Studies on the Biosynthesis of Dolichyl Phosphate: Evidence for the In Vitro Formation of 2,3-Dehydrodolichyl Phosphate

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SUMMARY: A particulate enzyme preparation from hen oviduct is shown to carry out the biosynthesis of a long chain polyprenyl phosphate from isopentenyl pyrophosphate and farnesyl pyrophosphate. The compound has the physical and chemical properties of 2,3-dehydrodolichyl phosphate. The enzyme system is inhibited by EDTA and stimulated by Triton X-100 and dithiothreitol. If the product of the reaction is 2,3-dehydrodolichyl phosphate, it may be derived from 2,3-dehydrodolichyl pyrophosphate, a likely intermediate in the biosynthesis of dolichyl phosphate.

Dolichol is a long chain polyisoprenoid alcohol which is widely distributed in both higher and lower eucaryotes (1). Depending on its biological source, the molecule consists of a linear polymer of 16-21 isoprene units, with the α-isoprene unit saturated (2). The phosphorylated monoester of dolichol has been shown to participate in glycoprotein biosynthesis, functioning as a lipid "carrier" in a manner analogous to its bacterial counterpart undecaprenyl phosphate (3,4). Indirect evidence for the role of dolichyl phosphate in the glycosylation of ovalbumin has been demonstrated recently (5).

In spite of its importance in cellular function, little is known of the biosynthesis of dolichol. Butterworth, et al. (6) demonstrated that mevalonic acid in vivo could be incorporated into dolichol in the livers of pigs and rabbits. Similar results were later obtained with rat liver (7). Based on the known structure of dolichol, it was proposed that dolichol shares a common biosynthetic pathway with cholesterol, branching off at the level of farnesyl pyrophosphate (8). Dolichol biosynthesis

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would continue at this point by sequential cis-addition of isoprene units, followed by stereospecific reduction of the α-isoprene unit and dephosphorylation. This pathway is analogous to that worked out in bacteria in which a single enzyme catalyzes the cis-addition of isoprene units to geranyl pyrophosphate to produce undecaprenyl pyrophosphate (9). With the bacterial system as a model we decided to investigate dolichol biosynthesis in hen oviduct, using the probable precursor substrates isopentenyl pyrophosphate and farnesyl pyrophosphate. In this communication we wish to report the in vitro biosynthesis of a long chain polyisoprenyl phosphate with the physical and chemical properties of dehydrodolichyl phosphate.

MATERIALS AND METHODS: Pig liver dolichol and dolichyl phosphate were from Sigma. Dolichol was isolated from hen oviduct as described previously (10). Dolichyl phosphate was prepared from hen oviduct dolichol by the method of Wedgwood, et al. (11). [1-^{3}H] dolichol (14.8 Ci/mmole) was obtained from New England Nuclear. [1-^{14}C] isopentenyl pyrophosphate (54 mCi/mmole) was from Amersham Searle. All trans farnesyl pyrophosphate was prepared chemically by the method of Popjak (12).

Thin layer chromatography was carried out on 20 cm plates (EM Labs) in the following solvents: A, 30% ether-petroleum ether; B, chloroform-methanol-water (60:25:4); C, chloroform-methanol-15 M NH_3-water (65:35:4:4).

Hen oviduct (Pel Freeze) was homogenized in five volumes of buffer (10 mM Tris-Cl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 6 mM β-mercaptoethanol, 0.25 m sucrose, 0.1% Triton X-100). The suspension was centrifuged at 1000 g for 5 min, washed once with homogenizing buffer, and suspended in 0.1 M Tris-Cl, pH 7.4 (1.5 ml/g original tissue). Incubations were performed at 37° with gentle shaking. Reaction conditions are given under the figures. Reactions were stopped by the addition of 2.5 volumes of chloroform-methanol (2:1), vortexing, and separating the layers by centrifugation. The upper layer was extracted with an equal volume of chloroform and the organic layers were combined and washed with methanol-water (1:1). The resulting organic phase was passed through a 1x2 column of DEAE (acetate), pre-equilibrated with chloroform-methanol (2:1) and eluted with 8 ml 0.3 M ammonium acetate in chloroform-methanol (2:1). The column eluates were washed once with 2 ml water to remove ammonium acetate prior to subsequent chromatography or liquid scintillation counting.

