triangle Institute, Durham, North Carolina, U.S.A., with a MS902 spectrometer at a source temperature of 190°C. The n.m.r. spectrum of fraction I was determined on a Varian A-60 spectrometer by using a 12.5% (w/v) solution in CCl₄. Its i.r.-absorption spectrum was determined as a thin film on a pellet of NaCl by using a Perkin-Elmer model 237B spectrometer.

Assay of radioactivity

Samples were plated at infinite thinness, and the radioactivity was determined in a thin-window gas-flow counter (Nuclear-Chicago Corp.) with a sensitivity of 6×10^5 c.p.m./μCi and with a background counting rate of 18 c.p.m.

Reagents

Grades and commercial sources of resins for chromatography are as follows: DEAE-cellulose, type DEAE (0.85 mequiv./g) A2930, Gallard-Schlesinger Chemical Mfg. Corp., Garden City, N.Y., U.S.A.; alumina, Brockmann Grade, E. Merck A.G., Darmstadt, Germany, and deactivated as described by Schneider *et al.* (1957); silicic acid (silicic acid for lipid chromatography, 325 mesh), Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Sephadex (R) LH-20, particle size 25–100 μm, Pharmacia, Uppsala, Sweden; t.l.c. plates, type K301R2, silica gel without fluorescent indicator, Distillation Products Industries, Rochester, N.Y., U.S.A. All other chemicals and reagents were as described by Durr & Habbal (1970).

Charcoal was treated as described by Morton (1954). The Kuhn–Roth degradation was done by the method of Cheronis & Ma (1964).

Results

Purification

The lipids derived from [2-¹⁴C]mevalonate by a cell-free preparation of *L. plantarum* were resolved by chromatography on DEAE-cellulose into five fractions (Fig. 1). The chromatographic behaviour of these lipids on DEAE-cellulose is close to that reported by Anderson *et al.* (1967) for the lipids of *M. lysodeikticus*. The first fraction (chloroform–methanol, 7:1, v/v) was subjected to t.l.c. (solvent 1) and radioautography, which showed three spots with *R_F* values of 0.47, 0.64 and 0.81. The first spot coincided with that for authentic mevalonate. The intensity of the mevalonate spot on the radioautography plates indicated that this fraction contained large amounts of [¹⁴C]mevalonate. When the various components were later resolved, [¹⁴C]mevalonate accounted for about 90% of the radioactivity of this fraction. When this fraction was subsequently saponified and extracted, the organic phase showed only the spots with *R_F* values of 0.64 and 0.81 on chromatography in the same system (solvent 1).

When the residue (115 mg, 2×10^5 c.p.m.) of the organic phase was chromatographed on deactivated alumina, only one radioactive fraction appeared, which still gave two spots on t.l.c. with *R_F* values of 0.64 and 0.81. The residue of this fraction (60 mg, 1.8×10^5 c.p.m.) was chromatographed on Sephadex LH-20, which resolved the applied material into fractions I (1.2×10^5 c.p.m.) and II (0.8×10^5 c.p.m.), with *R_F* values on t.l.c. of 0.81 and 0.64 respectively (Fig. 2). Each fraction was decolorized by treatment with charcoal. The residue of fraction I (10 mg, 1×10^5 c.p.m.) was chromatographed on silicic acid. Only one fraction could be eluted. Further chromatography of the residue (7 mg, 0.9×10^5 c.p.m.) on silicic

Fig. 1. Fractionation of $[2-^{14}\text{C}]$ mevalonate-derived lipids on DEAE-cellulose

Elution in 10ml fractions was as follows: (1) 600ml of chloroform-methanol (7:1, v/v); (2) 200ml of chloroform-methanol (7:3, v/v); (3) 200ml of methanol; (4) 200ml of chloroform-acetic acid (3:1, v/v) containing 1mmol of potassium acetate; (5) 100ml of acetic acid; (6) 200ml of chloroform-methanol (4:1, v/v) containing conc. NH_3 , sp.gr. 0.88 (10ml/l); (7) 200ml of methanol-1M-pyridinium acetate, pH4.2 (1:1, v/v); (8) 100ml of methanol-6M-pyridinium acetate, pH4.2 (1:1, v/v); (9) 100ml of methanol-6M-ammonium isobutyrate, pH4.2 (1:1, v/v). For experimental details see the text.

acid and Sephadex LH-20 did not change the specific radioactivity of the sample, which also retained the same R_F value (0.81).

