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The Transfer of Mannose from Guanosine Diphosphate Mannose to Dolichol Phosphate and Protein by Pig Liver Endoplasmic Reticulum

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When pig liver microsomal preparations were incubated with GDP-[^{14}C]mannose, 10–40% of the ^{14}C was transferred to mannolipid and 1–3% to mannoprotein. The transfer to mannolipid was readily reversible and GDP was one of the products of the reaction. It was possible to reverse the reaction by adding excess of GDP and to show the incorporation of [^{14}C]GDP into GDP-mannose. When excess of unlabelled GDP-mannose was added to a partially completed incubation there was a rapid transfer back of [^{14}C]mannose from the mannolipid to GDP-mannose. The other product of the reaction, the mannolipid, had the properties of a prenol phosphate mannose. This was illustrated by its lability to dilute acid but stability to dilute alkali, and by its chromatographic properties. Dolichol phosphate stimulated the incorporation of [^{14}C]mannose into both mannolipid and into protein, although the former effect was larger and more consistent than the latter. The incorporation of exogenous [^{3}H]dolichol phosphate into the mannolipid, and its release, accompanied by mannose, on treatment of the mannolipid with dilute acid, confirmed that exogenous dolichol phosphate can act as an acceptor of mannose in this system. It was shown that other exogenous polyprenol phosphates (but not farnesol phosphate or cetyl phosphate) can substitute for dolichol phosphate in this respect but that they are much less efficient than dolichol phosphate in stimulating the transfer of mannose to protein. Since pig liver contained substances with the chromatographic properties of both dolichol phosphate and dolichol phosphate mannose, which caused an increase in transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to mannolipid, it was concluded that endogenous dolichol phosphate acts as an acceptor of mannose in the microsomal preparation. The results indicate that the mannolipid is an intermediate in the transfer of mannose from GDP-mannose to protein. Some 4% of the mannose of a sample of mannolipid added to an incubation was transferred to protein. A scheme is proposed to explain the variations with time in the production of radioactive mannolipid, mannoprotein, mannose 1-phosphate and mannose from GDP-[^{14}C]mannose that takes account of the above observations. ATP, ADP, UTP, GDP, ADP-glucose and UDP-glucose markedly inhibited the transfer of mannose to the mannolipid.

The role of polyprenol phosphate sugar derivatives as intermediates in the biosynthesis of bacterial wall polymers is now well established and the evidence has been reviewed in several articles (e.g. Rothfield & Romeo, 1971; Heath, 1971). Caccam *et al.* (1969) studied the metabolism of GDP-mannose by various mammalian tissues and demonstrated the formation of a mannolipid and a mannoprotein. The mannolipid had some properties consistent with its being of the prenol phosphate mannose type, and Caccam *et al.* (1969) suggested that this was possibly a lipid intermediate in the formation of secreted glycoproteins but not in the synthesis of membrane glycoproteins. A similar mannolipid was detected in mouse brain microsomal fractions (Zatz & Barondes, 1969). Tetas *et al.* (1970) and Ikehara *et al.* (1971) reported the formation of galacto-, manno- and hexosaminolipids by rabbit liver microsomal preparations and suggested that they might be lipid intermediates in glycoprotein biosynthesis. Rat liver mitochondrial preparations have also been reported to incorporate mannose and glucose into endogenous lipid acceptors (Bosmann & Case, 1969), but whether any of these glycolipids are intermediates in mitochondrial glycoprotein synthesis (Bosmann & Martin, 1969) is not known.

In these reports on mammalian systems the nature of the glycolipid was not established and it was Behrens & Leloir (1970) who published the first significant pointers to the fact that mammalian microsomal preparations could form dolichol phosphate sugar derivatives, although even in this report the characterization of the lipid was not complete. These authors phosphorylated dolichol chemically

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and showed that pig liver contains a lipid that is chromatographically identical with this. When added to rat liver microsomal fractions both stimulated the transfer of glucose from UDP-glucose into a glucolipid. This glucolipid had properties consistent with its being dolichol phosphate glucose. Other work (Behrens *et al.*, 1971*a*) indicated that this rat liver preparation could also form a closely related mannolipid and an *N*-acetylglucosaminolipid, but no transfer of these sugar residues from the lipids to protein was observed. There was evidence that the glucolipid was able to pass on its glucose residues to a glucoprotein, but this suggestion has since been modified. It is suggested now (Behrens *et al.*, 1971*b*) that the final acceptor is not glycoprotein but an oligosaccharide linked to lipid that is bound tightly to protein. The lipid portion had some properties consistent with its being a dolichol pyrophosphate. There is no evidence for a possible function for this glycolipid.

The present paper is concerned with the transfer of mannose from GDP-mannose to a lipid by a preparation of endoplasmic reticulum of pig liver. In particular, the nature of the mannolipid formed and the properties of the transferase activity and of other relevant enzyme activities of the preparation have been studied. Some of the results have appeared in preliminary publications (Alam *et al.*, 1970; Richards *et al.*, 1971, 1972).

Materials and Methods

Pig liver microsomal preparation

Pig liver was obtained from the Liverpool Municipal Abattoir, Stanley, Liverpool, as soon after slaughter as possible, usually within 1 h. The microsomal preparation was isolated from the liver by essentially the same methods as that used by Caccam *et al.* (1969) for rabbit liver except that the fraction was not separated into smooth and rough endoplasmic reticulum components. The microsomal pellet resulting from the final centrifugation step of this method was suspended in tris–HCl (0.05M, pH7.1) containing sucrose (0.25M) to a final volume 0.35 times the weight of liver used. The preparation usually contained approx. 100mg of protein/ml when assayed by the biuret technique with serum albumin as standard (Gornall *et al.*, 1949). The preparation was divided into small volumes and stored at −20°C until required. Each sample was thawed only once, immediately before use.

Incubation conditions and recovery of lipid, protein and water-soluble constituents

Incubations were performed in 15ml centrifuge tubes, at 37°C, with shaking, in the medium described in Table 3. When phosphorylated polyprenols were added to incubation mixtures they were added as a complex with Mn^{2+} and EDTA in a similar manner to that with Mg^{2+} and EDTA described by Behrens & Leloir (1970). EDTA (0.025M, 5μl) was mixed with $MnCl_2$ (0.1M, 2μl) in a 15ml centrifuge tube and to this was added polyprenol phosphate dissolved in chloroform–methanol (2:1, v/v; 10μl). The solvents were evaporated under vacuum and then the other incubation constituents were added. In these experiments control incubation mixtures were prepared in exactly the same way but with the omission of the polyprenol phosphates.

When lipid was to be recovered the incubations were terminated by the addition of water (1ml) and butan-1-ol (1ml), followed by thorough mixing with a Vortex mixer; separation into an upper layer of butanol, a lower aqueous layer and a layer of protein at the interface was then aided by low-speed centrifugation (2000*g* for 10min). The upper layer was removed with a Pasteur pipette and the lower layer and interface were extracted twice in a similar manner but by using chloroform–methanol (2:1, v/v; 2ml each time) and removing the lower layer of chloroform. The chloroform and butanol extracts were combined, transferred to stoppered tubes and diluted to approx. 10ml by addition of chloroform–methanol (2:1, v/v). This solution was then washed twice by shaking vigorously with water (approx. 5ml) and by centrifugation as described above. The washed lipid extracts were evaporated to dryness under N_2 and stored at −20°C. The lipid was more stable if stored as a solution in chloroform–methanol (2:1, v/v; 1ml) than if stored dry. Samples of the lipid extract were usually assayed for radioactivity by liquid-scintillation counting as described below and a portion was checked by t.l.c. in system A or system B or both. This invariably confirmed the absence of contaminating water-soluble compounds such as GDP-[^{14}C]-mannose, [^{14}C]mannose or [^{14}C]mannose 1-phosphate.

Protein was recovered by adding an equal volume of trichloroacetic acid (10%, w/v) to the aqueous layer and interface, remaining after extraction of the lipid, and mixing thoroughly. The protein precipitate was recovered by centrifugation and was resuspended in trichloroacetic acid (5%, w/v) and recovered again by centrifugation. This washing was repeated twice with water. The protein was then dried thoroughly with a Genevac freeze-drier [General Engineering Co. (Radcliffe) Ltd., Manchester, U.K.]. The vibration of the freeze-drier ensured that the dried protein was in the form of very fine particles. After weighing, the protein could be suspended in a toluene solution of scintillator and assayed for radioactivity directly with very little loss in efficiency due to self-absorption. In experiments where it was particularly important to ensure that the protein was free of lipid, the precipitate

was also washed several times with chloroform–methanol (2:1, v/v).