Radioisotope counting was performed with a Packard Tri Carb Model 3390 liquid scintillation counter using a Triton X-100 based scintillation cocktail. Radiochromatograms were scanned with a Packard Model 7200 Radiochromatogram Scanner.

RESULTS AND DISCUSSION: Hen oviduct has been shown to contain large quantities of dolichol (10) and therefore seemed an appropriate tissue in which to investigate the biosynthesis of this molecule. When isopentenyl pyrophosphate and farnesyl pyrophosphate were incubated with oviduct particulate enzymes, a substance was formed with the chromatographic properties

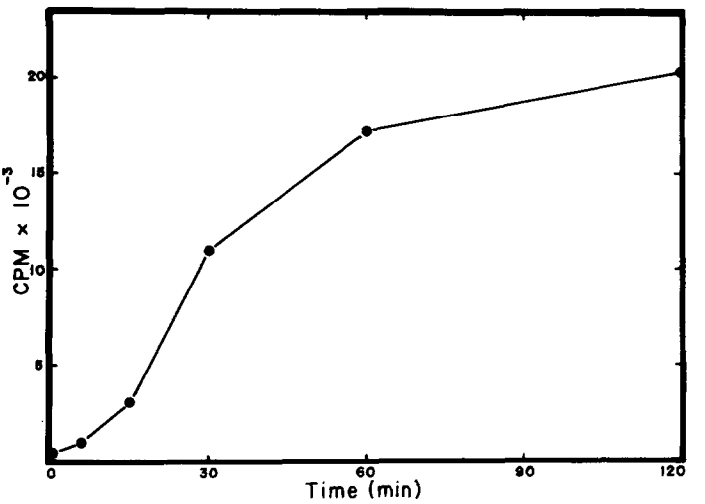

Fig. 1. Reaction mixtures contained: Tris-Cl, pH 7.4 (0.1 M); KF (0.05 M), ATP (2.5 mM); dithiothreitol (1 mM); farnesyl pyrophosphate (7 μM); [^{14}C]-isopentenyl pyrophosphate (24 μM, $2x10^6$ cpm); Triton X-100 (2%); and oviduct enzyme (4-6 mg) in a final volume of 0.8 ml. The mixtures were incubated at 37° with shaking and at the indicated times were terminated by the addition of 2.5 volumes of chloroform:methanol (2:1). The product was isolated as described in Materials and Methods.

of dolichyl phosphate. The time course of incorporation of ^{14}C into chloroform-methanol soluble material is shown in Fig. 1. The reaction is approximately linear up to 30 min of incubation time.

The requirements for incorporation are shown in Table I. KF and ATP were required, probably to inhibit the action of endogenous phosphatases which might degrade the reaction substrates. Triton X-100 provided a 25-fold stimulation at a concentration of 2%; increasing the concentration further was without effect. Although the addition of a divalent cation was not necessary, the strong inhibition by EDTA suggests that one is required, as has been shown for other prenyl transferase reactions (13). The particulate preparation was unstable in the Tris buffer in which it was suspended; storage at 4° led to a 50% decrease in enzymic activity after 16 hours.

After extraction of the reaction mixture as described in Methods,

Table I. Requirements for the biosynthesis of oviduct polyprenyl phosphate

	cpm incorporated	% control
Complete system*	27,000	100
-KF	300	1.1
-ATP	7,250	26.8
-Triton X-100	960	3.6
-dithiothreitol	8,520	31.6
-farnesyl-PP	1,480	5.5
+EDTA	2,100	7.7
Boiled enzyme control	320	1.2

* The composition of the complete system is given in Fig. 1. The incubation time was 2 hours and the product was isolated as described in Materials and Methods.

the product was chromatographed on thin layers of silica gel in solvent C. A single peak of radioactivity coinciding with authentic dolichyl phosphate, was observed (Fig. 2). The compound migrated faster (Rf = 0.51) than standard markers of farnesyl monophosphate (Rf = 0.27) and farnesyl pyrophosphate (Rf = 0.12), as well as that of dolichyl pyrophosphate (Rf = 0.33). Similar results were obtained with solvent B.