Fraction II was similarly treated. The residue (4mg, 0.5×10^5 c.p.m.) had an R_F value of 0.64. Fractions I and II were later shown by mass spectrometry to be an undecamer and a hexamer respectively of the polyprenol series.

A sample of fraction I was applied to t.l.c. sheets and gave R_F values in solvents (2), (3) and (1) of 0.30, 0.67 and 0.81 respectively. The compound gave a brown spot in iodine vapour, and a corresponding single radioactive spot by radioautography. Co-chromatography with an authentic sample of bactoprenol (kindly provided by Dr. K. J. I. Thorne) in the above solvents gave identical characteristics. A mixture of the unknown and bactoprenol behaved as a single component. A similar R_F value in solvent (2) was reported by Wright *et al.* (1967) for the C_{55} polyprenol with 11 unsaturated units isolated from *M. lysodeikticus*.

The fact that the specific radioactivity of fraction I was 10000 c.p.m./ μmol , which is one-ninth that of the starting substrate, when it should be eleven times as high, suggests that the newly synthesized radioactively labelled lipid was diluted by a large pool of endogenous undecaprenol present in the undialysed extract.

Spectroscopy

The mass spectrum of fraction I is typical of undecaprenol (11 unsaturated units) isolated from whole cells of *L. plantarum* by Gough *et al.* (1970). The relative intensities of the peaks (Table 1) agree very well with those reported by Higashi *et al.* (1967) for the undecaprenol isolated from *M. lysodeikticus*. The only striking variation in the relative intensities of peaks that accompany one another is the prominence of the peak ion at m/e 545 over that at m/e 543. Although conditions of analysis may affect the stability of ions, and hence their relative intensity, the peak ion at m/e 545 could be partly due to an octaprene fragment with one saturated isoprene unit. The minor peaks at m/e 768, 750, 681 and 613 support the possibility that undecaprenol is accompanied by a small amount of bactoprenol, which has one non-terminal saturated unit.

The n.m.r. spectrum also indicated that the compound is a polymer of isoprene units as shown by a broad peak at 4.95τ ($\text{CH}=\text{C}$), a doublet at 5.96 and 6.09τ ($-\text{CH}_2-$ adjacent to OH group), a sharp doublet at 7.5 and 8.02τ ($\text{CH}_2-\text{C}=\text{C}$) and a sharp triplet at 8.29 , 8.33 and 8.42τ ($\text{CH}_3-\text{C}=\text{C}$). The signal 8.29τ (methyl protons *cis* to an olefinic proton in the α residue) was strongly overlapped by the signal at 8.33τ (methyl protons *cis* to olefinic protons). The areas of the peaks for the olefinic protons:

Fig. 2. Resolution of free polyprenols on Sephadex LH-20 by using chloroform

For experimental details see the text.

methylene protons:methyl protons were in the proportions 1:4:3, which is typical of an isoprene unit. Computation of peak areas at 8.33 and 8.42 τ gave a ratio of about 4.4, thereby indicating that most of the methyl groups are *cis* to the olefinic protons, and that the polyprenol has a stereochemical configuration similar to that reported for the undecaprenol of *L. plantarum* (Gough *et al.*, 1970).

The i.r.-absorption spectrum confirmed that the compound is undecaprenol, because the strong bands present, including the signal at 1000 cm^{-1} [(OH) function on an unsaturated isoprene unit], were identical with those reported by Gough *et al.* (1970). An (OH) function on a saturated system will give a signal at 965 cm^{-1} .

In addition the n.m.r. spectrum showed a signal at 87.5 τ ($\text{C}=\text{CH}-$ and $\text{C}-\text{CH}_2-\text{C}$) and a small doublet at 9.1 τ (CH_3-C). Although accurate computation of the areas of these peaks was not possible, the number of protons resonating at these fields accounts for at least one saturated isoprene unit, thereby supporting the results obtained by mass-spectrum analysis.

Mass analysis of fraction II showed considerable thermal decomposition with several peak ions that are 14 mass units apart. The following peak ions at m/e 414, 343, 275, 205, 135 and 69 are consistent with a hexamer with three reduced isoprene units. Whether the hexamer was an alcohol could not be ascertained because no distinct parent ion at m/e 432 for the alcohol was apparent. Further analysis by additional methods was limited by the small amount of the sample.

Table 1. Mass spectrum of C_{55} polyprenols

For experimental details see the text.

