The Characterization of Undecaprenol of *Lactobacillus plantarum*

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Evidence for the presence of undecaprenol in the unsaponifiable lipid of *Lactobacillus plantarum* (N.C.I.B. 6376) is presented. Characterization of the compound was based mainly on mass, i.r. and n.m.r. spectrometry. The prenol was isolated at a concentration of 40 µg/g wet wt. of bacteria and contained over 90% (1.0-5.4% of the dose) of the ^{14}C present in the unsaponifiable lipid after incubation of the bacteria with $[2-^{14}\text{C}]$ mevalonate. N.m.r. spectrometry indicated the presence of two internal *trans*-, one α -*cis*- and seven internal *cis*-isoprene residues per molecule. The $^3\text{H}/^{14}\text{C}$ ratios of the prenol after incubation of the bacteria with $[2-^{14}\text{C}, (4R)-4-^3\text{H}_1]$ - and $[2-^{14}\text{C}, (4S)-4-^3\text{H}_1]$ -mevalonate were in agreement with this stereochemistry. There was no evidence of saturated isoprene residues in the molecule. The undecaprenol appeared to be accompanied by much smaller quantities of decaprenol and nonaprenol.

MVA† was originally discovered as a compound capable of replacing acetate as an essential growth factor for *Lactobacillus acidophilus* (Skeggs *et al.* 1956). The major metabolite of MVA in this organism and in the closely related *Lactobacillus casei* and *Lactobacillus arabinosus* proved to be a lipid material (Thorne & Kodicek, 1962*b*, 1963) which was shown later to be a polyprenol. This polyprenol was given the trivial name bactoprenol and the compound isolated from *L. casei* was shown to contain 11 isoprene residues, one of which is saturated (Thorne & Kodicek, 1966). The stereochemistry of the isoprene residues in bactoprenol has not been reported.

The elegant work of Wright, Dankert, Fennessey & Robbins (1967), Higashi, Strominger & Sweeley (1967) and Scher, Lennarz & Sweeley (1968) has demonstrated a role for polyprenol-phosphate-carbohydrate complexes as essential intermediates in the biosynthesis of some of the polysaccharide components of the cell walls of *Micrococcus lysodeikticus*, *Staphylococcus aureus* and *Salmonella newington*. In each case the polyprenol part was shown to contain eleven isoprene residues but only during the work of Scher *et al.* (1968) with *M. lysodeikticus* was sufficient intermediate isolated for the stereochemistry of the undecaprenol to be established. N.m.r. studies indicated the presence of two internal *trans*-isoprene residues, seven internal *cis*-isoprene residues and a *cis*- α -isoprene residue. This finding showed that this particular bac-

terial polyprenol is stereochemically different from those found in higher plants. The undecaprenol of higher plants contains three internal *trans*- and six internal *cis*-isoprene residues and a *cis*- α -isoprene residue (see e.g. Wellburn, Stevenson, Hemming & Morton, 1967). It is clear also that this polyprenol differs from that isolated from *L. casei* (Thorne & Kodicek, 1966).

Durr & Shwayri (1964) reported the incorporation of $[2-^{14}\text{C}]$ MVA into the unsaponifiable lipid of *Lactobacillus plantarum* but did not investigate the nature of the radioactive metabolite(s). The present authors have shown the main metabolite of MVA in *L. plantarum* to be a polyprenol and undertook its characterization with a view to comparing it with plant polyprenols, with bactoprenol and with the polyprenol shown to be involved in cell-wall biosynthesis of other bacteria. The results of this work are reported in this paper.

MATERIALS AND METHODS

Growth of L. plantarum. *L. plantarum* (N.C.I.B. 6376) was grown on slopes of tomato juice agar (5.2%, w/v; Oxoid) supplemented with agar (1%, w/v; Difco Bacto) at 30°C for 48 h. The culture was then transferred to 100 ml of semi-synthetic medium (Thorne & Kodicek, 1962*a*) in a 250 ml conical flask. After incubation at 30°C for 48 h on a rotary shaker this was used as the inoculum of a further 10 litres of medium. This final culture was grown for 48 h at 30°C with forced aeration.

Cells were harvested either by using the continuous-flow head in the MSE17 centrifuge at maximum speed or by centrifugation in 250 ml centrifuge tubes at 4500*g* for 15 min. The cells were washed twice with water. The yield was approx. 2 g wet wt. of cells/l of medium.

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† Abbreviation: MVA, mevalonate.