When subjected to gel filtration of columns of OR PVA-6000 (EM Laboratories), a porous polyvinyl acetate resin with a molecular weight exclusion limit of 6000, the biosynthetic product co-chromatographed with authentic [1-^{3}H] dolichyl phosphate [prepared by the method of Wedgwood, et al. (11)]. The results are given in Fig. 3. Substances of lower molecular weight such as ubiquinone-50 (MW 849), and cholesterol (MW 387), were clearly resolved in this system. Therefore, the molecular weight of the biosynthesized material falls within the same range as dolichyl phosphate (MW 1400).

Although the biosynthetic product behaved identically to dolichyl

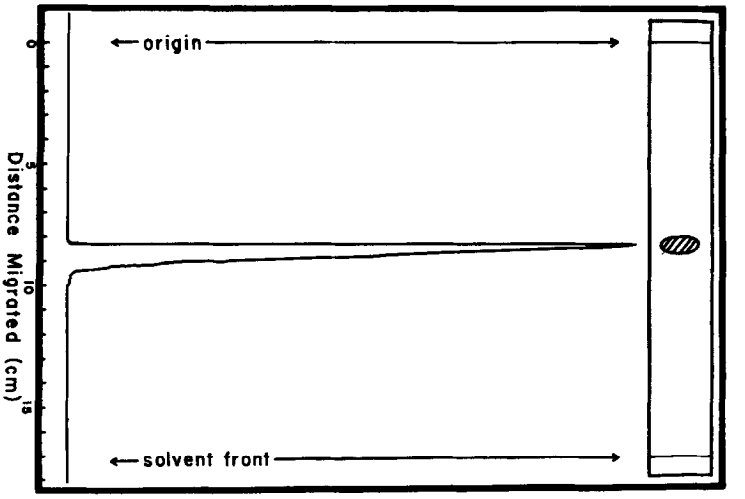

Fig. 2. Radioscan of biosynthesized material chromatographed on thin layers of silica gel G in solvent C. Authentic dolichyl phosphate migrated as shown at the top.

phosphate with respect to chromatographic and molecular weight properties, its chemical properties were quite distinct. Acid hydrolysis of the material in chloroform:methanol:0.8 M HCl (10:10:3) for 1 hour at 80°, followed by thin layer chromatography showed all of the compound to be converted to a form much less polar than dolichyl phosphate, migrating with the solvent front in solvent C.

In the less polar solvent A, two peaks of radioactivity were observed, with Rf values of 0.52 and 0.66, both migrating substantially faster than authentic dolichol (Rf = 0.32). Under identical conditions of hydrolysis authentic hen oviduct dolichyl phosphate was not degraded, in accordance with previous observations on pig liver dolichyl phosphate (11).

The acid lability of the compound strongly suggests the presence of an allylic phosphate group. Previous investigators have obtained similar results for the acid hydrolysis of undecaprenyl pyrophosphate and neryl phosphate (14,15), both of which are allylic phosphates containing an α-isoprene unit in the cis configuration. The degradation products of

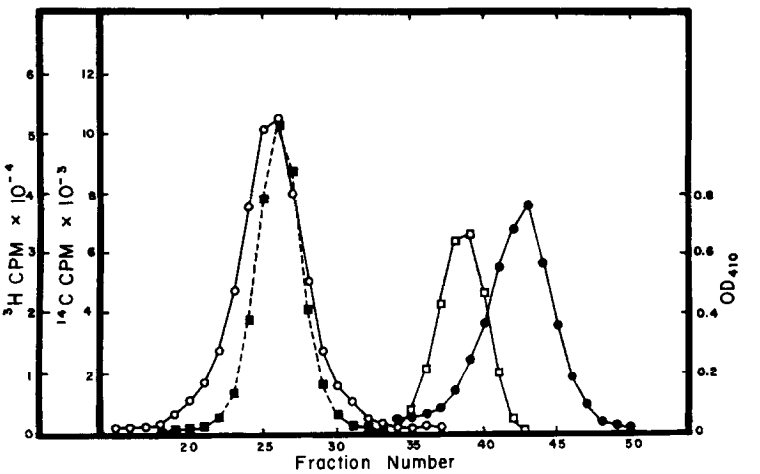

Fig. 3. The biosynthesized material was chromatographed on a 1x30 column of OR PVA-6000 equilibrated with chloroform methanol (2:1) containing 0.2 M ammonium acetate. 0.25 ml fractions were collected. ■--■, ^{14}C-labeled biosynthetic product; O——O, ^{3}H-dolichyl phosphate; □——□, ubiquinone (measured at 420 nm); ●——●, ^{3}H-cholesterol.