In those experiments in which it was desired to examine the water-soluble components of the incubation mixture the incubation was terminated by the addition of water (1 ml) and immediately heating for 90s in a bath of boiling water. After centrifugation the water layer was removed. The sediment was then resuspended in water (1 ml), mixed thoroughly, centrifuged and the water layer removed again. The sediment was again resuspended in water (1 ml) and the lipid and protein were recovered from the suspension as described above. The combined water layers were extracted with butan-1-ol (1 ml) and the extract was added to the lipid extract. The remaining water layer was freeze-dried before being assayed for radioactivity and being examined chromatographically.

In experiments designed to follow changes with time, one large incubation mixture (10 × quantities in Table 3) was usually set up and samples (0.2 ml) were removed at timed intervals and treated in the same way as for single incubations.

Isolation of total lipid from pig liver and its fractionation

The method used was essentially the same as that used by Behrens & Leloir (1970), except that after treatment of the chloroform–methanol extract with dilute alkali the solution was neutralized with acetic acid (0.1 M) and the acid-hydrolysis step was omitted. This avoided decomposition of any polyprenol phosphate sugars present in the extract.

Lipid was chromatographed on columns of silicic acid (100 mesh; A.R. Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.) in a manner similar to that described by Scher *et al.* (1968) (see Barr & Hemming, 1972). The fraction eluted by chloroform–methanol (1:1, v/v) was then chromatographed on DEAE-cellulose acetate by the method of Lahav *et al.* (1969) (see Barr & Hemming, 1972). The DEAE-cellulose (Whatman DE 52 from W. and R. Balston Ltd., Maidstone, Kent, U.K.) was acetylated as described by Rouser *et al.* (1963).

Synthesis of polyprenol phosphates

Pig liver dolichols (Burgos *et al.*, 1963; Dunphy *et al.*, 1967; Feeney & Hemming, 1967), ficaprenols (Stone *et al.*, 1967) and betulaprenols (Wellburn & Hemming, 1966) were isolated by the methods described in the original papers. Solanesol (Erickson *et al.*, 1959) was a gift from Hoffmann-La Roche, Basle, Switzerland. Farnesol was purchased from Koch–Light Laboratories (Colnbrook, Bucks., U.K.) and cetyl alcohol was from BDH Chemicals Ltd. (Poole, Dorset, U.K.). These alcohols were phosphorylated chemically and the product was purified essentially as described by Barr & Hemming (1972). The presence of a small amount of [^{32}P]phosphate in the phosphorylating reagent, of known specific radioactivity, enabled the quantity of prenol phosphates formed to be assayed accurately. The [^{32}P]phosphate had decayed by the time the prenol phosphates were added to incubations. In t.l.c. system B R_F values ranged from 0.5 for cetyl phosphate to approx. 0.7 for dolichol phosphate. In t.l.c. system A all of the phosphates remained close to the origin. Mild treatment with dilute acid (see below) failed to change the chromatographic mobility of the dolichol phosphate, confirming that it was in fact the monophosphate and not the pyrophosphate derivative.

General methods

T.l.c. systems. The absorbent used for systems A and B was Kieselgel G (E. Merck A.G., Darmstadt, Germany) and for systems C, D and E was cellulose (Whatman CC41; W. and R. Balston Ltd.). Solvents systems used were: system A, chloroform–methanol–water (65:25:4, by vol.); system B, di-isobutyl ketone–acetic acid–water (20:15:2, by vol.); system C, ethyl acetate–butan-1-ol–water–acetic acid (6:8:8:5, by vol.); system D, ethyl acetate–pyridine–water (10:4:1, by vol.); system E, ammonium acetate (1 M)–ethanol (1:1, v/v).

In preparative t.l.c. lipids were eluted with chloroform–methanol–water (6:3:1, by vol.).

Distribution of radioactivity on t.l.c. plates. This was usually followed by using a Panax RTLS-1A t.l.c. scanner or a Nuclear-Chicago Actigraph III radioscanner. The pattern of distribution was usually checked by radioautography as described by Barr & Hemming (1972). Accurate assay of radioactivity in relevant areas of chromatograms was made by transferring the adsorbent plus radioactive compound to a scintillation-counting vial. Lipid materials were then assayed in the toluene-based scintillation fluid and water-soluble materials in the dioxan solution of scintillators (see below). Non-radioactive areas of the chromatograms were usually added to other vials containing known quantities of radioactivity to check quenching. Only rarely was it necessary to correct for this effect.

Mild alkali treatment of lipids. This method was based on that of Dawson (1967) as used by Lahav *et al.* (1969). To the lipid dissolved in chloroform–methanol (1:4, v/v; 10 vol.) was added NaOH (1 M; 1 vol.). The mixture was incubated at 37°C for 10 min and then neutralized with ethyl formate (1 M; 1 vol.; Dawson, 1967). Chloroform–methanol–butanol–water (9:1:5:10, by vol.; 50 vol.) was added and after vigorous mixing the separation into layers was aided by low-speed centrifugation. The lower layer was

retained and washed with water–methanol (2:1, v/v; 10vol.) before evaporation to dryness.

Mild acid treatment of lipids. This method was similar to that used by Lahav *et al.* (1969). The radioactive lipid (usually 10–50% of the lipid extracted from a standard incubation) was mixed with methanol–water (1:1, v/v; 1ml) containing HCl (0.01M) and heated at 100°C for 10min. The mixture was then neutralized by adding NaOH (0.01M, in methanol; 1ml). To this was added water (2ml) and chloroform (2ml) and, after thorough mixing, the mixture was separated into two layers by low-speed centrifugation. The chloroform layer was evaporated to dryness and a portion was assayed for radioactivity by liquid-scintillation counting. The water layer was freeze-dried in a Genevac freeze-drier and a sample was assayed for radioactivity in the dioxan solution of scintillators (see below).

Radioassays. Radioactivity was measured by using a Beckman LS-100 or LS-200 liquid-scintillation counter. Samples were usually assayed by using a mixture of 2,5-diphenyloxazole (50mg) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (3mg) dissolved in toluene (10ml). Water-soluble samples were counted for radioactivity in vials containing 2,5-diphenyloxazole (50mg), naphthalene (1g), water (1ml) and dioxan (10ml). All measurements were checked and, if necessary, corrected for quenching. In experiments in which ^3H and ^{14}C were present in the same sample great care was taken to ensure the accuracy of the measurement of spill-over of ^{14}C into the ^3H channel and vice versa.

Other chemicals and solvents

Unlabelled nucleoside derivatives, bacitracin and chloramphenicol were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Vancomycin was obtained from Eli Lilly and Co. Ltd. (Basingstoke, Hants., U.K.).

GDP-[U-^{14}C]mannose was supplied in water–ethanol (49:1, v/v) from The Radiochemical Centre (Amersham, Bucks., U.K.) and was stored at −20°C. [^{32}P]Orthophosphate in sterilized hydrochloric acid at pH2–3 and [U-^{14}C]GDP were obtained from the same source. 3*RS*-[5-$^3H_2$]Mevalonic acid (1mCi/0.71mg) was purchased as its dibenzoylethylenediamine salt dissolved in methanol from New England Nuclear Corp. (Boston, Mass., U.S.A.). Acetone was dried over Hi-drite (Hi-drite Ltd., London, U.K.); acetonitrile was dried over anhydrous $CaCl_2$ and pyridine was dried over solid KOH. All of these solvents, and also methanol, ethanol, butan-1-ol, chloroform and trichloroacetonitrile, were distilled before use.

All other solvents and chemicals were used without purification.

Results and Discussion

(*a*) *Formation and properties of the mannolipid*

In initial experiments incubations (see Table 3 for details) were for 1h. Usually the incorporation of ^{14}C into lipid was between 10 and 40% of the GDP-[^{14}C]mannose presented to the preparation and that into protein was between 1 and 3%. The incorporation into lipid for any one microsomal preparation was normally consistent (±5% of that incorporated), but varied markedly from one preparation to another. The incorporation into protein was more variable (±20%). The incorporation into lipid and protein was time-dependent (see section *j*). The GDP-mannose–lipid mannosyltransferase activity of the preparation remained stable for several months at −20°C. The GDP-mannose–protein mannosyltransferase activity decreased slowly during this storage (see Table 3 and the Materials and Methods section for details).

When chromatographed on a column of silicic acid (10g) the ^{14}C-labelled lipid was not eluted by chloroform (100ml) or acetone (100ml) but was eluted completely by chloroform–methanol (1:1, v/v; 100ml). It could also be eluted from a column of DEAE-cellulose acetate by 7.5mM-ammonium acetate but not by chloroform–methanol (2:1, v/v) or methanol. In both these respects the lipid resembles undecaprenol phosphate mannose and differs, for example, from dimannosyl diglyceride, which is eluted from columns of silicic acid by acetone and which, since it is not acidic, would not be expected to be retained by DEAE-cellulose acetate (Lahav *et al.*, 1969; Lennarz & Talamo, 1966; Scher *et al.*, 1968).

