The Characterization and Stereochemistry of Biosynthesis of Dolichols in Rat Liver

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It was shown that rat liver contains a series of dolichols with chain lengths of from 17 (or possibly 16) to 21 isoprene residues, the main constituent of the mixture being dolichol-18. By using double-labelled radioactive mevalonates it was demonstrated that each of these dolichols possesses three biogenetically *trans*-isoprene residues and that the remaining residues are biogenetically *cis*, suggesting that these polyprenols are biosynthesized from all-*trans*-farnesyl pyrophosphate by the *cis* additions of isoprene residues, followed by saturation of the α -isoprene residue. The results obtained with these radioactive mevalonates also indicated that the activity of isopentenyl-pyrophosphate isomerase is low relative to the activity of prenyltransferase in rat liver.

The dolichols found in pig liver are a mixture of polyprenols with from 17 to 22 isoprene residues, the main constituent being dolichol-19 (Dunphy, Kerr, Pennock, Whittle & Feeney, 1967; Butterworth & Hemming, 1968). Each polyprenol has a saturated α -residue and two internal chemically *trans*-isoprene residues. The other internal residues are chemically *cis* (Feeney & Hemming, 1967). The concentration of dolichol in pig liver is approx. 100 mg of dolichol mixture/kg of tissue. About 60% of this mixture is esterified with fatty acid and associated mainly with a 'cell debris plus nuclei' fraction. Most of the free dolichols are associated with a mitochondrial fraction (Butterworth & Hemming, 1968). The relative proportions of the individual dolichols in pig dolichol mixtures are approximately the same whether the source was a mixture of esterified or free dolichols, or whether the tissue was liver, spleen or kidney (Dunphy *et al.* 1967; Butterworth & Hemming, 1968). Similar dolichol mixtures have been found in many mammalian tissues (Dunphy *et al.* 1967; Burgos, Hemming, Pennock & Morton, 1963; Butterworth, 1964).

It has been suggested (Feeney & Hemming, 1967) that these dolichols are probably formed from all-*trans*-farnesyl pyrophosphate by the *cis* additions of isoprene residues, followed by the saturation of the α -residue. Such a mechanism would require the ω -terminal and the two internal chemically *trans*-isoprene residues to be biogenetically *trans*, and the other internal residues and the

α -residue to be biogenetically *cis*. It would also require the two internal *trans*-residues to be directly adjacent to the ω -residue.

Since the incorporation of radioactive mevalonate into various isoprenoid compounds by rat liver slices has been reported (Gold & Olsen, 1966), it was decided to study the nature and stereochemistry of biosynthesis of polyprenols in this tissue. The use of double-labelled MVA† species for the latter aspect of the work is reviewed by Gough & Hemming (1970).

MATERIALS AND METHODS

Isolation and characterization of rat liver polyprenols. Livers (108 g) of 20 female rats were washed with water and macerated in methanolic pyrogallol in an Ultra-Turrax homogenizer, and saponified as described by Butterworth, Draper, Hemming & Morton (1966). The unsaponifiable lipid was subjected to chromatography on a column of alumina (M. Woelm, Eschwege, Germany; acid-washed, Brockmann grade III) essentially as described by Butterworth *et al.* (1966). The purification was followed by t.l.c. (on silica gel G with chloroform) with authentic samples of squalene, ubiquinone-9, pig liver dolichols-17–22, all-*trans*-farnesol and cholesterol as markers and with anisaldehyde as stain reagent (McSweeney, 1965). Dolichols were purified by further chromatography on a column of alumina followed by preparative t.l.c. (on silica gel G with chloroform) with an authentic sample of pig dolichols-17–22 as a marker. The prenols were detected by spraying with ethanolic fluorescein (Dunphy, Whittle & Pennock, 1965) and the rat dolichols (R_F 0.71) were extracted with ether. Pig dolichols-17–22 had R_F 0.73 in this system. A sample of

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† Abbreviation: MVA, mevalonate.

the partially purified rat prenols was subjected to reversed-phase t.l.c. [on kieselguhr with liquid paraffin-light petroleum (1:19, v/v)] with dry acetone as the mobile phase, with a sample of pig dolichols-17-22 as a marker and anisaldehyde as stain reagent. A series of prenols with the same R_F values as those of pig dolichols-17, -18, -19, -20 and -21 and possibly a trace of prenol corresponding in position to dolichol-16 was visible.

