The Involvement of Endogenous Dolichol in the Formation of Lipid-Linked Precursors of Glycoprotein in Rat Liver

By H. G. MARTIN* and KAREEN J. I. THORNE†

Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council,
Cambridge CB4 1XJ, U.K.

(Received 19 September 1973)

Endogenous dolichol was shown to function as a natural acceptor of mannose residues by using regenerating rat liver containing [^3H]dolichol. When subcellular fractions from this liver were incubated with GDP-[^{14}C]mannose a double-labelled lipid, which represented 30% of the total [^{14}C]mannolipid, could be isolated. This lipid was shown to be identical with the dolichol phosphate mannose formed from exogenous dolichol phosphate, by chromatography, stability to alkali and by chemical cleavage to mannose and dolichol derivatives. It was formed by the rough endoplasmic reticulum and mitochondria. If it is concerned in glycoprotein synthesis this would suggest that it functions in the formation of both secreted and mitochondrial glycoproteins. When both the dolichol and retinol of rat tissue were radioactive they made similar contributions to the synthesis of the lipid by liver microsomal fractions and intestinal epithelial cells.

The first evidence that dolichol might be concerned with the formation of lipid-linked glycosyl residues for glycoprotein synthesis in mammalian tissue, in a manner analogous to the involvement of prenols in the biosynthesis of bacterial cell-wall components (reviewed by Lennarz & Scher, 1972), came from the observation that dolichol phosphate stimulates the incorporation of glucose, mannose and *N*-acetylglucosamine from nucleotide precursors into lipid by rat liver microsomal fractions (Behrens & Leloir, 1970; Behrens *et al.*, 1971). Richards *et al.* (1971) and Richards & Hemming (1972) showed, by using [^3H]dolichol isolated from *Phytophthora cactorum*, that this stimulation of incorporation of [^{14}C]mannose resulted in the formation of a double-labelled lipid, from which [^{14}C]mannose and [^3H]dolichol phosphate could be recovered after acid hydrolysis.

Although liver microsomal fractions catalyse the reaction of exogenous dolichol phosphate with GDP-mannose the question remains whether this lipid is the natural acceptor of mannose *in vivo*. Considerable evidence is now accumulating that retinol participates in the formation of a manno-lipid intermediate in glycoprotein synthesis (De Luca *et al.*, 1971, 1973). The development of a method for rendering the dolichol of rat liver radioactive *in vivo*, by hepatectomy followed by injection of [4S- ^3H]mevalonate (Martin & Thorne, 1974), made it possible to discover whether endogenous dolichol

is used in the biosynthesis of manno-lipid and to compare its importance with that of retinol.

Experimental

Radioactive chemicals

[3R,(4S)-4- ^3H , 3S,(4R)-4- ^3H]Mevalonic acid lactone (250mCi/mmol) and GDP-[U- ^{14}C] mannose (143mCi/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [15- ^{14}C]Retinol (12.5mCi/mmol) was a generous gift from Dr. O. Wiss, Hoffman-La Roche, Basle, Switzerland.

Incorporation of [^3H]mevalonate into regenerating rat liver

Normal male and female Hooded rats (weight 250–350g) were fed from weaning on the stock 41B diet (E. Dixon and Son, Ware, Herts., U.K.).

Vitamin A-deficient rats were fed on a deficient diet (Moore, 1957) until eye lesions were first observed. At 5 days before experimentation 1% (w/w) cholesterol was added to the diet to inhibit cholesterol biosynthesis. Surgery, under light diethyl ether anaesthesia, involved the ligation and excision of the large median and left lateral liver lobes, some 60–70% of the total liver weight, while leaving the right lateral and caudate lobes to regenerate. Each rat was injected with 10 μCi of [^3H]mevalonate intraperitoneally in 0.9% sterile NaCl 48 h after hepatectomy and killed 12 h later.

Subcellular fractionation

Rough and smooth endoplasmic reticulum were prepared as described by Wagner & Cynkin (1969), by the method devised by Dallner (1963). Mito-

*Present address: H. J. Heinz Co. Ltd., Food and Nutrition Research Laboratory, Hayes Park, Hayes, Middx. UB4 8AL, U.K.