The ^{14}C-labelled lipid ran as a single radioactive compound in t.l.c. systems A and B, in which it usually had R_F values close to 0.31 and 0.41 respectively.

Glycolipids of the prenol phosphate sugar type are stable to mild alkali treatment but are extremely labile to mild acid treatment (Higashi *et al.*, 1967; Scher *et al.*, 1968). On the other hand glycolipids such as dimannosyl diglycerides are labile to alkali and require vigorous acid treatment before they are hydrolysed (Lennarz & Talamo, 1966). Hydrolysis of both types of glycolipid renders the [^{14}C]mannose present water-soluble. The major part (80–90%) of the ^{14}C of the [^{14}C]mannolipid could be recovered as lipid-soluble material after treatment with mild alkali (see the Materials and Methods section). When treated mildly with dilute acid (see the Materials and Methods section) 70% of the ^{14}C was recovered as water-soluble material, and most of this had chromatographic properties identical with those of mannose in systems C and D. Development of the latter system three times resulted in the ^{14}C and mannose marker (both R_F 0.33) being clearly distinguishable from glucose (R_F 0.25), galactose (R_F 0.22) and fucose (R_F 0.54) markers. A small amount of radioactive

material was observed with the same R_F value (0.71) as α-methyl D-mannoside.

These results indicate that the [^{14}C]mannolipid is acidic. It contains [^{14}C]mannose bound to a lipid moiety by a very acid-labile link. The molecule is stable to dilute alkali. These properties strongly suggest a molecule containing mannose linked through a phosphate to a lipid moiety, possibly a polyisoprenoid alcohol.

Since on no occasion was sufficient mannolipid obtained (even after accumulation of the lipid products of several incubations) to produce a stain on a chromatogram with any of the standard sugar, prenol or phosphate stain reagents, it was concluded that the isolation and purification of sufficient lipid for direct physicochemical characterization would be extremely difficult. The extraction of large quantities of liver also failed to provide sufficient purified material for this purpose. Because of this, less direct methods were used for identification of the lipid.

(b) Effect of adding exogenous prenol phosphates to incubations

If phosphorylated polyprenols are added to bacterial preparations they are utilized in the formation of polyprenol phosphate sugars as intermediates in mannan biosynthesis (Lahav *et al.*, 1969) and in peptidoglycan biosynthesis (Higashi *et al.*, 1970). Addition of dolichol monophosphate to incubations of rat liver preparations has been shown to increase the yield of glycolipids, presumably dolichol phosphate sugars (Behrens & Leloir, 1970; Behrens *et al.*, 1971*a*).

The main prenols of pig liver constitute a family of dolichols, of which dolichol-19 is the major component (see Table 1). A sample of each prenol listed in Table 1 and of cetyl alcohol was phosphorylated (see the Materials and Methods section). The fact that the chromatographic mobility of the preparation of dolichol phosphate was not altered by treatment with mild acid indicated that it was the monophosphate derivative.

Evidence that the stereochemistry of each prenol (Table 1) is decided when it is synthesized and that no isomerization occurs afterwards has been summarized (Hemming, 1970). In Table 1 the *trans*-isoprene residues of the *cis/trans* polyprenols have been placed at the ω-end of the chain of isoprene residues. Evidence for this has been summarized by Richards & Hemming (1972).

The effect of adding equimolar quantities of these prenol phosphates to standard incubations is shown in Table 2. Each of the long-chain prenol phosphates stimulated the incorporation of mannose into lipid, both dolichol phosphate and betulaprenol phosphate causing a fourfold increase, solanesol phosphate just over a threefold increase and ficaprenol phosphate just over a twofold increase. Neither farnesol phosphate nor cetyl phosphate caused any stimulation, the former in fact consistently causing a 30% inhibition. In view of evidence discussed in later sections it seems likely that these polyprenol phosphates were acting as acceptors of [^{14}C]mannose and that the transferase concerned has a specificity for long-chain prenol phosphates, but that this is not absolute to precise chain length, stereochemistry or degree of saturation. However, it is significant that the phosphate of the dolichol complex (the natural polyprenol complex of pig liver) and of the betulaprenol complex caused the greatest stimulation. It is relevant that the polyisoprenoid chains of betulaprenols-6 to -9 are identical with the first six to nine isoprene residues from the ω-end of the dolichols (Table 1) and also probably of bacterial undecaprenol. It may be that this stereochemistry is important for the proper functioning of the polyprenol phosphates in several sugar transferase reactions.

Occasionally the control value for incorporation of [^{14}C]mannose into mannolipid reached 35000–45000d.p.m. (30–40% of substrate added). In these cases the addition of dolichol phosphate again caused a further increase, often as high as 30000–40000d.p.m., to give a total incorporation of 60–70% of the substrate added. The variation in control values is probably due to differences in the amount of endogenous dolichol phosphate present in different microsomal preparations. Other workers have reported much lower incorporations of [^{14}C]mannose into microsomal preparations of rat liver and correspondingly greater percentage increases on adding dolichol phosphate (Behrens *et al.*, 1971*a*). This situation is consistent with rat liver containing a smaller concentration of dolichol phosphate than does pig liver.

Table 1. *Structures and sources of the prenols tested, as their phosphates, for stimulation of the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to lipid and protein*

See the Materials and Methods section for methods of isolation and phosphorylation. Abbreviations: ω, ω-isoprene residue; T, *trans*-isoprene residue; C, *cis*-isoprene residue; S, saturated isoprene residue.

Betulaprenol, $n = 1 \rightarrow 4$ (silver birch wood)	ω–T–T–C–[C]$_n$–C–OH
Dolichol, $n = 12 \rightarrow 16$ (mammalian)	ω–T–T–C–[C]$_n$–S–OH
Ficaprenol, $n = 3 \rightarrow 5$ (plant leaves)	ω–T–T–T–[C]$_n$–C–OH
Solanesol (plant leaves)	ω–T–T–T–T–[T]$_4$–T–OH
Farnesol	ω–T–T–T–OH

In this respect it should be noted that the unsaponifiable lipid of rat liver yields less dolichol than does that of pig liver (Hemming, 1970), although it may be that this is due to differences in the activity of phosphatase rather than simply a difference in concentration of substrate for the phosphatase. The presence of a polyprenol phosphate phosphatase in liver has been reported by Kurokawa *et al.* (1971).

Differences in the nature of polyprenol phosphate added to the incubations made negligible differences to the chromatographic properties of the [^{14}C]mannolipid formed when checked in t.l.c. systems A and B.

Exogenous dolichol phosphate caused a two- to three-fold increase in the incorporation of [^{14}C]mannose into protein (Table 2), although this effect varied markedly from preparation to preparation. The stimulation caused by betulaprenol phosphate and solanesol phosphate was 30% of that caused by dolichol phosphate, whereas ficaprenol phosphate, farnesol phosphate and cetyl phosphate caused little or no increase. Although generalizations made from these results can only be tentative it appears that the transferase using protein as an acceptor is much more specific to dolichol phosphate in its response to exogenous prenol phosphates than is the transferase using prenol phosphate as an acceptor.

The quantity of prenol phosphates (40nmol, corresponding to 179μM) always added to incubations in these experiments was the smallest amount that would give maximal increase of the incorporation into lipid. At higher concentrations the response remained more or less constant. Subsequent experiments indicated that the endoplasmic-reticulum preparation was rich in phosphatase activity and that much of the exogenous prenol phosphate was being decomposed before it could be effective in the transferase system. At 179μM the concentration of prenol phosphate was presumably sufficient to saturate the phosphatase. In the presence of F^- (80mM) the concentration of dolichol phosphate required to produce the same stimulation of incorporation of mannose into lipid and protein could be decreased at least tenfold. Incorporation of [^{14}C]mannose into lipid and protein in the absence of exogenous prenol phosphate was not affected by F^- in the medium up to a concentration of 100mM. This suggests that any endogenous prenol phosphate was much less susceptible to decomposition than exogenous prenol phosphate, possibly because the former was imbedded in the endoplasmic reticulum.

(*c*) *Transfer of* [^{14}*C*]*mannose from* [^{14}*C*]*mannolipid to protein*

[^{14}C]Mannolipid was recovered from several standard incubations to which no exogenous prenol phosphate had been added. This was combined and partially purified by treatment with dilute alkali. Chromatography of the lipid on DEAE-cellulose acetate was then followed by preparative t.l.c. in system B. A portion of this purified mannolipid (87000d.p.m.) was added to an incubation mixture exactly as described for the addition of prenol phosphates. GDP-mannose was omitted from the medium and the incubation lasted for 15min. The lipid, aqueous layer and protein were recovered in the usual way and the last two fractions were thoroughly washed with chloroform–methanol (2:1, v/v). In addition the protein was thoroughly washed with trichloroacetic acid (5%, w/v) and water. Of the ^{14}C added as mannolipid 72% was recovered unchanged and 3.7% (3600d.p.m.) was recovered as ^{14}C-labelled protein. That remaining was recovered from the aqueous layer. Although the percentage

Table 2. *Effect of adding exogenous prenol phosphates to incubation mixtures of GDP-*[^{14}*C*]*mannose with the pig liver microsomal preparation*

Expts. A and B were with two different samples of pig liver preparation. The incubation medium was as described in Table 3, minus F^-; the incubation time was 1h; concentration of prenol phosphate was 179μM, and that of GDP-[^{14}C]mannose was 1.56μM (0.05μCi).