Peak (m/e)	Relative intensity (% of m/e 69 peak)
135	21.9
203	7.8
271	4.4
273	2.6
339	4.0
407	2.0
409	1.2
475	1.5
477	0.9
543	0.5
545	1.1
546	0.9
547	0.7
611	1.1
612	0.9
613	0.8
614	0.8
679	0.8
680	0.7
681	0.5
748	3.3
749	2.4
750	1.2
766	0.8
768	0.3

Synthesis de novo

The incorporation of [2- ^{14}C]mevalonate into unsaponifiable lipids by the cell-free preparation may be either a synthesis *de novo* and/or elongation of pre-existing homologues of undecaprenols. If the extract catalysed only elongation, the *gem* methyl groups on the ω end of the chain will be unlabelled. But if synthesis *de novo* occurred, one of these methyl groups and only this methyl group of undecaprenol will be labelled. Hence a Kuhn-Roth degradation yields [^{14}C]acetic acid from undecaprenol only when synthesis of polyprenols *de novo* has occurred. It can also be shown that the liberated [^{14}C]acetic acid should not contain more than 5% of the total radioactivity in undecaprenol. This was borne out by results obtained on the degradation of a sample of undecaprenol containing 10000 c.p.m. In two trials the liberated [^{14}C]acetate contained 450 and 525 c.p.m.

Discussion

The cell-free preparation of *L. plantarum* catalyses the incorporation of mevalonate into polyprenols and more complex lipids. Since only one isomer of DL-mevalonate is metabolized it can be calculated that

7.5% of the added mevalonate is converted into these lipids, and this is of the same order of magnitude as the metabolism of mevalonate by whole cells (Durr & Shwayri, 1964; Gough *et al.*, 1970). The first fraction obtained by chromatography on DEAE-cellulose contained an undecamer and a hexamer. Their chromatographic behaviour on DEAE-cellulose, the fact that alkaline treatment did not change the R_F values of its components, and the results of analysis by mass spectrometry, n.m.r. and i.r. spectroscopy suggest that these polyprenes are unesterified. That the undecamer and hexamer respectively contain 4 and 2% of the total radioactivity in the lipids is not surprising, since the free alcohols are not the biologically active forms of these compounds. Most of the radioactivity is in complex lipids, which have chromatographic behaviour similar to that reported by Anderson *et al.* (1967). Further, preliminary investigations indicate that these fractions may be associated with peptides and sugars. The biosynthesis of polyprenes requires the presence of membranes, which are actually present in our enzymic preparation. With a gentle method for lysing *L. plantarum* and a cell-free preparation capable of synthesizing complex lipids, it is now possible to perform definitive studies on the role of mevalonate and the mechanism of its metabolism in lactobacilli.

Results of the Kuhn-Roth degradation showed that the *gem* methyl group is labelled, thereby establishing the ability of the extract to catalyse synthesis of the undecamer *de novo*. However, this does not exclude the possibility that elongation of short homologues can also take place. Enzymes capable of elongating various polymers have been isolated from several sources (Grob *et al.*, 1961; Nandi & Porter, 1964; Allen *et al.*, 1967).

As a pool of endogenous C_{55} polyprenols was present, the spectral analysis was unavoidably done on this pool. However, several lines of evidence support the identity of the radioactive material with undecaprenol. The newly synthesized radioactive lipids co-chromatographed with the endogenous material identified as undecaprenol, and with authentic bactoprenol. Further, the fact that the newly synthesized radioactive lipids were distributed among distinct fractions, and that it was possible to resolve one homologue from another, and that the specific radioactivity of these compounds did not change in the latter stages of chromatography, argue for the identity of the radioactive products and the endogenous pool which acted as the carrier during the

process of isolation. Further, on Kuhn-Roth degradation of a radioactive sample containing 10000 c.p.m., the isolated [^{14}C]acetic acid had 450-525 c.p.m. This is only possible if the newly synthesized material had 11, or close to 11, isoprene units.

The undecamer contains mainly the isomer with 11 unsaturated isoprene units (undecaprenol). The other compound may be bactoprenol with one reduced unit. Wright *et al.* (1967) have reported the same R_F value for undecaprenol and bactoprenol. Attempts to resolve C_{55} polyprenols differing in at least one reduced isoprene unit were not successful in our laboratory. However, it is not surprising that polyprenols exist at various degrees of oxidation and with various chain lengths. We have reported (Durr & Shwayri, 1964; Durr & Habbal, 1970) that molecular oxygen and reduced nicotinamide nucleotides greatly influence the synthesis of mevalonate-derived lipids in whole cells and cell-free preparations respectively.

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