The remaining portion of the rat prenol mixture was subjected to preparative reversed-phase t.l.c. [on kieselguhr with paraffin-light petroleum (1:19, v/v); dry acetone] with a sample of pig dolichols-17-22 as marker. Prenols were again located by spraying with ethanolic fluorescein. The individual rat polyprenol bands with the same R_F values as pig dolichols-17, -18, -19 and -20 were extracted with ether. Each prenol was rechromatographed in the reversed-phase system, sprayed with fluorescein and extracted with ether. Each rat dolichol was purified from paraffin and fluorescein by alumina column chromatography, and the lipid was weighed.

Incorporation of the radioactive MVA species. [$2\text{-}^{14}\text{C}$ -(4*R*)-4- $^3\text{H}_1$]- and [$2\text{-}^{14}\text{C}$ -(4*S*)-4- $^3\text{H}_1$]-MVA solutions were prepared as described by Wellburn, Stone & Hemming (1966), but with Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1964) instead of phosphate buffer, from mixtures of original specific radioactivities as follows: 3*R*[(4*R*)-4- $^3\text{H}_1$]- and 3*S*[(4*S*)-4- $^3\text{H}_1$]-MVA lactone, 134 mCi/mmol; 3*R*[(4*S*)-4- $^3\text{H}_1$]- and 3*S*[(4*R*)-4- $^3\text{H}_1$]-MVA lactone, 115 mCi/mmol; 3*R*,*S*-[2- ^{14}C]MVA lactone, 4.82 mCi/mmol.

Fresh liver (10g) from two female rats was placed in ice-cold oxygenated Krebs-Ringer bicarbonate buffer, pH 7.4. Portions of liver tissue were placed on filter papers soaked in ice-cold buffer and slices 0.4 mm thick were cut rapidly with a tissue slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.).

Tissue slices (approx. 5g) were placed in each of two 50 ml conical flasks containing radioactive MVA and buffer to a total volume of 10 ml. Incubation was carried out at 37°C for 3 h with constant shaking. The system was gassed with $\text{CO}_2 + \text{O}_2$ (5:95) throughout the incubation. The incubation was terminated by placing the flask in ice, the medium was decanted and the slices were washed with water.

The unsaponifiable lipid was isolated and subjected to chromatography on an alumina column as described above. T.l.c. indicated that most of the squalene was present in the fraction eluted by light petroleum (b.p. 40-60°C) and most of the dolichols in the fraction eluted by 8% diethyl ether in light petroleum (see Butterworth *et al.* 1966).

A sample (40%) of the squalene present in the petroleum fraction corresponding to each incubation was purified by preparative t.l.c. (on silica gel G with chloroform) followed by preparative t.l.c. (on silica gel G with light petroleum), with an authentic sample of squalene as a marker. Squalene was located by spraying with ethanolic fluorescein, extracted with ether, and finally freed from fluorescein by dissolving in light petroleum. Squalene was assayed for ^3H and ^{14}C as described by Stone & Hemming (1967) but by using the Beckman LS-200B liquid-scintillation spectrometer. Rat prenols (0.5 mg each) corresponding to pig liver dolichols-17, -18 and -19 were added to each of the fractions eluted by 8% diethyl ether

in light petroleum and the lipid was subjected to preparative reversed-phase t.l.c. on kieselguhr with paraffin-light petroleum (1:19, v/v) and dry acetone. The chromatograms were sprayed with ethanolic fluorescein. Those portions of the chromatograms between the individual prenol bands, i.e. the gaps, were assayed for ^3H and ^{14}C directly. The individual prenol bands were extracted with ether and freed from paraffin and fluorescein by alumina column chromatography. Each prenol was purified further by preparative t.l.c. (on silica gel G with chloroform) and the prenols were located by spraying with ethanolic fluorescein. They were finally freed from fluorescein by dissolving in light petroleum, and assayed for ^3H and ^{14}C by using the Beckman liquid-scintillation spectrometer.

