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Role of Mannosyl Phosphoryl Polyisoprenol in Biosynthesis of Mammary Glycoproteins

Inder K. Vijay and Steven R. Fram

Department of Dairy Science, University of Maryland, College Park, Maryland 20742

When a membrane preparation from the lactating bovine mammary gland is incubated with GDP-[^{14}C]mannose, mannose is incorporated into a [^{14}C]mannolipid, a [Man-^{14}C]oligosaccharide-lipid, and metabolically stable endogenous acceptor(s). The rate of mannosyl incorporation is the fastest into [^{14}C]mannolipid, intermediate in [Man-^{14}C]oligosaccharide-lipid, and least into]Man-^{14}C] endogenous acceptor(s).

The [^{14}C]mannolipid has been partially purified and characterized. Mild acid hydrolysis of this compound gives [^{14}C]mannose, whereas alkaline hydrolysis yielded [^{14}C]mannose phosphate as the labeled product. The $t_{1/2}$ of hydrolysis of the mannolipid under the acidic and basic conditions are comparable to values obtained for mannosyl phosphoryl dolichol in other systems. The mannolipid is chromatographically indistinguishable from calf brain mannosyl phosphoryl polyisoprenol and chemically synthesized β-mannosyl phosphoryl dolichol. Exogenous dolichol phosphate stimulates the synthesis of mannolipid in mammary particulate preparations 8.5-fold. Synthesis of mannolipid is freely reversible; in the presence of GDP, the transfer of mannosyl moiety from endogenously labeled mannolipid to GDP-mannose is obtained. All of these results indicate that the structure of mannolipid is mannosyl phosphoryl polyisoprenol. Even though the precise chain length of the polyisoprenol portion has not been established, it is tentatively suggested to be dolichol.

Partially purified [^{14}C]mannolipid can directly serve as a mannosyl donor in the synthesis of [Man-^{14}C]oligosaccharide-lipid and [Man-^{14}C] endogenous acceptor(s). Pulse and chase kinetics utilizing GDP-mannose to chase the mannosyl transfer from GDP-[^{14}C]mannose in the mammary membrane incubations caused an immediate and rapid turnover of [^{14}C]mannose from [^{14}C]mannolipid while the incorporation of label in [Man-^{14}C]oligosaccharide-lipid and radioactive endogenous acceptor(s) continued for a short period before coming to a halt.

Both gel filtration and electrophoresis indicate that the endogenous acceptor(s) are a mixture of 2 or more glycoproteins since incubation with proteases releases all of the radioactivity into water soluble low-molecular-weight components, perhaps glycopeptides.

All of the above evidence is consistent with the following precursor-product relationship:

GDP-mannose $\leftrightarrows$ mannosyl phosphoryl polyisoprenol $\rightarrow$ mannosyl-oligosaccharide-lipid $\rightarrow$ mannosyl-proteins.

The exact structure of the oligosaccharide-lipid and the endogenous glycoproteins is unknown.

Key words: mammary, glycoprotein, biosynthesis, mannosyl phosphoryl polyisoprenol

Abbreviations: C/M (2:1) – $CHCl_3$-CH_3OH, 2:1; C/M/W (10:10:3) – $CHCl_3$-CH_3OH-H_2O 10:10:3; NeuNAc – N-acetylneuraminic acid; Gal – galactose; GlcNAc – N-acetylglucosamine; Man, mannose.

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Presently, there are 2 types of mechanisms known for the assembly of carbohydrate moieties of glycoproteins. The process whereby single carbohydrate residues are transferred directly from their respective nucleotide donors to the distal end of the incomplete growing portions of glycoproteins containing complex heterooligosaccharide chains has been thoroughly documented (1, 2). The terminal carbohydrate sequence of many glycoproteins is the trisaccharide residue NeuNAc→Gal→GlcNAc. By sequential treatment of a number of glycoproteins with neuraminidase, β-galactosidase, and hexosaminidase, the stepwise and orderly transfer of terminal sugars from nucleotide donor substrates to the modified acceptor proteins, catalyzed by specific glycosyltransferases, has been established (1, 2).

