Biochem. J. (1971) **124**, 957–959
Printed in Great Britain

Dolichol Phosphates as Acceptors of Mannose from Guanosine Diphosphate Mannose in Liver Systems

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(*Received* 13 *August* 1971)

When preparations of endoplasmic reticulum of pig liver (Alam, Barr, Richards & Hemming, 1970), rat liver (Behrens, Parodi, Leloir & Krisman, 1971; De Luca, Rosso & Wolf, 1970), rabbit liver (Caccam, Jackson & Eylar, 1969) or chicken liver (P. J. Evans & F. W. Hemming, unpublished work) are incubated with GDP-mannose, a mannolipid is formed. The mannolipid has many of the properties expected of dolichol phosphate mannose, and its yield can be increased by the addition of dolichol phosphate to the incubation medium (Alam *et al.* 1970; Behrens *et al.* 1971). However, insufficient of the mannolipid has been isolated in a pure state to enable it to be characterized unequivocally, and demonstration of the incorporation of radioactive dolichol phosphate into the lipid requires a specific radioactivity greater than has been available. The latter difficulty has been largely overcome, and the present paper reports the incorporation of exogenous [^{3}H]dolichol phosphate into the mannolipid of pig liver. The formation of a similar lipid containing endogenous [^{3}H]dolichol phosphate in chicken liver is also reported.

In *Phytophthora cactorum*, which does not form sterols, one of the major metabolites of mevalonate is a family of dolichols in which dolichol-15 predominates (J. B. Richards & F. W. Hemming, unpublished work). The fungus was grown for 25 days on a synthetic medium (Elliot, Hendrie & Knights, 1966) containing [5-$^3H_2$]mevalonic acid (200μCi; 0.53μmol) and the dolichol was recovered in the usual way (Stone, Butterworth & Hemming, 1967). This was phosphorylated chemically and purified (Alam *et al.* 1970). The [^{3}H]dolichol phosphate (2×10^5 d.p.m.; approx. 90pmol) was added to a microsomal preparation of pig liver as the MgEDTA complex (Behrens & Leloir, 1970). Incubation of this in a total volume of 245μl containing enzyme preparation (0.15ml), EDTA (2.2mM), GDP-[^{14}C]mannose (0.05μCi; 1.56μmol), manganese chloride (8.8mM), sodium fluoride (80mM), tris–HCl buffer, pH7.1 (26.4mM) and Triton X-100 (0.08%) was carried out for 40min at 37°C. The reaction was stopped by adding butan-1-ol (1ml) and the lipid was extracted with chloroform–methanol (2:1, v/v) (2×2ml). Treatment of the lipid with alkali (Dawson, 1967) was followed by preparative t.l.c. with system *A* (silica gel G; di-isobutyl ketone–acetic acid–water, 20:15:2, by vol.). Radioassay of portions of 0.5cm bands of the chromatogram showed the presence of four areas containing ^{3}H. A small amount of ^{3}H ran just in front of the origin, but larger amounts corresponded to the [^{14}C]mannolipid (R_F 0.41; 6900 d.p.m. of ^{14}C; 7800 d.p.m. of ^{3}H), unchanged [^{3}H]dolichol phosphate (R_F 0.67) and an impurity in the original [^{3}H]dolichol phosphate (R_F 0.55). Rechromatography of half of the material eluted from the mannolipid region confirmed that most of the ^{3}H remained associated with the ^{14}C. When the other half was treated with dilute acid (pH2 for 10min at 100°C) and the chloroform-soluble products were chromatographed, 98% of the ^{14}C formerly present in the mannolipid region had disappeared, having been liberated as mannose. Of the ^{3}H that disappeared from the same region, 90% reappeared in the dolichol phosphate region. This shows that the mannolipid contains dolichol phosphate linked to mannose by an acid-labile link.

Analogy with bacterial systems suggests the possibility of two alternative reactions forming this lipid:

$$\text{GDP-mannose} + \text{dolichol phosphate} \rightleftharpoons \text{GDP} + \text{dolichol phosphate mannose} \quad (1)$$

$$\text{GDP-mannose} + \text{dolichol phosphate} \rightleftharpoons \text{GMP} + \text{dolichol diphosphate mannose} \quad (2)$$

