SYNTHESIS OF DOLICHOL PHOSPHATE BY A CELL-FREE EXTRACT FROM PEA

G. R. DALEO and R. PONT LEZICA+

Departamento de Biología, Fundación Bariloche, Casilla de Correo 138, 8400 San Carlos de Bariloche, Argentina

Received 5 January 1977

1. Introduction

Dolichols are long-chain polyprenols found in eucaryotes made up of 16–22 isoprene residues linked head to tail. The molecule has *cis* and *trans* double-bonds and the α-isoprene unit is saturated. Usually polyprenols are isolated as mixtures differing from each other only in one or two isoprene residues [1,2]. The phosphorylated polyprenols appear as biologically active carriers of sugars in the biosynthesis of bacterial wall polysaccharides and eucaryotic glycoproteins [1,3]. Bacterial undecaprenol has been synthesized in vitro with preparations of *Micrococcus lysodeikticus* [4], *Salmonella newington* [5] and *Lactobacillus plantarum* [6,7]. The *cis*-undecaprenyl pyrophosphate synthetase from *L. plantarum* has recently been partially purified and the reaction requirements were studied [8].

Little is known about the biosynthesis of polyprenols in higher organisms. Incorporation of mevalonate into betulaprenol by woody tissue of *Betula verrucosa* [9] and into dolichol by rat liver [10] has been achieved in vivo.

We report here the first evidence for the synthesis of dolichol phosphate by a cell-free preparation from pea epicotyls and the ability of this compound to accept glucose from UDP–glucose.

2. Materials and methods

Δ^3-[1-^{14}C]Isopentenyl pyrophosphate (IPP) (60 mCi/mmol) was obtained from Amersham/Searle Corporation. [^{3}H]UDP–glucose (2.42 Ci/mmol) was donated by the Instituto de Investigaciones Bioquímicas 'Fundación Campomar'. Dolichol (Dol) and dolichyl monophosphate (DMP), isolated from liver and partially purified, were a generous gift from N. H. Behrens. Ficaprenol (Fol) and ficaprenyl monophosphate (FMP) were offered by P. A. Romero. Dolichol monophosphate [^{14}C]glucose (DMPG) was a gift from R. Staneloni. Alkaline phosphatase from *Escherichia coli* was obtained from Sigma Chemical Co. All other chemicals were analytical reagent grade.

2.1. *Enzyme preparation and assay*

Dwarf *Pisum sativum* L. seedlings (cv. Cuarentona) were grown in the dark for 5–7 days at 25°C. The epicotyls were homogenized in an Omnimixer with 0.1 M Tris–HCl, pH 7.4, 20 mM β-mercaptoethanol. The homogenate was strained through cheese-cloth and centrifuged at 1000 × *g* for 15 min. The supernatant was then centrifuged at 25 000 × *g* for 1 h and the resulting supernatant was used as enzyme source.

Standard incubations were carried out for 30 min at 30°C, containing the following mixture in a final volume of 0.05 ml: 50 mM Tris–HCl, pH 7.3, 1 mM $MgCl_2$, 80 μM [1-^{14}C]IPP (60 mCi/mmol) and pea enzyme (150–200 μg protein).

The enzymatic hydrolysis of prenyl phosphates was performed with alkaline phosphatase at 37°C for 4 h in the following medium: 50 mM Tris–HCl, pH 8.4, 10 mM $MgCl_2$, 0.1% Triton X-100, *E. coli* alkaline phosphatase (100 μg protein) in 50% methanol. The resulting free alcohols were extracted with petroleum ether.

The ability of polyprenyl phosphates to act as acceptors of sugars was assayed at 20°C for 15 min in a reaction mixture containing: 100 mM Tris–HCl, pH

+Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina

7.5, 10 mM $MgCl_2$, 0.6% Triton X-100, 12 μM [^{3}H]-UDP–glucose (2.42 Ci/mmol), a crude membrane preparation from peas [11] and chloroformic extract.

2.2. *Analytical methods*

The products were separated following the scheme of fig.1.

A Sephadex LH-20 column (1 X 20 cm) was prepared and equilibrated with 0.1 M ammonium acetate buffer in 99% methanol [13]. Prenyl phosphates resistant to acid hydrolysis (0.1 ml) were mixed with standards of DMP and FMP and poured into the column. Elution was carried out with the same buffer and 0.4 ml fractions were collected. The eluates were analyzed for radioactivity, acid-labile phosphate and total phosphate.