†Present address: Strangeways Research Laboratory, Wort's Causeway, Cambridge CB1 4RN, U.K.

chondria were prepared and purified as described in the preceding paper (Martin & Thorne, 1974). Preparations were suspended in 0.05M-Tris-HCl buffer (pH 7.0) at a concentration of about 10mg of protein/ml. The purity of the fractions was checked by measuring the activities of the following enzymes: glucose 6-phosphatase (Hübscher & West, 1965), 5'-nucleotidase (Michell & Hawthorne, 1965), acid phosphatase (Hübscher & West, 1965), succinate dehydrogenase (Pennington, 1961). The RNA content was also measured (Ceriotti, 1955). Protein was determined by the Folin-Lowry method (Lowry *et al.*, 1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard.

Incorporation of mannose into lipid

Except where otherwise stated GDP-[U-¹⁴C]-mannose (1×10^4 d.p.m.) was incubated in 4ml of 10mM-MnCl₂-2.5mM-EDTA-30mM-Tris-HCl (pH 7.0)-3mM-NaF with approx. 20mg of tissue protein for 15min at 37°C. The reaction was terminated by cooling to 4°C and adding 15ml of ice-cold chloroform-methanol (2:1, v/v). The suspension was transferred to a separating funnel with 15ml of water and a further 35ml of chloroform-methanol (2:1, v/v) and extracted at 4°C for 16h. The organic layer was collected and the aqueous layer extracted twice more with 25ml of chloroform-methanol (2:1, v/v). The organic layers were combined and evaporated to dryness by distillation under reduced pressure.

Measurement of radioactivity

Radioactive lipids were dissolved in 10ml of toluene containing (per litre) 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (Packard Instrument Co., Downer's Grove, Ill., U.S.A.). Silica from thin-layer chromatograms was counted directly for radioactivity in the same scintillant. Paper chromatograms were cut into strips (1cm wide) which were then immersed in 1ml of water for 1h at room temperature and the paper in water was counted for radioactivity with 10ml of Kennedy's (1969) scintillation fluid. Radioactivity was measured in low-potassium glass vials in a Packard Tri-Carb 3375 liquid-scintillation spectrometer. All radioactivity measurements were corrected for quenching and expressed as d.p.m. Corrections were made for the overlapping of the ¹⁴C spectrum into the ³H channel.

Thin-layer chromatography

Solvents: A, chloroform-methanol-water (65:25:4, by vol.); B, di-isobutyl ketone-acetic acid-water (60:45:6, by vol.); C, chloroform-methanol-formic acid-water (70:18.5:0.5, by vol.); D, chloroform-methanol-35% (w/v) NH₃-water (80:30:0.5:3,

by vol.); E, hexane-ethyl acetate (9:1, v/v) were used for t.l.c. on silica gel G (E. Merck, Darmstadt, Germany). Radioactive spots were detected by radioautography with Kodirex X-ray film (Kodak Ltd., London W.C.2, U.K.) and by direct counting of the silica for radioactivity.

Column chromatography

(a) *Silicic acid.* By using a modification of the method of Lennarz & Talamo (1966) the sample was applied in chloroform to a column (1.5cm × 20cm) of silicic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) equilibrated with chloroform, and was eluted with a linear gradient of increasing concentration of methanol in chloroform. Fractions (5-8ml) were collected.

(b) *DEAE-cellulose.* DEAE-cellulose (preswollen DE-52; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) was acetylated by the method of Dankert *et al.* (1966). The column (1cm × 30cm) was equilibrated with 99% (v/v) methanol, the sample was applied in 15-20ml of methanol and was eluted with 20ml of methanol followed by a gradient of increasing concentration of ammonium acetate in methanol [100ml of 0.4M-ammonium acetate in methanol containing 3% (v/v) acetic acid, mixed with 100ml of methanol] (Dankert *et al.*, 1966). Fractions (6ml) were collected.

Chemical cleavage of the lipid

(a) *Acid hydrolysis.* Lipid eluted from silicic acid columns was incubated in 0.5ml of propan-1-ol with 0.5ml of 0.1M-HCl at 100°C for 15min. The mixture was cooled, 1ml of water was added and the solution was extracted with 2 × 1ml of diethyl ether, followed by 2 × 1ml of butan-1-ol. The aqueous layer was then neutralized with 0.1M-NaOH and further extracted with 2 × 1ml of butan-1-ol.