	Recovery of ^{14}C (d.p.m.)			
	Expt. A		Expt. B	
Addition	Lipid	Protein	Lipid	Protein
None	12000	1670	10200	1680
Dolichol phosphate	48400	4600	43400	4200
Betulaprenol phosphate	47600	2390	42000	2495
Solanesol phosphate	35800	2380	34800	2340
Ficaprenol phosphate	22950	1780	27200	1715
Farnesol phosphate	8550	2320	6790	1700
Cetyl phosphate	12960	1720	11680	1795

transfer of [^{14}C]mannose to protein was low it does represent genuine formation of mannoprotein. It is likely that the low extent of transfer was due in part to a shortage of endogenous protein and also to the difficulty of ensuring that all of the mannolipid reached the membrane-bound enzyme.

Approximately one-fifth of the ^{14}C added as mannolipid was recovered as water-soluble materials, including GDP-mannose, mannose 1-phosphate and mannose. This was probably a consequence of the ready reversibility of the reaction forming mannolipid from GDP-mannose (see sections *g–j*).

(*d*) *Constitution of the incubation medium*

The effect of omitting various components of the medium was investigated (Table 3). Mannolipid synthesis in the absence of exogenous dolichol phosphate increased slightly when EDTA was omitted and decreased in the absence of Triton X-100. However, in the presence of exogenous dolichol phosphate the lowering of the concentration of either of these two components caused a significant decrease in incorporation into lipid. Probably both components increase the solubility, and availability to the enzyme, of the dolichol phosphate. Scher & Lennarz (1969) noted that, in *Micrococcus lysodeikticus*, EDTA selectively inhibited the transfer of mannose from prenol phosphate mannose to mannan. There is little evidence from Table 3 for extending these conclusions to the pig liver system.

The results from Expt. A (Table 3) show that the system is dependent upon Mn^{2+} for full activity and that Mg^{2+} substitutes only poorly. Expt. B presents a complication in this respect because the dolichol phosphate was accompanied by Mn^{2+}. It was observed in a separate experiment that the addition of Mg^{2+} (8.2mM) to a standard incubation mixture containing Mn^{2+} (8.2mM) had no effect on the transfer of mannose.

This requirement for Mn^{2+} by mammalian liver sugar transferases agrees with the findings of Caccam *et al.* (1969) for a rabbit liver system but is at variance with the requirement for Mg^{2+} by the rat liver system used by Behrens & Leloir (1970).

The prenol phosphates were added to incubation mixtures as complexes with Mn^{2+} and with EDTA. When dolichol phosphate alone was added to incubation mixtures the stimulation of transfer of mannose to lipid was only 50% of that when the complex with Mn^{2+}–EDTA was used. This suggests that complex-formation possibly aided solubility and chelation with the enzyme. In controls in which only $MnCl_2$ (0.90mM) and EDTA (0.56mM) were added to the standard medium no change in the incorporation of ^{14}C into lipid or protein was observed.

(*e*) *Incorporation of* [^{3}*H*]*dolichol phosphate into the mannolipid*

Phytophthora cactorum contains small concentrations of a dolichol complex of which the major components are dolichols-14, -15 and -16 (Richards & Hemming, 1972), whereas the major components of

Table 3. *Effect of changing the concentration of or omitting various components of the incubation medium on the transfer of* [^{14}*C*]*mannose from GDP-*[^{14}*C*]*mannose to lipid and protein by the pig liver preparation of endoplasmic reticulum*

The standard incubation mixture contained $MnCl_2$ (8.2mM), EDTA (2.0mM), tris–HCl, pH7.1 (25mM), NaF (80mM), Triton X-100 (0.08%), suspension of endoplasmic reticulum (150μl), GDP-[^{14}C]mannose (1.43μM, 0.05μCi) and water to 225μl. The incubation time was 15min. Expt. A, no exogenous dolichol phosphate added; Expt. B, plus exogenous dolichol phosphate (179μM) and additional EDTA (0.56mM) and $MnCl_2$ (0.90mM).

Component varied	Concn. (mM)	Recovery of ^{14}C (d.p.m.) Expt. A Lipid	Expt. A Protein	Expt. B Lipid	Expt. B Protein
None		13000	3850	30400	3880
EDTA	0.00	15800	3560		
	0.56			20800	3340
Triton X-100	0.00	9200	3340	10200	3240
Mn^{2+}	0.00	3420	1930		
	0.90			15500	2800
Mn^{2+} replaced by Mg^{2+}	8.20	5400	2190		
Mn^{2+} plus Mg^{2+}	0.90 + 8.20			18100	2800

the pig liver preparation are dolichols -18, -19 and -20 (Feeney & Hemming, 1967). Each complex contains very small quantities of the major components of the other. In view of the relatively low specificity to prenol chain-length shown by the transfer of mannose to lipid when stimulated by polyprenol phosphates (section *b*), it seemed reasonable to assume that the dolichol complex of *P. cactorum* should substitute well for the dolichol complex.

P. cactorum was grown for 25 days in the presence of [5-$^3H_2$]mevalonate (200μCi, 0.53μmol) and the [^{3}H]dolichol was isolated (Richards & Hemming, 1972). Chemical phosphorylation (see the Materials and Methods section) of the [^{3}H]dolichol (4μCi) with a large excess of di(triethylammonium) monohydrogen [^{32}P]phosphate (43μCi/μmol) yielded [^{3}H]dolichol [^{32}P]phosphate, which, after chromatographic purification, contained 0.12μCi of ^{3}H and 4.3nCi of ^{32}P, corresponding to 0.1nmol of phosphate. When required for the incorporation experiment the ^{32}P in the material had decayed to an almost negligible quantity.

A sample of this [^{3}H]dolichol phosphate (210000d.p.m., 82pmol) was incubated with the microsomal preparation and GDP-[^{14}C]mannose in the normal way for 45min. The [^{14}C,^{3}H]mannolipid formed was treated with dilute alkali and separated (R_F 0.41) from unchanged [^{3}H]dolichol phosphate (R_F 0.67), [^{3}H]dolichol (liberated by hydrolysis of the phosphate and running close to the solvent front) and from other impurities by preparative t.l.c. in system B. At this stage the mannolipid contained 6900d.p.m. of ^{14}C and 7800d.p.m. of ^{3}H. A portion of the mannolipid was then chromatographed again in system B. An equal portion was treated with dilute acid and the lipid-soluble material recovered was also chromatographed in system B. The acid treatment resulted in the loss of 98% of the ^{14}C formerly present in the mannolipid region of the chromatogram, owing to its release as [^{14}C]mannose. Of the ^{3}H that disappeared from the same region 90% reappeared in the dolichol phosphate region. In a repeat experiment the corresponding value was 50%. The experiment was complicated by the presence of a tritiated impurity in the [^{3}H]dolichol phosphate. Nevertheless these results confirm that the dolichol phosphate from *P. cactorum* was acting as an acceptor of mannose and that acid treatment of the mannolipid so formed released mannose and dolichol phosphate.

Table 4. *Effect of adding chromatographic fractions of pig liver lipid to incubations of GDP-*[^{14}C]*mannose with the pig liver microsomal preparation*

In Expt. A the fractions added were portions of those eluted from a column of DEAE-cellulose acetate (see the text). In Expt. B the fractions added were portions of the lipids from bands obtained by preparative t.l.c. (see the text). These were added to the medium as described for dolichol phosphate (see the Materials and Methods section). The incubation medium was as described in Table 3. In Expt. A incubation was for 30min and in Expt. B it was for 40min. The liver preparation used for Expt. A was different from that used for Expt. B.

	Addition			
	Portion added to incubation		Incorporation of ^{14}C (d.p.m.)	
Chromatographic fraction	% of fraction isolated	Wt. (μg)	Lipid	Protein
Expt. A				
Methanol	2	400	30800	2280
7.5mM-Ammonium acetate	0.4	400	79400	4600
0.1M-Ammonium acetate	2	1200	64300	3900
None	—	—	31800	2750
Expt. B				
Band 1	2.5	25	14800	3300
Band 2	2.5	38	10300	960
Band 3	2.5	80	11800	1120
Band 4	2.5	75	21400	1110
Band 5	2.5	118	10400	1060
None	—		10300	1040

(f) Endogenous dolichol phosphate and dolichol phosphate mannose in pig liver

A compound chromatographically indistinguishable from dolichol phosphate can be isolated from pig liver and this compound will stimulate the incorporation of glucose from UDP-glucose into glucolipid (Behrens & Leloir, 1970). It has also been reported that chicken liver contains a chromatographically identical compound that incorporates ^3H from [5-$^3H_2$]mevalonate and which gives rise to a [^{14}C,^3H]mannolipid identical chromatographically with dolichol phosphate mannose when a microsomal preparation of the same chicken liver is incubated with GDP-[^{14}C]mannose (Richards *et al.*, 1971). It was decided to investigate the lipids of pig liver by checking their activity in the pig liver mannose transferase system.