Spectrometry. I.r.-absorption spectra were determined on a Perkin-Elmer 'Infracord' model 137 spectrometer as solvent-free films between rock-salt discs. Mass spectra were determined by Mr P. Kelly at the University of Newcastle upon Tyne, using an MS9 spectrometer.

RESULTS AND DISCUSSION

Characterization of rat liver prenols. The amounts of purified prenols obtained from the rat mixture corresponding to pig dolichols-17, -18, -19 and -20 were 1.5 mg, 2.4 mg, 1.9 mg and 1.0 mg respectively. This represents a total yield of 63 mg of polyprenol mixture/kg of liver.

The R_F values of the rat prenols when subjected to reversed-phase partition chromatography (see the Materials and Methods section) were 0.55, 0.50, 0.45 and 0.40. These corresponded to pig liver dolichols-17, -18, -19 and -20 respectively.

A plot of the R_m values [$\log(1/R_F - 1)$] against the number of isoprene residues (based on the number of residues/molecule in the pig dolichols with corresponding chromatographic properties) resulted in a straight-line graph, indicating that the rat prenols constitute a homologous series.

The i.r. spectrum of the rat prenol corresponding to pig dolichol-18 was essentially the same as that of the pig dolichol, showing the rat prenol to be a primary alcohol with most of its isoprene units in the *cis* configuration (see Burgos *et al.* 1963).

A sample of the rat prenol corresponding to pig dolichol-19 was subjected to mass spectrometry. This resulted in a molecular ion (M^+) peak at m/e 1312, consistent with the rat prenols possessing 19 isoprene units, one of which is saturated. The intensity of the molecular ion was much greater than the intensity of ($M - 18$) $^+$ corresponding to the loss of water from the molecular ion. The greater intensity of M^+ was probably due to the saturation of the α -terminal isoprene residue: A similar observation was made in the mass spectra of the hexahydropolyprenols (Stone, Butterworth & Hemming, 1967). However, there appear to be three series of peaks in the mass spectrum (Fig. 1) as follows: (i) where there is no loss of water from

Fig. 1. Histogram of the most prominent peaks, with m/e values greater than 100, for rat dolichol-19. Intensities are expressed relative to 100 for the peak at m/e 135. Between m/e values of 330 and 540 relative intensities have been increased by $10\times$ and between 540 and 1350 by $100\times$.

the molecular ion. The first unit lost is the ω -residue, to give $(M-69)^+$, m/e 1243, followed by the successive loss of isoprene units from the ω -end to give peaks at m/e 1175, 1107 etc.; (ii) where water is lost from the molecular ion to give $(M-18)^+$, m/e 1294. This is then followed by the loss of the ω -residue to give a peak at m/e 1225 and the successive loss of isoprene units to give peaks at m/e 1157, 1089 etc.; (iii) where water and two hydrogen atoms are lost from the molecular ion to give $(M-20)^+$, m/e 1292. This is then followed by the loss of the ω -residue to give an ion at m/e 1223 and the successive loss of isoprene residues to give m/e 1155, 1087 etc.

As the value of m/e decreases (below 1100) series (ii) becomes more abundant than series (i). As m/e decreases still further (below 600), series (iii) becomes the most abundant. The most abundant ion in series (iii) at m/e 135 is derived from the α - and β -isoprene residues. The relatively high abundance of this ion may be due to rearrangement of the double bonds to give a stable ion, such as $[\text{CH}_2\cdot\text{C}(\text{CH}_3):\text{CH}\cdot\text{CH}_2\cdot\text{CH}:\text{C}(\text{CH}_3)\cdot\text{CH}:\text{CH}_2]^+$. The fracture of the bond between adjacent methyl-

ene groups (see Fig. 1) gives rise to the characteristic series of peaks 68 mass units apart. The rearrangement of the ion m/e 135 as shown above results in there no longer being two adjacent methylene groups, hence the ion is stabilized. An abundant ion was also evident at m/e 69. This is due to the retention of the charge in some cases by the ω -terminal residues. (The lower part of the mass spectrum showed the presence of some contaminating hydrocarbon, probably paraffin, in the sample.) Hence the mass spectrum obtained for the rat prenol is consistent with its being a polyprenol possessing 19 isoprene residues, with the α -residue saturated.