(b) *Acetolysis (Renkonen, 1965).* The purified lipid was dissolved in 2.0ml of anhydrous acetic acid-acetic anhydride (3:2, v/v) and heated at 145°C for 4h in a sealed ampoule. The mixture was cooled, acetylating reagents were removed by evaporation under a stream of N₂ and the dry residue was redissolved in 2.0ml of chloroform-methanol-water (8:4:3, by vol.) After partition of the solvent system by centrifugation the lower organic layer was collected and the aqueous layer further extracted with 1.0ml of chloroform.

(c) *Hydrogen fluoride.* About 1.0ml of liquid HF (British Drug Houses Ltd., Poole, Dorset, U.K.) was added to the dry purified lipid at 0°C and left at this temperature for 3h. Then 1.0ml of ice-cold chloroform was added, followed by 1.0ml of ice-cold water. The lower organic layer was collected and the aqueous layer was further extracted with 1.0ml of chloroform. The chloroform layers were combined and washed twice with 2.0ml of water and the

aqueous layer was neutralized with a solution of 20% (w/v) KOH and discarded.

Results

Incorporation of mannose into lipid by different cell fractions

Both mitochondrial and microsomal preparations catalysed the incorporation of mannose from GDP-mannose into lipid (Table 1). Fractionation of the microsomal preparation into rough endoplasmic reticulum and smooth endoplasmic reticulum indicated that the rough endoplasmic reticulum was more active, although it was apparent from the glucose 6-phosphatase activity that this fraction was less pure than the smooth endoplasmic reticulum (Table 2). The ability of the microsomal fractions to synthesize mannanlipid could not be explained by mitochondrial contamination, because the activity of the mitochondrial marker enzyme, succinate dehydrogenase, was low. Plasma-membrane contamination, as measured by 5'-nucleotidase activity, was fairly high in the smooth endoplasmic reticulum, but as this fraction is relatively inactive in mannose incorporation it seems unlikely that the plasma membrane is the site of mannanlipid formation. Both rough endoplasmic reticulum and smooth endoplasmic reticulum were contaminated with lysosomal debris, both fractions exhibiting fairly high acid phosphatase activities. The ratio of RNA to protein

by weight was seven times greater in the rough endoplasmic reticulum than in the smooth endoplasmic reticulum.

Characteristics of mannose incorporation

The time-course of mannose incorporation into lipid by rough endoplasmic reticulum and smooth endoplasmic reticulum is shown in Fig. 1. The incorporation of mannose into lipid proceeded in a manner characteristic of the formation of lipid intermediates of glycopolymers (Scher *et al.*, 1968). It was greatest at the earliest time measured, 3 min, and then declined. This resembles the results obtained by Richards & Hemming (1972) for pig liver microsomal preparations.

The requirements for mannose incorporation into lipid are shown in Table 3. Both MnCl₂ and EDTA were essential, whereas Triton X-100 had no effect. Richards & Hemming (1972) found with pig liver microsomal preparations that EDTA was not necessary and that Triton X-100 stimulated the reaction; Caccam *et al.* (1969), with rabbit liver smooth endoplasmic reticulum, demonstrated a requirement for EDTA. If the concentration of EDTA exceeded that of MnCl₂ the incorporation of mannose was greatly inhibited, owing to the chelation of the Mn²⁺ ions. The amount of mannose incorporated into lipid after a 15 min incubation was directly proportional to the protein concentration.

Table 1. *Incorporation of mannose into lipid by different cell fractions*

GDP-[U-¹⁴C]mannose (4.4 × 10⁵ d.p.m.) in 4.0 ml of 10 mM-MnCl₂-2.5 mM-EDTA-30 mM-Tris-HCl (pH 7.0)-3 mM-NaF was incubated with 2.0 ml of membrane suspension at 37°C for 15 min. The lipid was extracted as described in the Experimental section.

Cell fraction	Protein (mg)	[¹⁴ C]Mannose incorporated into lipid	
		(d.p.m.)	(d.p.m./mg of protein)
Mitochondria	72.1	13 675	190
Microsomal fraction	80.9	19 641	243
Rough endoplasmic reticulum	86.5	29 670	343
Smooth endoplasmic reticulum	108.4	3802	35

Table 2. *Purity of rough and smooth endoplasmic reticulum determined by activity of marker enzymes*

The units of enzyme activity were µg of P released/15 min per mg of protein for glucose 6-phosphatase, 5'-nucleotidase and acid phosphatase and change in E₄₉₀/15 min per mg of protein for succinate dehydrogenase.