Pig liver lipid was extracted with chloroform–methanol (2:1, v/v) and treated with dilute alkali. The resulting lipid was chromatographed on silicic acid and DEAE-cellulose acetate (see the Materials and Methods section). The results of adding fractions eluted from the latter column to standard incubations are shown in Table 4. The fractions eluted by 7.5mM- and 0.1M-ammonium acetate both contained material capable of stimulating incorporation of mannose into lipid and protein. The mannolipid formed was chromatographically identical with dolichol phosphate mannose.

The 7.5mM-ammonium acetate fraction contained most of the stimulatory activity and this was fractionated further by preparative t.l.c. in system A. Lipid from five successive bands of the chromatogram was recovered. Band 1 (the slowest) corresponded in R_F value to dolichol phosphate and band 4 to dolichol phosphate mannose. Considerable loss of material occurred during this preparative chromatography and some of the materials present decomposed. Difficulties in preparative t.l.c. of similar lipids have also been reported by Behrens & Leloir (1970). Table 4 shows that the lipid of band 1 caused an increase in the transfer of [^{14}C]mannose into both lipid and protein. Lipid from band 4 caused an increased transfer into lipid only. The other three bands yielded lipid which had no stimulatory effect on the transfer to either lipid or protein.

The results are consistent with band 1 containing dolichol phosphate, although the stimulation of the incorporation into lipid was smaller and into protein was larger than is usually obtained with synthetic dolichol phosphate. The result with the lipid of band 4 was particularly interesting. It suggests that the transfer of mannose from GDP-mannose to dolichol phosphate mannose is readily reversible, so that [^{14}C]mannose rapidly exchanges between GDP-mannose and dolichol phosphate mannose (see also sections *g–j*). The addition of dolichol phosphate mannose, present in band 4 lipid, would increase the pool of dolichol phosphate mannose, which, on equilibration, would lead to the presence of a larger quantity of ^{14}C in the lipid. It appears that in this experiment the effect of increasing the size of the [^{14}C]mannolipid pool on the transfer of mannose to protein was balanced by the effect of lowering the specific radioactivity, so that the transfer of ^{14}C to protein was not changed. Variations observed in the transfer of mannose to protein in different microsomal preparations and in their response to exogenous dolichol phosphate may be due in part to differences in concentrations of endogenous dolichol phosphate mannose as well as to differences in concentrations of acceptor protein (see section *c*).

(g) Reversibility of the formation of mannolipid

The reaction of dolichol phosphate with GDP-mannose is likely to liberate either GDP or GMP, depending on which of the two alternative reactions (1) and (2) is operative:

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

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

Both types of reaction are known to occur in bacterial systems.

Table 5 (Expt. A) shows that GDP, but not GMP, when present in excess at the beginning of a standard incubation inhibited the incorporation of mannose into both lipid and protein. GTP also caused a slight inhibition, presumably owing to partial hydrolysis to GDP. These incubations contained F^- at 80mM. In the absence of F^- all three guanosine phosphates had a negligible effect on the mannose transfer. It was assumed that F^- partially inhibited phosphatase activity. These results show that it is reaction (1) that is concerned and that the dolichol is linked to mannose through a monophosphate and not a diphosphate bridge. This last point was further supported by investigating the products of a short-term (1min) dilute acid hydrolysis of the [^{14}C]mannolipid. T.l.c. (system C) indicated that only [^{14}C]mannose (R_F 0.43) was formed, there being no radioactivity associated with marker mannose 1-phosphate (R_F 0.32).

In a second experiment [Expt. B (*i*), Table 5] the addition of GDP to the incubation mixture after 5min caused a reversal of the reaction and did not simply inhibit it. By 5min the incorporation of mannose into lipid was high and by 15min it had fallen to 60% of this value (see also section *j*). However, when GDP was added at 5min the recovery of ^{14}C in the lipid at 15min was down to 5% of the 5min value and only 8% of the control (15min) result. The effect of adding GDP at 5min was almost

Table 5. *Effects of adding excess of nucleotides to incubations of GDP-[^{14}C]mannose with the pig liver microsomal preparation*

The incubation medium was as described in Table 3. In Expts. B (*ii*) and C dolichol phosphate was added as described in the Materials and Methods section. Expts. A, B and C were with different liver preparations. In Expt. A 50nmol of nucleotide was added; in Expts. B and C 100nmol was used. n.d., Not determined.

Nucleotide added		Total incubation time (min)	Recovery of ^{14}C (d.p.m.)		
Compound	Time (min)		Lipid	Protein	Aqueous
Expt. A					
GMP	0	5	26800	1520	n.d.
GDP	0	5	9500	1140	n.d.
GTP	0	5	18900	1370	n.d.
None	—	5	25700	1790	n.d.
Expt. B (*i*)					
None	—	5	35800	2540	n.d.
None	—	15	22250	2930	n.d.
GDP	0	15	800	760	n.d.
GDP	5	15	1880	2680	n.d.
Expt. B (*ii*)					
None	—	5	49750	2600	n.d.
None	—	15	37100	3140	n.d.
GDP	0	15	1390	625	n.d.
GDP	5	15	3820	2480	n.d.
Expt. C					
None	—	2	38000	n.d.	54400
None	—	5	40000	n.d.	51500
GDP	2	5	11500	n.d.	83000

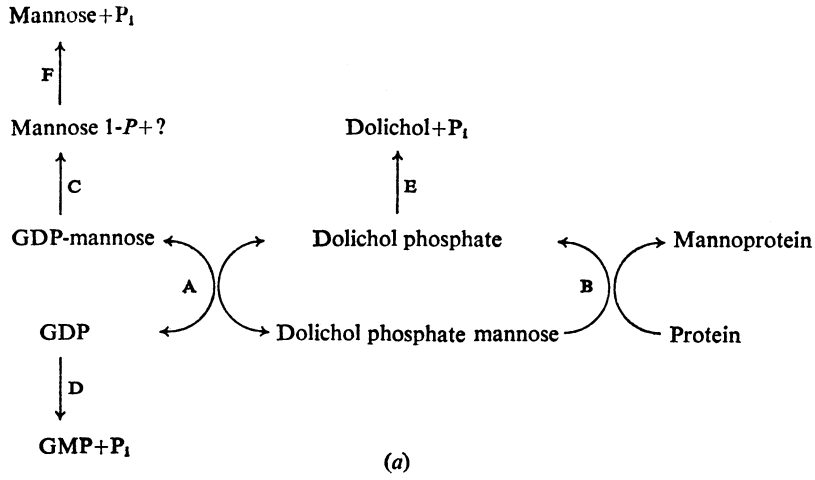
as great as having the GDP present from the beginning.

The mannosylation of protein continued over the 5–15min period. In contrast to the effect on lipid the addition of GDP at 5min did not cause a decrease in [^{14}C]mannose content of the protein, although it did inhibit the slight increase previously observed during this period. The presence of GDP from the start markedly inhibited the incorporation of mannose into protein. It appears that although GDP inhibits the transfer of mannose from GDP-mannose to protein it does not reverse the process. The results are consistent with the mannolipid being an intermediate in the transfer (see also section *j* and Scheme 1*a*).

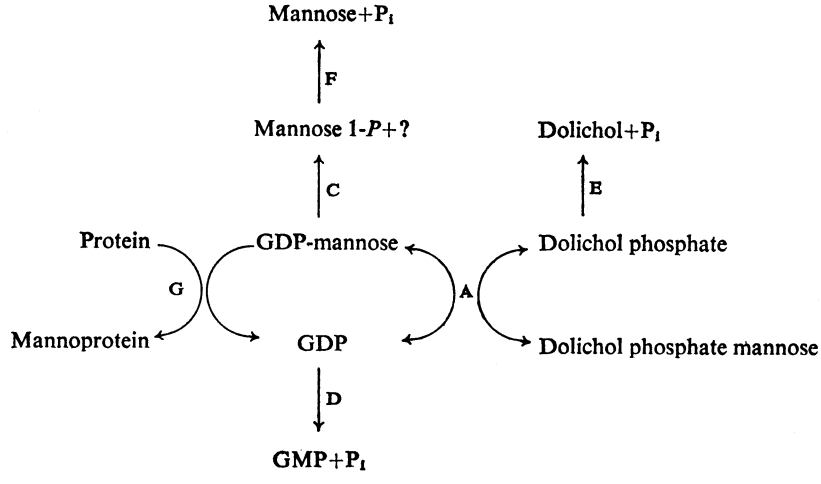
In the presence of dolichol phosphate [Expt. B (*ii*)] the picture was essentially the same, although in general the recoveries of ^{14}C, especially in the mannolipid, were higher. This experiment involved the use of a large excess of GDP. Two further experiments were performed which showed the rapid reversibility of reaction (1) without using high concentrations of GDP.