Incorporation of radioactive MVA. Radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. 3-*R,S*-[2-¹⁴C]MVA lactone (3.25 μ Ci, 0.68 μ mol) was converted into the sodium salt by dissolving in 5 ml of 0.1 M-sodium citrate, pH 7. [2-¹⁴C,(4*R*)-4-³H₁]- and [2-¹⁴C,(4*S*)-4-³H₁]-MVA lactone were prepared by mixing 3-*R,S*-[2-¹⁴C]MVA lactone (4.82 mCi/mmol) with 3-*R*-[(4*R*)-4-³H₁]- and 3-*S*-[(4*S*)-4-³H₁]-MVA lactone (134 mCi/mmol) and with 3-*R*-[(4*S*)-4-³H₁]- and 3-*S*-[(4*R*)-4-³H₁]-MVA lactone (115 mCi/mmol) respectively. The original ³H/¹⁴C ratio of the [2-¹⁴C,(4*S*)-4-³H₁]MVA lactone was 8.57:1 and of the [2-¹⁴C,(4*S*)-4-³H₁]MVA lactone was 5.88:1. A portion of each mixture was converted into its sodium salt as above for the incorporation experiment.

Incorporation of radioactive MVA was essentially according to the optimum conditions described by Durr & Shwayri (1964). [2-¹⁴C]MVA (3.25 μ Ci) was added to 8 g of wet cells suspended in 100 ml of 0.3 M-sodium citrate buffer, pH 5.0. After incubation on a rotary shaker at 30°C for 3 h the cells were recovered by centrifugation at 4500g for 15 min. Incubation of the double-labelled MVA species differed only in that 5 μ Ci of ¹⁴C of each was used and the weight of cells in each 100 ml of buffer was 5 g.

Isolation of unsaponifiable lipid. Wet cells were suspended in a solution of pyrogallol in methanol (1%; 1 ml/g of cells) and aqueous KOH (60%, w/v; 0.5 ml/g of cells) and the mixture was boiled under reflux for 45 min. The mixture was cooled and diluted threefold with water before being extracted three times with diethyl ether-light petroleum (b.p. 40–60°C) (1:1, v/v). The combined extracts were washed free of alkali with water and the organic solvent was evaporated, finally under N₂. The yield of unsaponifiable lipid from the wet cells was approx. 0.17%.

Chromatographic methods. Quantities of unsaponifiable lipid greater than 50 mg were usually subjected to a crude separative procedure on columns of alumina before t.l.c. Smaller quantities, as obtained for example in the incorporation studies, were subjected to chromatography directly on thin layers of adsorbent.

Unsaponifiable lipid (50–100 mg each time) was subjected to chromatography on 10 g of alumina (M. Woelm, Eschwege, Germany; acid-washed, Brockmann grade III) by using 100 ml portions of diethyl ether-light petroleum (b.p. 40–60°C) as solvents. A 1:49 (v/v) mixture of these solvents was followed by a 1:9 mixture that eluted polyprenol. The final eluent was diethyl ether.

The second fraction from the column of the total unsaponifiable lipids was subjected to preparative adsorption chromatography on layers (250 μ m thick) of silica gel G impregnated with Rhodamine as adsorbent, with chloroform as solvent (system A). After the developed chromatogram had been viewed under u.v. light a fraction was recovered running fractionally ahead of a ficaprenols marker (*R_F* 0.59). This was usually subjected to preparative chromatography on kieselguhr G impregnated with paraffin [by immersion in a mixture (1:20, w/v) of liquid paraffin in light petroleum (b.p. 40–60°C)] with acetone–water, (19:1, v/v) as developing solvent (system B). The polyprenol had *R_F* 0.69 compared with 0.53, 0.64 and 0.74 for ficaprenols-12, -11 and -10 respectively in this reversed-phase partition system.

The purity of the polyprenol was followed analytically by using systems A and B and also on silica gel G with diisopropyl ether–light petroleum (1:4, v/v) (system C) and in system B modified so that the acetone/water ratio was 93:7. Anisaldehyde was used as stain reagent (McSweeney, 1965) for analytical t.l.c. Fluorescein was used as a non-destructive detection spray for preparative chromatography in system B (Dunphy, Whittle & Pennock, 1965). Material was eluted from the adsorbent by diethyl ether. Preparative chromatography in system B was followed by chromatography on alumina to remove fluorescein and paraffin from the polyprenol (see, e.g., Wellburn *et al.* 1967).