	Enzyme activity			
	Glucose 6-phosphatase	5'-Nucleotidase	Acid phosphatase	Succinate dehydrogenase
Total homogenate	0.55	4.55	4.30	0.34
Rough endoplasmic reticulum	4.20	4.22	5.34	0.084
Smooth endoplasmic reticulum	7.04	14.95	3.50	0.095

Table 3. *Requirements for incorporation of mannose into lipid*

The complete system was 10mM-MnCl₂, 2.5mM-EDTA, 30mM-Tris-HCl (pH 7.0), GDP-[U-¹⁴C]mannose (1 × 10⁴ d.p.m.) and 3mM-NaF in 100 μl, incubated with 50 μl of membrane suspension (about 1 mg of protein) for 15 min at 37°C. The lipid was extracted as described in the Experimental section.

Incubation system	[¹⁴ C]Mannose incorporation into lipid (d.p.m./mg of protein)	
	Smooth endoplasmic reticulum	Rough endoplasmic reticulum
Complete	94.4	524.7
– MnCl ₂	24.6	58.8
– EDTA	31.6	85.4
+ Triton X-100 (0.1%)	72.8	548.4
Boiled enzyme	1.9	8.0

By analogy with the bacterial system (Lennarz & Scher, 1972) it would be expected that dolichol phosphate, rather than free dolichol, would react with GDP-mannose to form a lipid intermediate. To inhibit endogenous phosphatase activity, which might hydrolyse dolichol phosphate, F[–] was included in the incubation system (Hübscher & West, 1965). The effect of F[–] is shown in Fig. 2. NaF, at a concentration of 3 mM, maintained the incorporation of mannose into lipid at the rate observed after 3 min (Fig. 1) for a further 30 min. It thereby prevented the rapid decay in activity. A higher concentration of NaF (10 mM) was less effective. In pig liver the concentration of exogenous dolichol phosphate required to stimulate mannose incorporation could be decreased at least tenfold if 80 mM-NaF was present,

but mannose incorporation into endogenous lipid was unaffected by F[–] (Richards & Hemming, 1972).

Chromatography of mannoslipid

The characteristics of mannose incorporation into lipid refer to total mannoslipid. Further work revealed that more than one compound was formed. Mannolipid from GDP-[¹⁴C]mannose was prepared by using rough endoplasmic reticulum from regenerating rat liver which contained [³H]dolichol as described in the Experimental section. Chromatographic methods were used to discover if a lipid was formed which contained both [¹⁴C]mannose and [³H]dolichol. On silicic acid columns two [¹⁴C]-mannolipid components were obtained (Fig. 3). The first, representing 60–70% of the total ¹⁴C radioactivity, was alkali-labile, being hydrolysed by 0.05 M-NaOH after 20 min at 37°C. As glycosylated phosphoprenols are usually stable to mild alkali (Scher *et al.*, 1968) this compound was not investigated further. All the identification studies were performed with the purified second compound. The presence of ³H in the [¹⁴C]glycolipid suggested that it did indeed contain dolichol.

When the second lipid from the silicic acid column was analysed on a column of DEAE-cellulose (Fig. 4) both ¹⁴C and ³H were recovered in a single peak, eluted with 0.05 M-ammonium acetate. The binding of the lipid to DEAE-cellulose is evidence for the presence of phosphate in the molecule.

¹⁴C and ³H radioactivities co-chromatographed on thin layers of silica developed with the five solvents A (*R_F* 0.35), B (*R_F* 0.20), C (*R_F* 0.13), D (*R_F* 0.08) and E (*R_F* 0). The values for solvents A and B are identical with those obtained for prenol phosphate mannose isolated from *Micrococcus lysodeikticus* (Lahav *et al.*, 1969) and are somewhat similar to those reported by Richards & Hemming (1972) for dolichol phosphate mannose from pig liver.

The mannoslipid from mitochondria had the same chromatographic characteristics as the lipid from the rough endoplasmic reticulum.