Ascending paper chromatography was performed on Whatman No. 1 paper developed with butanol/water (4:1 v/v).

Reverse phase thin-layer chromatography of free alcohols was carried out on Kieselguhr G plates, immersing the plate in a 5% (v/v) solution of paraffin oil in light petroleum [14] developed with acetone/water (98:2 v/v).

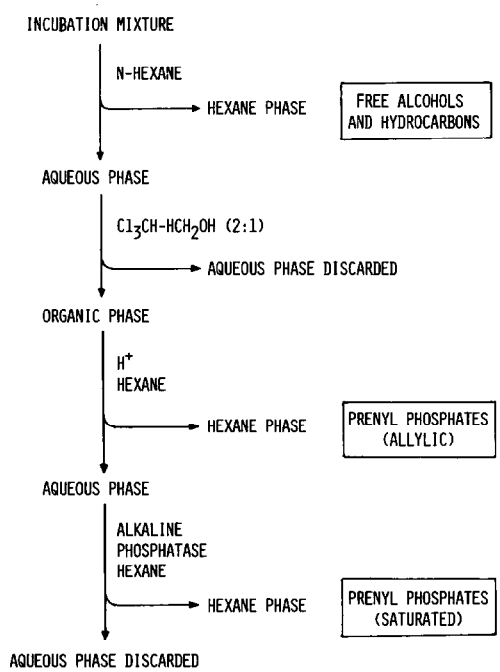

Fig.1. Scheme of extraction and separation of products.

Polyprenyl phosphates stable to mold acid hydrolysis, were treated for 30 min at 70°C under nitrogen in 60% aqueous KOH/absolute ethanol (1:5 v/v) [15]. The resulting free alcohols were extracted by addition of water and petroleum ether.

Phosphate was determined by the method of Fiske and Subbarow [16] and the Lowry method [17] was used for protein determination.

3. Results and discussion

When pea extracts were incubated with [1-^{14}C]-IPP, radioactive free alcohols appeared in the hexane phase. Those alcohols probably were liberated by the activity of endogenous phosphatase. The analysis of this fraction by reverse phase thin-layer chromatography (fig.2A) showed that only short chain prenols were the products.

A significant amount of the radioactivity (8%) could be extracted from the incubation mixture by chloroform/methanol (2:1), suggesting the presence of polyprenol phosphates. Differences in the incorporation of label to this fraction were observed among different preparations. The enzyme system was very unstable, and only fresh preparations synthesized long chain polyprenols.

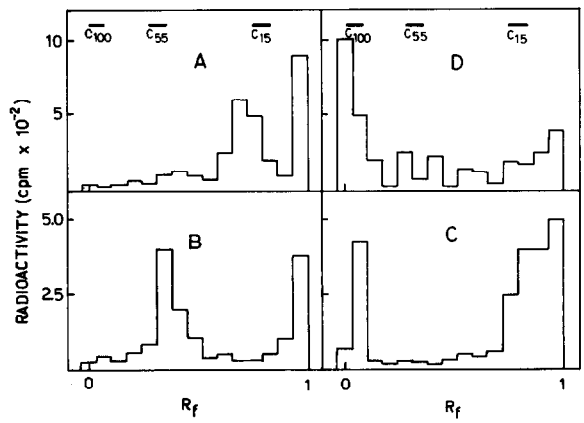

Fig.2. Reverse phase thin-layer chromatography of the hexane soluble products. A, free alcohols and hydrocarbons. B, alcohols hydrolyzed by acid treatment. C, alcohols hydrolyzed by alkaline phosphatase. D, alcohols liberated by strong alkaline treatment. Standards of farnesol (C_{15}), ficaprenol (C_{55}) and dolichol (C_{100}) were detected by iodine vapours.

Allylic polyprenol phosphates were hydrolyzed by acid treatment. Twenty percent of the total polyprenyl phosphates were liberated by this method. The analysis of this fraction showed (fig.2B) that radioactivity was distributed between short chain prenols and a compound with the chromatographic properties of Fol (C_{55}) standard. No long chain polyprenol similar to Dol (C_{100}) was liberated by acid hydrolysis.