In the first of these experiments, a normal incubation mixture was set up in which unlabelled GDP-mannose (0.35nmol) was used instead of GDP-[^{14}C]mannose. After incubation for 3min [U-^{14}C]-GDP (0.406nmol, 445000d.p.m.) was added and the incubation continued for a further 3min before termination. The aqueous fraction was examined by t.l.c. in system E. Of the initial [^{14}C]GDP, 85–90% was usually recovered and of this 23% was chromatographically identical with GDP-mannose (R_F 0.37). Of the recovered ^{14}C 32% was in the form of unchanged GDP (R_F 0.15) and 45% was recovered as GMP (R_F 0.24), presumably owing to hydrolytic activity. T.l.c. in system C confirmed this pattern. In a control containing boiled enzyme the ^{14}C was recovered as unchanged [^{14}C]GDP. In view of the results in Table 5 it seems obvious that the ^{14}C recovered as GDP-mannose was due to the rapid reversal of reaction (1).

When exogenous dolichol phosphate (40nmol) was present in the incubation medium, the recovery of added [^{14}C]GDP was 27% as GDP, 58% as GMP and 15% as GDP-mannose. It is not clear how significant the differences in results in the absence and presence of exogenous dolichol phosphate are. However, it is relevant that the ratio GDP-mannose/GMP (1:2) becomes lower (1:4) when exogenous



(*a*)



(*b*)

Scheme 1. *Possible explanations of the relationship between GDP-mannose, dolichol phosphate mannose, water-soluble derivatives and mannoprotein in the pig liver microsomal preparation*

(*a*) The proposed scheme to explain the observations reported; see the Results and Discussion sections (*g*)–(*k*). (*b*) A theoretically possible alternative scheme not supported by the observations; see the Results and Discussion sections (*j*) and (*k*).

dolichol phosphate is present. Assuming that GDP was involved either in the reversal of reaction (1) or alternatively in hydrolysis to GMP (see also Scheme 1*a*) a change of ratio in this direction would be expected if the equilibrium of reaction (1) (reaction A in Scheme 1*a*) was pushed to the right (downwards in Scheme 1*a*) by an increase in the concentration of dolichol phosphate.

The third experiment of this section is described in Table 6. The addition of excess of unlabelled GDP-mannose at 2min caused a transfer of lipid-soluble radioactivity to water-soluble radioactivity as assayed 3min later. Radioscanning after t.l.c. of the water-soluble materials in system C showed that in each case virtually all of the ^{14}C was present as GDP-[^{14}C]mannose (R_F 0.12) with just a little (less than a

Table 6. *Effect of adding excess of unlabelled GDP-mannose to incubations of GDP-[^{14}C]mannose with the pig liver microsomal preparation*

The incubation medium was as described in Table 3. GDP-[^{14}C]mannose from start: 110000d.p.m.; 0.35nmol. GDP-[^{12}C]mannose added: 70nmol.

Incubation time (min)	Time of addition of GDP-[^{12}C]mannose (min)	Recovery of ^{14}C (d.p.m.)	
		Lipid	Aqueous
2	—	35000	51400
5	2	11100	77000
5	—	34000	52000

total of 2000d.p.m.) corresponding to mannose 1-phosphate (R_F 0.27). This was interpreted as good evidence of the rapidity and ready reversibility of reaction A (Scheme 1). By 2min the pool of mannolipid was more or less saturated and in equilibrium with the GDP-[^{14}C]mannose (see also section *j*). The sudden increase in pool size of GDP-mannose and consequent decrease in its specific radioactivity was followed by a rapid re-equilibration until the specific radioactivities of the two pools were equal. Assuming the final pool size of GDP-mannose to be greater than that of mannolipid, it follows that most of the ^{14}C should be recovered in the form of GDP-mannose.

Thus from the experiments described so far it appears that the endoplasmic reticulum catalyses the reactions summarized in Scheme 1(*a*). In this scheme reaction A appears to be rapid and readily reversible whereas reaction B is slow, possibly owing to a shortage of acceptor protein. Reactions D and E, especially in the absence of F^- (for exogenous but not endogenous substrate), may be quite rapid. Reactions C and F are hardly detectable in short incubations (see sections *h* and *j*).

(h) Effect of excess of GDP on the nature of the water-soluble ^{14}C-labelled compound formed from [^{14}C]mannolipid

In the experiments illustrating the reversibility of reaction A (Scheme 1) it was shown that the addition of excess of GDP caused a fall in the amount of ^{14}C associated with lipid (Table 5, Expts. A and B). An experiment was designed to check if this decrease in lipid-soluble ^{14}C was compensated by an increase in water-soluble ^{14}C. The results (Table 5, Expt. C) show that a compensatory increase does in fact occur. However, t.l.c. in system C revealed that most of the water-soluble radioactivity resulting from the presence of excess of GDP was in the form of mannose 1-phosphate. The increase in the ^{14}C associated with mannose 1-phosphate (approx. 50000d.p.m.) was much greater than the decrease in ^{14}C associated with lipid (approx. 28000d.p.m.), and clearly the mannose 1-phosphate had been formed at the expense of both GDP-mannose and mannolipid. The results suggest that mannose 1-phosphate was formed by reaction C (Scheme 1), which was in some way activated by the presence of excess of GDP. As the GDP-mannose was broken down it was replaced at the expense of dolichol phosphate mannose through reaction A. That the slow formation of mannose 1-phosphate in standard incubation mixtures was primarily from GDP-mannose was confirmed by time-studies in the presence and the absence of exogenous dolichol phosphate (see section *j*). Whether or not the production of mannose 1-phosphate was accompanied by the formation of GMP was not determined.

(i) Effect of other nucleotides and nucleoside diphosphate sugars on the formation of mannolipid

Since it was observed that GDP and (slightly) GTP inhibit the formation of [^{14}C]mannolipid from GDP-[^{14}C]mannose it was decided to investigate the effect of a range of nucleoside phosphate derivatives. Table 7 shows that ATP, ADP, UTP and GDP cause significant inhibition. In this experiment the effect of GDP was less than is usually experienced. The most marked inhibition was by ATP. This was not affected by the presence of either dolichol or dolichol phosphate (179μM).

Behrens & Leloir (1970) have reported that dolichol phosphate acts as an acceptor of glucose from UDP-glucose in rat liver. It was therefore relevant to check the effect of unlabelled UDP-glucose and various other nucleoside diphosphate sugars on the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to mannolipid. The results (Table 7) show that both ADP-glucose and UDP-glucose markedly inhibit the transfer to the acceptor. This situation can be explained on the basis of the transferases for these donors competing with GDP-mannose transferase for dolichol phosphate. Alternatively the donors, ADP-glucose and UDP-glucose (which are present

Table 7. *Effect of nucleoside phosphates on the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to lipid during incubations with the pig liver microsomal preparation*

The incubation medium was as described in Table 3. Amount of nucleotides added was 50nmol. Incubation time was 15min. Expts. A and B were with different liver preparations.

	Nucleotide added	Lipid recovered (d.p.m.)
Expt. A		
	—	25500
	GMP	23600
	GDP	13700
	GTP	21600
	UMP	26100
	UDP	26800
	UTP	13600
	AMP	26400
	ADP	15200
	ATP	9050
Expt. B		
	—	21400
	ADP-mannose	23400
	ADP-glucose	6500
	UDP-glucose	10400
	UDP-mannose	20300
	UDP-*N*-acetyl-glucosamine	19500

in large excess), may compete with GDP-mannose for GDP-mannose–lipid mannosyltransferase without necessarily acting as a substrate for the transferase. In view of the work with rat liver preparations, showing the transfer of glucose from UDP-glucose to dolichol phosphate (Behrens & Leloir, 1970), and the evidence in favour of transferases being specific to one donor (Bosmann *et al.*, 1968), the former explanation is favoured for UDP-glucose. Since no glucosyl transferase has yet been demonstrated in mammalian liver that utilizes ADP-glucose as donor the latter explanation is more likely for ADP-glucose.