Assay of radioactivity. The ³H and ¹⁴C contents of fractions in the experiment with double-labelled MVA species were determined as described by Stone & Hemming (1967) but with the Beckman liquid-scintillation system (model LS-200B; Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). Quenching was shown to be negligible in each fraction. In the calculations of ³H/¹⁴C ratios it has been assumed that 3*S*-MVA had no biological activity (Cornforth, Cornforth & Popják, 1962). The 'corrected ratio' is the ³H/¹⁴C ratio determined (a) assuming that the ³H/¹⁴C ratio of the original MVA lactone was 1:1 [the actual ratios 8.57:1 and 5.88:1 (see above) were determined at the same time as the ratios for the polyprenol samples to avoid the complication of ³H decay] and (b) having multiplied the result by the number of C-2 atoms from MVA expected to be incorporated into the polyprenol molecule (in this case 11). Unfortunately in this organism insufficient label was incorporated into all-*trans*-polyisoprenoid compounds for a satisfactory internal control to be available. Nevertheless the ³H/¹⁴C ratios of the polyprenol were calculated in exactly the same way as in previous experiments that gave the expected ratios for 'internal control' compounds such as squalene (see e.g. Stone & Hemming, 1967; Gough & Hemming, 1970a) and there is every reason to believe that the ratios obtained in this experiment are equally reliable.

In assays involving ¹⁴C but not ³H the Packard Tri-Carb liquid-scintillation counter was used with a counting efficiency for ¹⁴C of 62%.

Some thin-layer chromatograms were scanned for the presence of radioactivity by using a Nuclear-Chicago Actigraph III radiochromatogram scanner. Radioactivity was also occasionally located on thin-layer chromatograms by radioautography with Kodirex no-screen medical X-ray film (Eastman-Kodak Corp., Rochester, N.Y., U.S.A.) over 2 weeks.

Spectroscopy. The i.r.-absorption spectrum of the polyprenol was determined as a solvent-free film between rock salt discs in a Perkin-Elmer Infracord model 137 spectrometer. Its mass spectrum was determined on an MS902 spectrometer at the Physico-Chemical Measurements Unit, Harwell, Didcot, Berks., U.K. The n.m.r. spectrum of the polyprenol was determined on a Varian HA100 spectrometer in the Department of Organic Chemistry, University of Liverpool.

RESULTS AND DISCUSSION

The incorporation of radioactive MVA into the unsaponifiable lipid varied from 1.0 to 5.4%. Of the ¹⁴C incorporated into the unsaponifiable lipid

over 90% was associated with a material (presumed to be a polyprenol) that had chromatographic properties similar to those of ficaprenol. Small quantities of radioactivity were associated with other areas of thin-layer chromatograms after chromatography of total unsaponifiable lipid in system A. Chromatographic evidence indicated the presence in these areas of menaquinones-8 and -9, farnesol, sterol (possibly cholesterol) and possibly squalene. The concentration of these other materials was very low, being between 1 and 5 $\mu\text{g/g}$ of wet cells. The concentration of polyprenol was approx. 40 $\mu\text{g/g}$ of wet cells.

The mass spectrum of the polyprenol fraction was typical of that of a polyisoprenoid alcohol containing 11 isoprene residues linked head to tail (see e.g. Wellburn *et al.* 1967). The molecular ion appeared at m/e 766. It was approximately two-thirds as intense as the peak at m/e 748, which corresponds to the $(M - \text{H}_2\text{O})^+$ ion. The next major peak in the spectrum was at m/e 679, which corresponds to $(M - \text{H}_2\text{O} - 69)^+$, i.e. the additional loss of the ω -isoprene residue. [A smaller peak at m/e 697 for $(M - 69)^+$ was also apparent.] The main peaks in the rest of the spectrum were at m/e values of 611, 543, 475, 407, 339, 271, 203, 135 and 69. The first eight of these confirm further sequential loss of isoprene units (m/e 68) from the ω -end of the chain to produce finally the stable ion $\text{CH}_2 \cdot \text{C}(\text{CH}_3) : \text{CH} \cdot \text{CH}_2 \cdot \text{CH} : \text{C}(\text{CH}_3) \cdot \text{CH} : \text{CH}_2]^+$ with m/e 135 and being equivalent to $[M - 18 - 69 - (8 \times 68)]^+$. The intense peak at m/e 69 corresponded to $[\text{CH}_3 \cdot \text{C}(\text{CH}_3) : \text{CH} \cdot \text{CH}_2]^+$ derived from the ω -end of the chain.

An i.r.-absorption spectrum confirmed the polyisoprenoid nature of the compound (see e.g., Wellburn *et al.* 1967), for strong bands were present at 835 cm^{-1} (C-H deformation of a trisubstituted olefin), 1660 cm^{-1} (C=C stretching) and at 3025 cm^{-1} (C-H stretching of =CH) as well as at 1450 cm^{-1} (C-H deformation of $-\text{CH}_2-$ and $-\text{CH}_3$), 1365 cm^{-1} (C-H deformation of $-\text{CH}_3$) and at approx. 2850, 2920 and 2960 cm^{-1} (C-H stretching of $-\text{CH}_2-$ and $-\text{CH}_3$). The O-H deformation band was strong and at 1000 cm^{-1} rather than at 965 cm^{-1} , which is

good evidence that the α -residue is not saturated. In addition, the presence of minor peaks at 1307, 1236, 1153, 1126, 1089 and 889 cm^{-1} with shoulders at 1326 and 1222 cm^{-1} and the absence of a shoulder at 795 cm^{-1} was consistent with the compounds containing more *cis*- than *trans*-isoprene residues (Wellburn *et al.* 1967).