It was found that when GDP (240μM) was added to the incubation medium, from the beginning or after 5min incubation, the amount of mannolipid formed after a total of 15min incubation was 4 and 8% respectively of a control, whereas GMP (240μM) had no effect. That the addition of GDP reverses the reaction rather than just inhibits it was confirmed by incubating with unlabelled GDP-mannose (1.44μM) instead of GDP-[^{14}C]mannose and after 3min adding [U-^{14}C]GDP (4.4×10^5 d.p.m.; 1.66μM) to the incubation medium. After a

further 3min incubation, water (1ml) was added and the mixture was boiled for 90min. The denatured protein was removed by centrifugation and washed once with water (1ml). The supernatant and washings were combined and the volume was made up to 8ml with water. The lipids were removed by extraction with butan-1-ol (1ml), and the aqueous layer was recovered and freeze-dried. Radioassay of a portion of this material from different experiments by liquid-scintillation counting indicated recoveries of 85–90% of the initial ^{14}C added to the incubation mixture. T.l.c. of portions of the material alongside standard marker compounds on cellulose with ethyl acetate–butan-1-ol–acetic acid–water (6:8:5:8, by vol.) as solvent showed that 20% of the ^{14}C present was in the form of GDP-mannose (R_F 0.07); 30% remained as GDP (R_F 0.13) and 40% was recovered as GMP (R_F 0.28). This pattern was confirmed by t.l.c. on cellulose with ethanol–1M-ammonium acetate (1:1, v/v) as solvent. In this system the R_F values were 0.38 for GDP-mannose, 0.15 for GDP and 0.25 for GMP. The evidence is clearly in favour of the formation of [^{14}C]GDP-mannose. No [^{14}C]-GDP-mannose was formed in a boiled-enzyme control. This is interpreted as illustrating the presence of an enzyme capable of catalysing reaction (1), which is reversible.

Although it is established that the enzyme will use exogenous dolichol phosphate as an acceptor, the argument that it uses endogenous dolichol phosphate would be strengthened if the incorporation into the mannolipid of a radioactive precursor of dolichol phosphate could be demonstrated. In normal mammalian tissue the rate of biosynthesis of dolichol compared with that of sterols is low (Butterworth, Draper, Hemming & Morton, 1965; Gough & Hemming, 1970), and it seemed that the chick embryo might be a more useful organism for this experiment since, although chicken liver contains dolichol (J. Burgos & F. W. Hemming, unpublished work), the egg contains little or no dolichol but relatively large quantities of cholesterol (Pennock, Neiss & Mahler, 1962). Six fertile chicken eggs were inoculated with [5-$^3H_2$]-mevalonic acid (200μCi; 0.53μmol in 0.6ml of 50mM-tris–HCl buffer, pH7.1) during the first day of development. The eggs were incubated at 37°C with frequent turning for a further 20 days. Livers of the 1-day-old chicks provided the enzyme preparation, as for pig liver, which was incubated (3×quantity of medium used above) with (*a*) GDP-[^{14}C]mannose (0.6μCi; 420nmol) or (*b*) unlabelled GDP-mannose (420nmol) for 15min. The extracted lipid was then chromatographed on a column of silica gel (10g). Chloroform (100ml) removed neutral lipids and chloroform–methanol (1:1, v/v) (100ml) eluted polar lipids, portions of which were subjected to t.l.c. in system *A*. In experiment (*a*) 75% of the ^{14}C on the chromatogram (1500 d.p.m.) corresponded in position to dolichol phosphate mannose. A little ^{14}C remained close to the origin. [The formation of this lipid had been shown previously to increase in the presence of exogenous dolichol phosphate (P. J. Evans & F. W. Hemming, unpublished work).] The major peak of ^3H (1270 d.p.m.) coincided precisely with that of ^{14}C, and most of the remaining ^3H ran near the origin. Chromatography of polar lipids from a second incubation (*a*) on silica gel G with solvent system *B* (chloroform–methanol–water, 65:25:4, by vol.) again showed that most of the ^3H (80%; 3500 d.p.m.) remained associated with ^{14}C at an R_F (0.33) expected for dolichol phosphate mannose. The distribution of ^3H on the chromatogram after t.l.c. (system *A*) of the polar lipids of incubation (*b*) was precisely the same as in incubation (*a*). When [5-$^3H_2$]mevalonic acid was replaced by [2-^{14}C]mevalonic acid (20μCi; 3.5μmol) and incubation (*b*) repeated the distribution of ^{14}C after t.l.c. in system *A* was essentially the same as that of ^3H described above. These results leave little doubt that the endoplasmic reticulum of chicken liver uses endogenous dolichol phosphate as an acceptor of mannose.

It is clear from the above experiments that in pig liver exogenous dolichol phosphate will accept mannose residues from GDP-mannose according to reaction (1) and that in chicken liver both exogenous and endogenous dolichol phosphate fulfil this role. It is likely that all animal systems will use dolichol phosphate in this way. The relevance of these observations to brief reports of a possible similar role for a metabolite of retinol (De Luca, Rosso & Wolf, 1971; Griffin, Dickenson, Chiba & Johnson, 1971) is of great interest but at present uncertain.

J. B. R. and P. J. E. are in receipt of Research Studentships from the Medical Research Council and from the Science Research Council respectively. The work was aided by a grant from the Science Research Council.

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