Polyprenols resistant to mild acid hydrolysis (80% of total polyprenyl phosphates) were submitted to enzymatic hydrolysis by alkaline phosphatase. Only 10% of this fraction could be hydrolyzed by this treatment. In this case, radioactivity was associated with Dol standard (fig.2C). Also short chain alcohols appeared at the front of the chromatographic plate due to a contamination (2%) of the chloroform/methanol fraction by the substrate (IPP). The results obtained by this treatment were only qualitative. It is known [4] that long chain polyprenyl phosphates are hardly hydrolyzed by alkaline phosphatase, the short chain ones being a better substrate. This differential affinity of the enzyme could explain the ratio between long and short chain prenols observed in fig.2C.

To confirm the chain-length of the polyprenol phosphates synthesized with pea preparations by a different method, column chromatography on Sephadex LH-20 was performed. An aliquot of the fraction containing the acid stable polyprenyl phosphates was chromatographed together with DMP and FMP carriers. The elution profile is shown in fig.3. The radioactive peak elutes with the acid stable phosphate in DMP (mol. wt ~ 1400) and far from the labile one corresponding to the standard of FMP (mol. wt ~ 700). When fractions 25–32 were pooled, concentrated under N_2 and treated with strong alkali, a quantitative recovery of radioactivity was obtained in the hexane phase. Reverse phase chromatography (fig.2D) revealed a radioactive peak with the chromatographic properties of dolichol. After treatment of the plate with iodine vapours, a spot of nonradioactive Dol arising from the DMP used as standard in the column was observed at the same place.

In order to check the ability of the biosynthesized polyprenyl phosphate to accept glucose, an aliquot of the [^{14}C]polyprenyl phosphate coming from the Sephadex column was incubated with [^{3}H]UDP–glucose and an enzyme system from peas. Paper

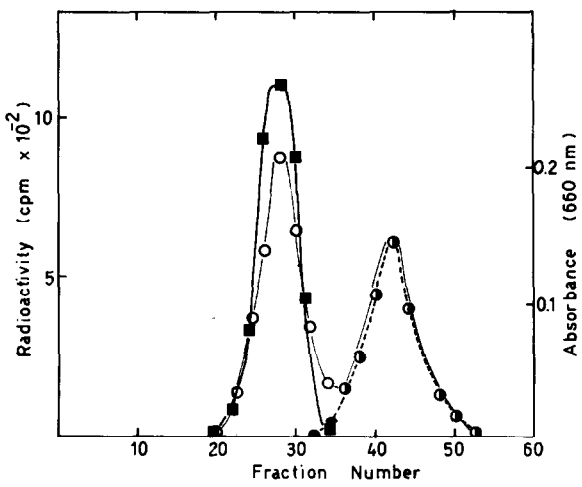

Fig.3. Column chromatography on Sephadex LH-20. Standards of dolichyl phosphate and ficaprenyl phosphate were detected by total phosphate (○——○) and by acid labile phosphorous (●- -●), respectively. [^{14}C]polyprenyl phosphate was detected by radioactivity (■——■).

chromatography of the butanol soluble material (fig.4) showed several peaks. At the origin, a peak accounting for [^{14}C]DMP and [^{3}H]glucose-1-phosphate was observed. Free [^{14}C]polyprenols and tritiated steryl glucosides formed from endogenous unlabelled

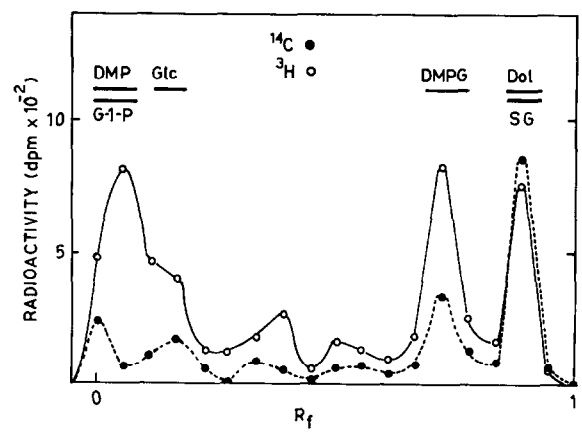

Fig.4. Paper chromatography of the butanol soluble products from the incubation of [^{14}C]polyprenyl phosphate with [^{3}H]-UDP–glucose. DMP, dolichyl monophosphate. Glc, glucose. G-1-P, glucose-1-phosphate. DMPG, dolichyl monophosphate glucose. Dol, dolichol; SG, steryl glucosides.

steroids and [^{3}H]UDP–glucose [11] migrated at the solvent front. Immediately before appeared a double labelled compound with the same R_F as DMPG. The amount of endogenous acceptor was negligible in this preparation and assuming a chain-length of 20 isoprene units, a molar ratio of tritiated glucose to [^{14}C]dolichyl phosphate of 1.15 can be calculated. When this material was eluted from the paper strip, acid treated and partitioned, 73% of tritium was obtained in the aqueous phase and 71% of ^{14}C was found in the butanolic phase. This is consistent with the properties of DMPG.