Fig. 1. Time-course of incorporation of [¹⁴C]mannose into lipid by microsomal membrane fractions

GDP-[¹⁴C]mannose (1.0 × 10⁴ d.p.m.) in 50 μl of 10mM-MnCl₂–2.5mM-EDTA–30mM-Tris-HCl (pH 7.0) was incubated with 50 μl (about 1 mg of protein) of membrane suspension or cell homogenate at 37°C. Incubations were for measured time-intervals and the reaction was stopped by cooling and the lipid extracted with 3 × 3 ml of ice-cold chloroform-methanol (2:1, v/v). ○, Rough endoplasmic reticulum; △, smooth endoplasmic reticulum; ●, total homogenate

Fig. 2. Effect of NaF on the incorporation of [¹⁴C]mannose into lipid

GDP-[¹⁴C]mannose (1.0×10^4 d.p.m.) in 50 μ l of 10 mM-MnCl₂-2.5 mM-EDTA-30 mM-Tris-HCl (pH 7.0) was incubated with 50 μ l (about 1 mg of protein) of membrane suspension at 37°C. Tubes were incubated for measured time-intervals and the reaction was stopped by cooling and the lipid extracted with 3 $\times$ 3 ml of ice-cold chloroform-methanol (2:1, v/v). $\circ$, No NaF; $\bullet$, 3 mM-NaF; $\blacktriangle$, 10 mM-NaF. (a) Rough endoplasmic reticulum; (b) smooth endoplasmic reticulum.

Fig. 3. Column chromatography of mannlipid from [¹⁴C]mannose and [³H]mevalonate on silicic acid

Mannlipid was prepared as described in the Experimental section. —, ¹⁴C; ·····, ³H. Fractions (5–8 ml) were collected.

Identification of [¹⁴C]mannose in mannlipid

[¹⁴C]Mannose was liberated from the purified mannlipid by acid hydrolysis as described in the Experimental section. The aqueous fraction was freeze-dried, dissolved in pyridine and a sample taken for paper chromatography (Fig. 5). The [¹⁴C]-labelled water-soluble hydrolysis products co-chromatographed almost exclusively with the authentic mannose marker.

Fig. 4. Column chromatography of mannlipid from [¹⁴C]mannose and [³H]mevalonate on DEAE-cellulose

The second peak from the silicic acid column (Fig. 3) was collected and chromatographed on DEAE-cellulose as described in the Experimental section. —, ¹⁴C; ·····, ³H; ----, ammonium acetate concn. Fractions (6 ml) were collected.

Identification of [³H]dolichol in mannlipid

The presence of an α -saturated residue in dolichol makes the dolichol-phosphate bond stable to acid hydrolysis (Shah *et al.*, 1965). It is therefore difficult to recover substantial amounts of free dolichol from

a mannoside containing it. Three methods for chemical cleavage were, however, found which gave derivatives of dolichol, together with small amounts of the free compound (Table 4). These methods have all been used successfully for the cleavage of glycosylated phosphoprenols from *Lactobacillus plantarum* (Thorne, 1973). The identities of the ^3H -labelled lipid products were determined by co-chromatography with samples of dolichol (a generous gift from Dr. F. W. Hemming, University of Liverpool), chemically synthesized dolichol acetate (Thorne & Kodicek, 1966), dolichol phosphate (Thorne, 1973) and HF-treated dolichol. In agreement with Richards & Hemming (1972) acid hydrolysis liberated dolichol phosphate. Renkonen (1965) developed a method for cleavage of phospholipids between phosphate and glycerol by

Fig. 5. Paper chromatography of water-soluble hydrolysis products from $[^{14}\text{C}]$ mannoside

Ascending chromatography in propanol-ethanol-water (5:3:2, by vol.) on Whatman no. 1 paper was used. Marker sugars were detected by the periodate-Schiff method (Shaw, 1968).

acetolysis. This procedure liberated dolichol acetate and free dolichol, but in rather small yield. HF has been used to hydrolyse the phosphate bond linking 1-kojibiosyl diglyceride to teichoic acid (Toon *et al.*, 1972). It proved very effective in the cleavage of the mannoside. It is concluded that the ^3H -labelled component of the mannoside is dolichol.

Inhibition and reversal of mannose incorporation by guanine nucleotides

The attachment of the mannoside to DEAE-cellulose and its hydrolysis by acid to dolichol phosphate showed that the molecule contains phosphate. Reaction of GDP-mannose with dolichol phosphate could give either dolichol phosphate mannose and GDP, or dolichol pyrophosphate mannose and GMP. The stability of the lipid to alkali suggests the former (Dankert *et al.*, 1966). This was confirmed by testing the inhibitory activity of GDP and GMP on the reaction (Table 5). GDP inhibited the incorporation of mannose into lipid even at 0.5 mM. Inhibition by GMP was much less significant: these results are consistent with the formation of dolichol phosphate mannose and GDP. After mannose incorporation into lipid for 5 min, further incubation for 10 min with GDP decreased the radioactivity of the lipid to 9% of the value obtained on incubation without GDP. GDP has evidently reversed the incorporation of mannose into lipid. The reversibility of this reaction has been described in detail by Richards & Hemming (1972). It should be remembered that two mannosides are present and are both affected by GDP.