From the chromatographic characteristics of the rat liver prenols and the i.r. and mass spectra, it appears that these rat prenols are identical with the pig dolichols, the only difference being that dolichol-18 is the main constituent of the rat liver mixture and dolichol-19 is the main constituent of the pig mixture. However, these results give no direct evidence as to the stereochemistry of the rat dolichols, and insufficient rat dolichol-18 was obtained for a satisfactory n.m.r. spectrum.

Table 1. *Incorporation of radioactive MVA species*

	From [2- ¹⁴ C,(4 <i>R</i>)-4- ³ H ₁]MVA			From [2- ¹⁴ C,(4 <i>S</i>)-4- ³ H ₁]MVA		
	³ H (d.p.m.)	¹⁴ C (d.p.m.)	Corrected ³ H/ ¹⁴ C ratio	³ H (d.p.m.)	¹⁴ C (d.p.m.)	Corrected ³ H/ ¹⁴ C ratio
Squalene	146 000	15 700	5.89:6	2063	21 380	0.08:6
Dolichol-17	54	36	2.68:17	195	36	13.73:17
gap 17/18	87	20	—	134	29	—
Dolichol-18	91	65	2.66:18	353	63	15.08:18
gap 18/19	71	15	—	95	23	—
Dolichol-19	59	40	2.94:19	232	41	15.95:19

The corrected ³H/¹⁴C ratios of squalene and rat dolichols formed from [2-¹⁴C,(4*R*)-4-³H₁]MVA and [2-¹⁴C,(4*S*)-4-³H₁]MVA are recorded in Table 1. The results for squalene are in agreement with squalenes being biogenetically all-*trans*. The corrected ratios for dolichol-19 are in agreement with this dolichol possessing three biogenetically *trans*-isoprene residues and 16 biogenetically *cis*-isoprene residues. The results obtained from the [2-¹⁴C,(4*R*)-4-³H₁]MVA incubation for dolichols-17 and -18 show slightly low corrected ratios, but these also suggest the presence of three biogenetically *trans* residues in each dolichol. The corrected ratio from the incubation with [2-¹⁴C,(4*S*)-4-³H₁]MVA for dolichol-18 shows that this dolichol possesses 15 biogenetically *cis*-isoprene units. The ratio for dolichol-17 from this incubation is slightly low, but indicates the presence of 14 biogenetically *cis*-isoprene units. In each case most of the radioactivity associated with the rat liver dolichols-17–20 was found in dolichol-18, which has been shown to be the main constituent of the rat mixture. Since the sum of the two corrected ratios for dolichols-17, -18 and -19 approach 17, 18 and 19 respectively, the activity of any isopentenyl pyrophosphate isomerase (EC 5.3.3.2) under the conditions of this experiment must be low relative to the activity of prenyltransferase (EC 2.5.1.1) (see Gough & Hemming, 1970).

Pig liver dolichols have been shown by n.m.r. spectroscopy to possess two internal chemically *trans* residues with the other internal residues being chemically *cis* (Feeney & Hemming, 1967). Biogenetic evidence has shown that the rat liver dolichols possess three biogenetically *trans*-isoprene residues, and that the remaining residues are biogenetically *cis*. Assuming that the ω -terminal residue is biogenetically *trans* (which it seems to be in all polyprenols studied so far), then two of the internal residues are most probably biogenetically *trans*, again suggesting that the pig and rat dolichols are identical in structure, and that the saturated α -residue is biogenetically *cis*. Since the rat dolichols, and probably the pig dolichols, possess

three biogenetically *trans*-isoprene residues they are most probably formed from all-*trans*-farnesyl pyrophosphate by the *cis* additions of isoprene residues, followed by saturation of the α -residue. However, the experiment with the double-labelled MVA species does not give any information as to the positions of the biogenetically *trans* residues within the dolichol molecules. For the dolichols to be biosynthesized from all-*trans*-farnesyl pyrophosphate the three biogenetically *trans* units must be grouped together at the ω -end of the dolichol molecule, one of them being the ω -residue itself.

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