T.l.c. (system D, developed three times) of the water-soluble fraction of the microsomal preparation before incubation showed the presence of small amounts of glucose. This was presumably formed by the hydrolysis of the sucrose present in the preparation (see the Materials and Methods section). It is possible that the inhibition by ATP and UTP (Table 7) was due to the formation of ADP-glucose and UDP-glucose. UDP-glucose pyrophosphorylase is known to be present in mammalian liver, but the presence of ADP-glucose pyrophosphorylase has not been described. The inhibition by high concentrations of ADP (Table 7) is not understood but could possibly be due to activation of reaction C (Scheme 1) in the same way as that proposed for high concentrations of GDP (section *h*).

(j) Variation in production of mannolipid, mannoprotein, mannose 1-phosphate and mannose from GDP-mannose with time

The results of time-studies on the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to lipid and protein by the pig liver microsomal preparation are shown in Fig. 1. The incorporation into lipid reached a peak at 2–3min and in the presence of exogenous dolichol phosphate this peak was enhanced and delayed slightly. Although the amount of ^{14}C associated with lipid then fell markedly in both cases to level off after 40min, the fall was less marked in the presence of exogenous dolichol phosphate. The transfer of mannose to protein was much slower than to lipid and, although the rate slowed down throughout the experiment, the incorporation was still increasing slightly after 1h. The presence of exogenous dolichol phosphate caused an increase in the rate of transfer resulting in approximately twice as much [^{14}C]mannose being associated with protein at 90min compared with that in the absence of exogenous dolichol phosphate. Fig. 1 shows that the decrease in ^{14}C associated with lipid between 2 and 30min is much greater than the increase in ^{14}C associated with protein. In view of this and of the results of experiments discussed in sections (*g*) and (*h*) it was decided to repeat the experiment, paying regard also to the changes in ^{14}C associated with water-soluble components. The results of this experiment, which was with a different microsomal preparation from that used in the experiment reported in Fig. 1, are shown in Fig. 2. Scheme 1(*a*) allows adequate explanation of all of the important features of Figs. 1 and 2.

In the absence of exogenous dolichol phosphate the initial rapid rise in mannolipid radioactivity is explained by the readily reversible reaction A, equilibrium being reached rapidly to give a peak of lipid radioactivity soon after 2min. This peak coincides with a momentary levelling off in the rapidly falling amount of ^{14}C in the form of GDP-mannose. The metabolism of GDP-mannose then continues at a slower rate via reaction C to give a slow increase in the concentration of [^{14}C]mannose 1-phosphate. The change in concentration of [^{14}C]mannose 1-phosphate with time is followed closely by changes in [^{14}C]mannose as a result of reaction F. The GDP-[^{14}C]mannose metabolized via reaction C is then replaced at the expense of [^{14}C]mannolipid through reaction A. A small part of the decrease in [^{14}C]mannolipid is due to the transfer of [^{14}C]-mannose to protein via the slow reaction B, but most is lost through the reaction A. The increase in

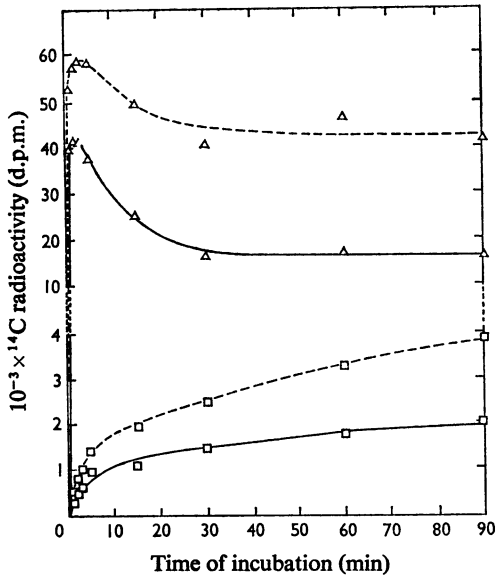


Fig. 1. *Variation with time of the amount of* ^{14}C *recovered in the form of dolichol phosphate mannose* (△) *and mannoprotein* (□)

Recovery was measured after incubation of GDP-[^{14}C]mannose with the pig liver microsomal preparation in the absence (——) and in the presence (– – – –) of exogenous dolichol phosphate (179 μM). The preparation used for this experiment was different from that used in the experiment illustrated in Fig. 2.

[^{14}C]mannose plus [^{14}C]mannose 1-phosphate between 2 and 90min was approx. 60000d.p.m. and this is balanced by the decrease in GDP-[^{14}C]mannose (40000d.p.m.) plus the decrease in [^{14}C]mannolipid (21000d.p.m.). During the same period the [^{14}C]-mannoprotein increased by 1000d.p.m.

That the loss of ^{14}C from the mannolipid to mannose and mannose 1-phosphate was via reaction A and GDP-[^{14}C]mannose was confirmed by the effect of adding dolichol phosphate to the system. This shifted the equilibrium of reaction A towards the formation of [^{14}C]mannolipid, so that more ^{14}C became associated with the mannolipid than in the absence of exogenous dolichol phosphate at the peak of incorporation. In this particular experiment the increase in [^{14}C]mannolipid and [^{14}C]mannoprotein in the presence of dolichol phosphate was less than is usually observed. The result shown in Fig. 1 is more typical. This in turn caused a small increase in the rate of transfer of [^{14}C]mannose to protein. On the other hand the high concentration of dolichol phosphate slowed down the loss of mannose from mannolipid through reaction A. This led to an initially more rapid disappearance of GDP-[^{14}C]mannose and a slower and eventually smaller production of mannose 1-phosphate and mannose during the 90min period of the incubation. The total production of [^{14}C]mannose plus [^{14}C]mannose 1-phosphate was 46000d.p.m., as against 68000d.p.m. in the absence of exogenous dolichol phosphate. Had, for example, the mannose 1-phosphate been formed directly from dolichol phosphate mannose one would have expected an increase in its production after elevation of the concentration of dolichol phosphate mannose by exogenous dolichol phosphate. A similar argument indicates that the [^{14}C]-mannose is unlikely to have been derived directly from either dolichol phosphate [^{14}C]mannose or [^{14}C]mannoprotein.

(k) Evidence in favour of mannose being transferred from GDP-mannose to protein through dolichol phosphate mannose

This paper is mainly concerned with studying the transfer of mannose from GDP-mannose to mannolipid, and most of the experiments were designed to this end. However, in the course of these experiments observations relating to the transfer of mannose to protein were also made. Some of these observations are consistent with the formation of mannoprotein according to either Scheme 1(*a*) or 1(*b*). On the other hand some of the results can be explained by Scheme 1(*a*) but not by 1(*b*). These points are discussed below.

The results in this paper show that the addition of dolichol phosphate to an incubation usually causes an increased transfer of mannose to protein. The results vary from one preparation to another, and sometimes the effect is negligible, but there is no evidence that dolichol phosphate causes a decreased transfer of mannose to protein. Had the [^{14}C]mannoprotein been formed directly from GDP-[^{14}C]mannose and not via the [^{14}C]mannolipid (Scheme 1*b*), a decrease in [^{14}C]mannoprotein would have been the expected result owing to competition between the two systems (reactions A and G) for the limited and rapidly dwindling amount of GDP-[^{14}C]mannose. The observed results are readily explained on the basis of Scheme 1(*a*).

It was observed in section (*b*) above that other prenol phosphates can act as acceptors for mannose but that they do not stimulate transfer of mannose to protein as well as does dolichol phosphate. If in fact Scheme 1(*b*) was operating and the increase in mannoprotein was due to a pool of prenol phosphate mannose maintaining the pool of GDP-mannose there would be no obvious reason why betulaprenol phosphate, which causes as large a stimulation of [^{14}C]mannolipid formation as does dolichol phosphate, should not cause as large an increase in

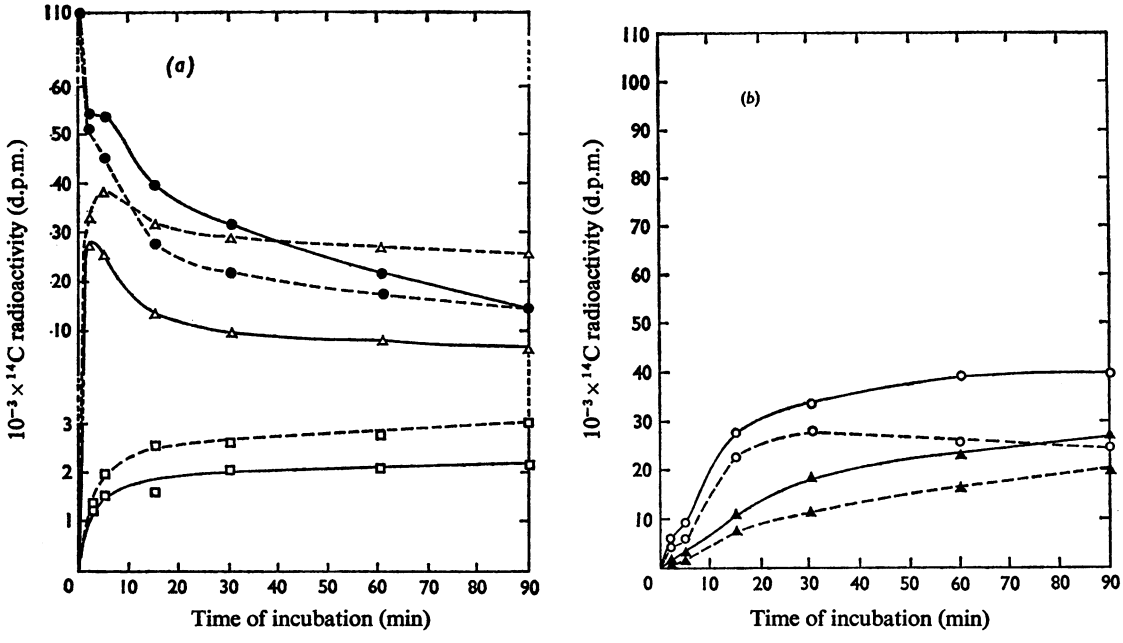


Fig. 2. *Variation with time of the amount of ^{14}C recovered in the form of various components*