The precise number of *cis* and *trans* residues was determined in the usual way (see e.g. Feeney & Hemming, 1967), from an n.m.r. spectrum at 100 MHz of 6.5 mg of the polyprenol in carbon tetrachloride. Integration of the areas under the peaks at 8.42 τ (protons of methyl groups *trans* to olefinic hydrogens), at 8.34 τ (protons of methyl groups *cis* to olefinic hydrogens) plus 8.29 τ (protons of methyl group *cis* to olefinic hydrogen in the α -residue) and at 8.00 τ plus 7.97 τ plus 7.93 τ (all protons of methylene groups) gave values of 2.8 *trans*-methyl groups and 9.2 *cis*-methyl groups for every 20 methylene groups (excluding that one methylene group next to the hydroxyl group) in the molecule. Since the ω -residue will provide one *cis*- and one *trans*-methyl group it was concluded that the polyprenol contains two internal *trans*-isoprene residues, seven internal *cis*-isoprene residues and that the α -isoprene residue is also in the *cis* configuration (because of the peak at 8.29 τ).

The n.m.r. spectrum showed no peaks at τ values higher than 8.5 and thus confirmed the evidence from mass spectrometry and i.r. spectrometry that none of the isoprene residues was saturated. The rest of the spectrum illustrated the fully isoprenoid nature of the compound for there were present a broad peak at 4.95 τ (olefinic protons) accompanied by a weak resonance centred at 4.64 τ (olefinic proton of α -residue) and a doublet at 6.02, 6.09 τ (protons of methylene group adjacent to hydroxyl group).

Evidence in support of the stereochemical conclusions was provided by the $^3\text{H}/^{14}\text{C}$ ratios in the polyprenol formed from the double-labelled MVA species (Table 1). Although the ratio after incorporation of the 4*S*-MVA was higher than expected by 6% it is still clear that the results approximate to the situation that three of the 11 isoprene residues

Table 1. Incorporation of ^3H and ^{14}C from the double-labelled MVA species into the polyprenol

Details of the determination of the corrected $^3\text{H}/^{14}\text{C}$ ratio are given in the Materials and Methods section. In each case the radioactivity of 5% of the fraction was counted.

	Incorporation		Corrected $^3\text{H}/^{14}\text{C}$ ratio
	^3H (d.p.m.)	^{14}C (d.p.m.)	
From $[2\text{-}^{14}\text{C}, (4R)\text{-}4\text{-}^3\text{H}_1]\text{MVA}$	21210	8640	3.15:11
From $[2\text{-}^{14}\text{C}, (4S)\text{-}4\text{-}^3\text{H}_1]\text{MVA}$	34900	7680	8.48:11

are biogenetically *trans* and eight are biogenetically *cis*. One of the biogenetically *trans* residues is almost certainly the ω -residue (see, e.g., Gough & Hemming 1970b) and it is likely that the α -residue is biogenetically *cis*. Thus the number of internal chemically *trans* residues and the number of internal biogenetically *trans* residues are in agreement. The same conclusion can be drawn for the internal *cis* residues.

The evidence presented indicates that in *L. plantarum* the major metabolite of MVA that can be recovered in the unsaponifiable lipid of the micro-organism is an undecaprenol containing a *cis*- α -residue, seven internal *cis* residues and two internal *trans* residues. It is probably identical with the prenol present in the mannose-phosphate-prenol complex isolated from *M. lysodeikticus*. It is clearly different from the prenol of *L. casei* described by Thorne & Kodicek (1966). It also differs from ficaprenol-11 (castaprenol-11) in having one less *trans* residue and one more *cis* residue. It may be noted that this difference is sufficient to cause the prenol to travel between ficaprenols-11 and -10 during reversed-phase partition chromatography, i.e. it is slightly more polar than ficaprenol-11. Radioautography of a reversed-phase chromatogram of a radioactive prenol fraction after preparative chromatography in system A also illustrated this point and, in addition, gave clear evidence of small amounts of two other radioactive substances running just ahead of ficaprenols-10 and -9 respectively. That running ahead of ficaprenol-10 gave a very slight positive colour with the anisaldehyde reagent, whereas the undecaprenol gave a strong positive colour. It is probable that traces of decaprenol and nonaprenol accompany the undecaprenol and that all three differ from each other only in the number of internal *cis* residues. Most other natural sources of prenols yield families of poly-

prenols the members of which differ from each other only in this way (Hemming, 1970).

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