these two compounds have been identified as a mixture of tertiary alcohols and hydrocarbons which have a faster mobility on thin layer chromatography than the parent alcohols.

A structure for the biosynthesized material, compatible with the above observations, is that of 2,3-dehydrodolichyl phosphate, distinguished from dolichyl phosphate by the presence of an unsaturated α-isoprene unit. This molecule could arise by pyrophosphatase cleavage of 2,3-dehydrodolichyl pyrophosphate, the logical product of cis-isoprene addition to farnesyl pyrophosphate. Attempts on our part to effect the reduction of the α-isoprene unit of the biosynthesized material have thus far been unsuccessful. Addition of NADPH, NADH, or reduced methyl viologen to the incubation mixture or subsequent to incubation has led to no decrease in acid labile products. One possible explanation is that the substrate for the α-isoprene reductase contains a pyrophosphate ester rather than a monophosphate ester and that a potent pyrophosphatase degrades the molecule to the mono-

phosphate before reduction is accomplished. Further investigations on this hypothesis are in progress.

While these investigations were in progress, Daleo and Lezica reported the biosynthesis of dolichyl phosphate in pea seedlings (16). The enzyme preparation that was utilized was a soluble one and apparently synthesized authentic dolichyl phosphate as judged by the lack of acid lability of the product. The marked differences between their observations and those presented in this paper probably reflect the difference in the source of enzyme employed.

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REFERENCES:

1. Hemming, F. W. (1974) in MTP International Reviews in Science, Biochemistry, Series 1. Biochemistry of Lipids (Goodwin, T. W., ed.), Vol 4, pp. 39-98, Butterworth, London.
2. Dunphy, P. J., Kerr, J. D., Pennock, J. F., Whittle, K. J., and Feeney, J. (1967) Biochim. Biophys. Acta 136, 136-147.
3. Waechter, C. J. and Lennarz, W. J. (1976) Ann. Rev. Biochem. 45, 95-112.
4. Wright, A., Dankert, M., Fennessey, P., and Robbins, P. W. (1967) Proc. Nat. Acad. Sci. 57, 1798-1803.
5. Struck, D. K. and Lennarz, W. J. (1977) J. Biol. Chem. 252, 1007-1013.
6. Butterworth, P. H. W., Draper, H. H., Hemming, F. W., and Morton, R. A. (1966) Arch. Biochem. Biophys. 113, 646-653.
7. Martin, H. G. and Thorne, K. J. I. (1974) Biochem. J. 138, 277-280.
8. Gough, D. P. and Hemming, F. W. (1970) Biochem. J. 118, 163-166.
9. Allen, C. M., Keenan, M. V., and Sack, J. (1976) Arch. Biochem. Biophys. 175, 236-248.
10. Keller, R. K. and Adair, W. L. (1977) Biochim. Biophys. Acta, in press.
11. Wedgwood, J. F., Strominger, J. L., and Warren, C. D. (1976) J. Biol. Chem. 249, 6316-6324.
12. Popjak, G., in Methods in Enzymology (R. B. Clayton, ed.), Vol 15, pp. 393-453, Academic Press, New York.
13. Dorsey, J. K., Dorsey, J. A., and Porter, J. W. (1966) J. Biol. Chem. 241, 5353-5360.
14. Keenan, M. V. and Allen, C. M. (1974) Arch. Biochem. Biophys. 161, 375-383.
15. Valenzuela, P. and Cori, O. (1967) Tetrahedron Letts. 132, 3089-3094.
16. Daleo, G. R. and Lezica, R. P. (1977) FEBS Letts. 74, 247-250.