Recovery was measured after incubation of GDP-[^{14}C]mannose with the pig liver microsomal preparation: ——, in the absence of exogenous dolichol phosphate; – – – –, in the presence of exogenous dolichol phosphate (179μM). (*a*) ●, GDP-mannose; △, dolichol phosphate mannose; □, protein. (*b*) ○, Mannose; ▲, mannose 1-phosphate. Details of the incubation medium are given in Table 3 and in the Materials and Methods section.

[^{14}C]mannoprotein as does dolichol phosphate. On the other hand, if one assumes that reaction B (Scheme 1*a*) is highly specific to dolichol phosphate mannose and accepts mannose from other prenol phosphates much less readily, the observed results fit Scheme 1(*a*).

If Scheme 1(*b*) was correct, it would also be difficult to understand why excess of GDP present from the beginning of an incubation caused a 75% inhibition of the incorporation of [^{14}C]mannose into protein but that, as expected from the nature of the mannose linkages concerned, the same concentration of GDP did not reverse the incorporation when it was added after 5min (section *g*). Scheme 1(*a*) offers an explanation of this observation, for it is seen that GDP present from the beginning of the incubation is effective through the readily reversible reaction A and not reaction B. Since little ^{14}C reaches the mannolipid only a little also reaches the mannoprotein. However, reaction B would not be expected to be readily reversible, and once [^{14}C]mannose has been incorporated into protein the addition of GDP is unlikely to reverse the process, even if the mannolipid were to lose all of its radioactivity through reaction A.

(*l*) *Incubations in the presence of antibiotics*

An understanding of the function of prenol phosphate in bacterial wall biosynthesis has been assisted by the inhibitory effect of several antibiotics. Undecaprenol pyrophosphate sugar derivatives donate the sugar derivative moiety to polymer, leaving undecaprenol pyrophosphate. The terminal phosphate group is then removed by a phosphatase and the undecaprenol monophosphate is available to take up a sugar phosphate and enter the cycle again. Bacitracin binds irreversibly with undecaprenol pyrophosphate (Stone & Strominger, 1971) and inhibits its conversion into monophosphate and so inhibits the incorporation of radioactive sugar into lipid intermediate and into polymer (Siewert & Strominger, 1967). This antibiotic also binds with farnesyl pyrophosphate, thus inhibiting squalene biosynthesis (Stone & Strominger, 1972). Vancomycin inhibits the formation of peptidoglycan by blocking the transfer of the disaccharide unit

from the lipid intermediate to the existing polymer (Anderson & Strominger, 1966). A third antibiotic, chloramphenicol, inhibits the transfer of glucose from UDP-glucose to lipid intermediate involved in the formation of certain teichoic acids (Stow *et al.*, 1971).

Incubations were performed with a range of concentrations of bacitracin up to 400μM in both the presence and the absence of exogenous dolichol phosphate (179μM). The antibiotic was found to have no effect on the transfer of mannose to either lipid or protein. This may be interpreted as confirming that the pig liver lipid intermediate is dolichol monophosphate mannose rather than dolichol pyrophosphate mannose. However, since it is not certain that bacitracin would inhibit the removal of a phosphate group from dolichol pyrophosphate this essentially negative evidence cannot be conclusive.

Even when added to standard incubation mixtures (see Table 3) relatively large quantities of both vancomycin (30μg) and chloramphenicol (200μg) had no detectable effect on the incorporation of mannose into lipid and protein.

General discussion

The studies on the transfer of mannose from GDP-mannose to lipid show that this step is readily reversible and produces GDP. This is consistent with the mannolipid being a prenol phosphate mannose. The chromatographic properties and response to dilute acid and alkali constitute supporting evidence. Further confirmation of this point is presented by the response of the transfer of mannose to lipid to exogenous prenol phosphates, which appear to act as acceptors of mannose by substituting for endogenous prenol phosphate. That the prenol phosphate concerned is almost certainly dolichol phosphate is shown by the presence in pig liver of dolichol and of two compounds corresponding chromatographically and in mannose-acceptor (or -exchange) activity to dolichol phosphate and dolichol phosphate mannose respectively. The incorporation of radioactive dolichol phosphate into the mannolipid, and the liberation of dolichol phosphate and mannose on acid hydrolysis of this mannolipid, are in keeping with this situation. The evidence available is strongly in favour of dolichol phosphate acting as an acceptor of mannose from GDP-mannose in the pig liver microsomal preparation. Unequivocal proof of this must await isolation and purification of sufficient mannolipid for its complete characterization. This is especially important in view of reports of the ability of a retinol derivative to form a mannolipid (De Luca *et al.*, 1970).

Behrens *et al.* (1971*b*) observed the formation of an oligosaccharide–lipid complex when a glucolipid corresponding to dolichol phosphate glucose was incubated with a rat liver microsomal preparation. This complex was tightly bound to the protein and could be removed efficiently only by extraction with certain precisely defined mixtures of chloroform, methanol and water. They conclude that this complex probably accounts for the glucose previously reported to have been transferred to protein when the microsomes were incubated with UDP-glucose (Behrens & Leloir, 1970). No evidence has been found in the present authors' laboratory for the formation of a complex with similar solubility properties from GDP-[^{14}C]mannose or [^{14}C]mannolipid when it was incubated with pig liver microsomal preparations. The [^{14}C]mannose recovered in the protein fraction remains associated with the protein in all separative procedures investigated so far (P. J. Evans & F. W. Hemming, unpublished work). All the indications are that the [^{14}C]mannose is part of a glycoprotein.

The relationship between the mannolipid and mannoprotein is less certain than that between the mannolipid and GDP-mannose. However, the evidence available is in favour of the mannolipid acting as an intermediate between GDP-mannose and the mannoprotein. In this respect the analogy between this system and the function of prenol phosphate sugars in bacterial systems is obvious. The reconciliation of such observations from relatively crude pig liver microsomal preparations with the results of studies of several purified soluble transferases, which appear to function efficiently in the absence of prenol phosphates (e.g. Roseman, 1970; Bosmann, 1970), is less clear. It is possible that a solution to this problem will emerge from studies on the nature of the protein acceptor(s) involved. It is, for example, conceivable that prenol phosphate sugars are more efficient as donors to hydrophobic proteins, or parts thereof, than are nucleoside diphosphate sugars. Possibly the need for such hydrophobic donors lessens with decreasing hydrophobicity of the acceptor, a fact which may become apparent, for example, as glycosylation of a protein becomes more extensive.

An alternative possibility is that two pools of GDP-mannose exist, one in the cytoplasm and the other inside the microsomal vesicles (inside the lumen of the endoplasmic reticulum *in vivo*), and that mannose can be transferred from the extravesicular GDP-mannose to the intravesicular GDP-mannose only through the intermediate dolichol phosphate mannose in the membrane of the vesicles. Given that the GDP-mannose–protein mannosyltransferase is on the inside of the microsomal membrane, and that the membrane is impermeable to relevant water-soluble compounds such as GDP, it becomes possible to accommodate the observations reported in the present paper and to reconcile them with results obtained with purified solubilized transferases. Although there is no direct evidence for the presence

of nucleoside diphosphate sugars inside the lumen (or vesicles *in vitro*) of the endoplasmic reticulum it is relevant that such an arrangement, coupled with their export in the vesicles of the Golgi apparatus when these become everted as they join the plasma membrane (Morré *et al.*, 1971), would explain the source of nucleoside diphosphate sugars and glucosyl transferases required for Roseman's (1970) theory of cell adhesion and separation. A similar arrangement in higher plants would also explain the source of glucose donors and transferases required for cellulose biosynthesis at the cell surface (e.g. Northcote, 1969).

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