Simultaneous utilization of retinol and dolichol for mannoside formation

The experiments described above demonstrate that endogenous $[^3\text{H}]$ dolichol reacts with GDP-mannose to form dolichol phosphate mannose.

Table 4. Chemical treatment of mannoside to liberate dolichol derivatives

Chemical treatment of the isolated mannoside is described in more detail in the Experimental section. The products were analysed by t.l.c. on silica gel G with solvent E [ethyl acetate-hexane (1:9, v/v)] or solvent B [di-isobutyl ketone-acetic acid-water (20:15:2, by vol.)] and radioactive substances were detected by direct counting of the silica with toluene scintillation fluid.

Treatment	Product	Identification (R_f in solvent E)	Yield (%)
0.05 M-HCl, 100°C, 15 min	Free dolichol	0.4	3.8
	Dolichol phosphate	0.7*	17
Acetic anhydride-acetic acid (2:3, v/v), 145°C, 4 h	Free dolichol	0.4	4.3
	Dolichol acetate	0.75	6.6
HF, 0°C, 3 h	Free dolichol	0.4	10
	Fluoro derivative of dolichol	0.8	43.5

* Solvent B used for dolichol phosphate identification.

Table 5. *Inhibition and reversal of mannoslipid synthesis*

The complete system was 10mM-MnCl₂, 2.5mM-EDTA, 30mM-Tris-HCl (pH7.0), GDP-[U-¹⁴C]mannose (5 × 10⁴ d.p.m.) and 3mM-NaF in 0.5ml, incubated with 0.2ml of microsomal membrane suspension (approx. 0.8mg of protein) at 37°C for 15min (inhibition experiment) or 5 min (reversal experiment). Nucleotides were included in the incubation for 15min (inhibition) or, after 5 min incubation, for 10 min (reversal). The lipid was extracted as described in the Experimental section.

Incubation conditions	[¹⁴ C]Mannose incorporation into lipid			
	Inhibition experiment		Reversal experiment	
	(d.p.m./mg of protein)	%*	(d.p.m./mg of protein)	%*
Complete system	2475	100	824	100
+5mM-GMP	318	13	887	107
+0.5mM-GMP	1338	49	511	62
+5mM-GDP	41	1.5	74	9
+0.5mM-GDP	51	1.9	132	17

* Percentage of complete system.

Fig. 6. *Relative contribution of dolichol and retinol to mannoslipid formation*

Four vitamin A-deficient rats were fed with 125μg of [15-¹⁴C]retinol each by stomach tube 40h after partial hepatectomy and were injected intraperitoneally with 50μCi of [³H]mevalonate each 52h after hepatectomy. They were killed 12h later. Homogenized epithelial cells in 50mM-Tris-HCl buffer (pH7.0) and microsomal fractions were incubated with 0.1mM-GDP-mannose unlabelled under the conditions described in the Experimental section. The lipid was extracted and chromatographed on silicic acid as described in the Experimental section. —, ¹⁴C; ·····, ³H. (a) Liver microsomal fractions (80mg of protein); (b) intestinal epithelial cells (270mg of protein). Fractions (5–8ml) were collected.

They do not, however, exclude the possibility that GDP-mannose may react with other lipids as well. DeLuca *et al.* (1971, 1973) demonstrated that rat liver cells can synthesize a double-labelled lipid from [^3H]retinol and GDP-[^{14}C]mannose. To test for the formation of both dolichol and retinol mannosides simultaneously rat liver and intestinal preparations were made containing [^3H]dolichol and [^{14}C]retinol. Vitamin A-deficient rats were hepatectomized and then fed with [^{14}C]retinol and injected with [^3H]mevalonate to produce [^3H]dolichol. Liver microsomal fractions and homogenized intestinal epithelial cells were prepared and incubated with unlabelled GDP-mannose. The lipid was extracted and analysed by chromatography on silicic acid (Fig. 6). Both [^3H]dolichol and [^{14}C]retinol contributed to the formation of a lipid with the appropriate chromatographic characteristics (peak II) in both tissues.