The identity of the long chain polyprenyl phosphates with DMP is based on the following evidence:

(i) The acid stability of the phosphorylated product formed is similar to that of DMP.

(ii) The molecular size obtained by gel filtration is coincident with DMP.

(iii) The chromatographic properties of the radioactive products liberated by alkaline phosphatase or strong alkali corresponds to that of Dol.

(iv) The ability of the phosphorylated product to form a glucosylated lipid with the chromatographic properties and acid lability of DMPG.

These results confirm the polyprenylic nature of the lipid-glucose acceptor detected in the same system [11,18] as well as the early assumption that the lipid carrriers in higher plants were similar to pig liver DMP. The hypothesis that the sugar acceptor in eucaryotic cells is DMP [18] seems at present supported by the results obtained in mammals [10,19], yeast [20] and plants. The results obtained in insects also support this idea [21] even when the identification of the insect lipid as dolichol has not been achieved.

This is the first report of the in vitro synthesis of dolichyl phosphate with sugar acceptor capacity. Work on the stereochemistry of the compound and the enzymes involved is in progress.

Acknowledgements

The authors are indebted to their colleagues in the Departamento de Biología for helpful discussions and criticism. This work was partly supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

References

[1] Hemming, F. W. (1974) in: Biochemistry of lipids, MTP International Review of Science (Goodwin, T. W. ed) Vol. 4, pp. 39–97, Butterworths, London.
[2] Beytia, E. D. and Porter, J. W. (1976) Annu. Rev. Biochem. 45, 113–142.
[3] Waechter, C. J. and Lennarz, W. J. (1976) Annu. Rev. Biochem. 45, 95–112.
[4] Kurokawa, T., Ogura, K. and Seto, S. (1971) Biochem. Biophys. Res. Commun. 45, 251–257.
[5] Christenson, J. G., Gross, S. K. and Robbins, P. W. (1969) J. Biol. Chem. 244, 5436–5439.
[6] Durr, I. F. and Habbal, M. Z. (1972) Biochem. J. 127, 345–349.
[7] Keenan, M. V. and Allen, C. M. (1974) Arch. Biochem. Biophys. 161, 375–383.
[8] Allen, C. M., Keenan, M. V. and Sack, J. (1976) Arch. Biochem. Biophys. 175, 236–248.
[9] Gough, D. P. and Hemming, F. W. (1970) Biochem. J. 117, 309–317.
[10] Martin, H. G. and Thorne, K. J. I. (1974) Biochem. J. 138, 281–289.
[11] Pont Lezica, R., Romero, P. A. and Dankert, M. A. (1976) Plant Physiol. in press.
[12] Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509.
[13] Dankert, M., Wright, A., Kelley, W. S. and Robbins, P. W. (1966) Arch. Biochem. Biophys. 116, 425–435.
[14] Dunphy, P. J., Kerr, J. D., Pennock, J. F., Whittle, K. J. and Feeney, J. (1967) Biochim. Biophys. Acta 136, 136–147.
[15] Baynes, J. W., Hsu, A. F. and Heath, E. C. (1973) J. Biol. Chem. 248, 5693–5704.
[16] Fiske, C. H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Pont Lezica, R., Brett, C. T., Romero Martínez, P. and Dankert, M. A. (1975) Biochem. Biophys. Res. Commun. 66, 980–987.
[19] Evans, P. J. and Hemming, F. W. (1973) FEBS Lett. 31, 335–338.
[20] Jung, P. and Tanner, W. (1973) Eur. J. Biochem. 37, 1–6.
[21] Quesada Allué, L. A., Belocopitow, E. and Maréchal, L. R. (1975) Biochem. Biophys. Res. Commun. 66, 1201–1208.