The absolute amount of radioactivity contributed by the two lipids was similar in intestinal cells and fourfold greater for retinol than for dolichol in liver microsomal fractions. The molar specific radioactivity of the original retinol was one-twentieth of that of the original mevalonate or one-four-hundredth of that for dolichol of 20 isoprene units synthesized from it. However, the final specific radioactivity of the tissue dolichol is considerably decreased, owing to dilution by synthesis from other sources of mevalonate. The concentration of dolichol in rat liver is $60\mu\text{g/g}$ wet wt. (Gough & Hemming, 1970). The concentration of dolichol in the liver microsomal fractions may therefore be calculated from the analyses reported in the preceding paper (Martin & Thorne, 1974). At 60 h after hepatectomy the protein concentration was 0.21g/g wet wt. As the concentration of dolichol in the microsomal fraction was 29% of that of the whole liver, this represents about $80\mu\text{g/g}$ of protein. Two-thirds of the dolichol from 80 mg of microsomal protein co-chromatographed with 16000 d.p.m. of retinol (Fig. 6). This peak therefore contains of the order of $40\mu\text{g}$ (3 nmol) of dolichol and 0.6 nmol of retinol. Although this is of necessity only an approximate calculation, it may be deduced that both retinol and dolichol made important contributions to the formation of this lipid.

Only one peak of the lipid was eluted from silicic acid at this position instead of the two seen when [^{14}C]mannose was incorporated. This is partly because only the isoprenoid lipid and not the sugar was labelled. The absence of the isoprenoid radioactivity seen in the first peak of Fig. 3 cannot be explained.

Discussion

The incorporation of [$4\text{-}^3\text{H}$]mevalonate by regenerating rat liver into dolichol provided a system

for demonstration that liver microsomal fractions and mitochondria utilize endogenous dolichol for the formation of mannoside. One of the lipids formed from GDP-[^{14}C]mannose was a double-labelled compound from which [^{14}C]mannose and [^3H]dolichol or a derivative thereof could be recovered. Chemically the lipid seemed to be identical with the dolichol phosphate mannose formed when exogenous dolichol phosphate and GDP-mannose were incubated with pig liver microsomal fractions (Richards & Hemming, 1972). The two systems differed only in small details which could be due to species variation or to the difference between utilization of endogenous and exogenous dolichol phosphate.

Other evidence that dolichol phosphate is the natural acceptor of mannose has come from the work of Evans & Hemming (1973) who isolated a [^{14}C]mannoside from a bulk preparation of pig liver microsomal fractions incubated with GDP-[^{14}C]mannose and showed it to be identical, both chromatographically and by its i.r. and n.m.r. spectra, with chemically synthesized dolichol phosphate mannose.

It appears, however, that dolichol phosphate is not the only natural acceptor of mannose. DeLuca *et al.* (1973) have described in detail the utilization of endogenous retinol phosphate for the formation of mannoside. Our system has allowed us to detect the presence of both dolichol and retinol at somewhat similar amounts in this lipid. It is difficult to ascribe a functional difference to the two lipids at this stage. They do not appear to be tissue-specific, since both retinol and dolichol were utilized in intestine and in liver.

Analogy with the bacterial system suggests that these mannosides are lipid intermediates in the synthesis of extracellular glycoprotein. The high activity for mannoside formation in the rough endoplasmic reticulum correlates well with the observations of Molnar *et al.* (1969) that the rough endoplasmic reticulum is the site of mannose incorporation into secreted glycoprotein in rabbit liver. The dolichol concentration is, however, higher in the mitochondria than in the microsomal fractions (Burgos & Morton, 1962; Martin & Thorne, 1974) and mitochondria were nearly as active as microsomal fractions in the formation of dolichol phosphate mannose. This could be explained if dolichol functions in the formation of lipid intermediates in the biosynthesis of mitochondrial glycoprotein. Martin & Bosmann (1971) have reported that mitochondria are capable of autonomous glycoprotein biosynthesis.

We thank Dr. D. R. Fraser for performing the hepatectomies, Dr. F. W. Hemming for a gift of dolichol, Dr. O. Wiss for a gift of [^{14}C]retinol and Dr. E. Kodicek for his interest and encouragement.

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