A number of glycoproteins have the sequence . . . . . $(Man)_n$ -GlcNAc-GlcNAc-Asn, n = 2–5, for a portion of their prosthetic groups. This oligosaccharide region has been termed the "core" region to distinguish it from the commonly found NeuNAc→Gal→GlcNAc "terminal" trisaccharide. The attachment of core region of some glycoproteins has been shown to involve the transfer of sugars from sugar-nucleotides to a "carrier" lipid, dolichol phosphate, preassembly or oligomerization on this lipid, and finally a transfer en bloc to the acceptor protein (3). The identify of proteins glycosylated by this mechanism has not been established for most systems and the evidence indicates that endogenous glycosyl acceptor proteins are membrane bound. Recently, evidence has come forth from the laboratories of Heath and Lennarz to support that an oligosaccharide-lipid can indeed serve as an intermediate in the transfer of core region sugar residues to the nonglycosylated soluble proteins. With the mouse myeloma tumor system, 2-deoxy-D-glucose was used to block the first step in the glycosylation of K-46 immunoglobulin light chain (4). The secreted nonglycosylated protein was then shown to serve as an exogenous acceptor of core region sugars from the oligosaccharide-lipid intermediate. In the hen oviduct system, it was shown that the antibiotic tunicamycin inhibits the synthesis of N-acetylglucosaminyl pyrophosphoryl dolichol and oviduct slices incubated in the presence of this antibiotic synthesized nonglycosylated ovalbumin (5). Further, the enzymatic activity in the oviduct membrane preparation is capable of transferring the oligosaccharide moiety from the oligosaccharide-lipid intermediate to several exogenously added proteins after the latter have been denatured to expose potential sites for glycosylation (6).

We have recently initiated studies for the biosynthesis of glycoproteins in the lactating bovine mammary gland. The lactating mammary was chosen as an interesting tissue for the following reasons: i) this tissue elaborates large amounts of proteins including several glycoproteins into its secretion, i.e., milk; ii) it provides an excellent system for investigations into membrane glycoprotein biosynthesis. The milk fat globule membrane which is known to arise from the apical plasma membrane of the mammary secretory cell (7, 8), contains a number of glycoproteins (7–11). This report presents evidence that the lactating bovine mammary possesses all the basic parameters for a study of glycoprotein synthesis via lipid intermediates. A membrane preparation of this tissue catalyzes the transfer of [^{14}C] mannose from GDP-[^{14}C] mannose through the following sequence of steps:

$$\text{GDP-[}^{14}\text{C] mannose} \overset{Mn^{2+}}{\rightleftarrows} \text{[}^{14}\text{C] mannosyl phosphoryl polyisoprenol}$$

$$\rightarrow \text{[Man-}^{14}\text{C] oligosaccharide-lipid} \rightarrow \text{[Man-}^{14}\text{C] protein(s).}$$

MATERIALS AND METHODS

The bovine mammary tissue from a freshly slaughtered cow was routinely obtained from Frederick County Products, Frederick, Maryland. When sliced, the lactating gland oozes milk rather profusely. The tissue was placed on ice and transported to the laboratory immediately. The secretory tissue was substantially freed from the collagenous connective material and diced into pea sized cubes in the cold room (4°C). Unless stated otherwise, all subsequent operations were performed at ice temperature. After several washes in buffer consisting of 50 mM Tris-HCl, pH 7.2, 0.25 M sucrose, 1 mM EDTA, and 5 mM mercaptoethanol (homogenizing buffer) to remove milk and blood, the tissue was suspended in an equal volume of homogenizing buffer and homogenized in a Polytron homogenizer (Brinkmann) equipped with PT 10 ST head and operating at setting 8 for 60 sec. Since homogenization in Polytron results in excessive foaming and possible protein denaturation, some experiments were conducted using Lourdes MM-1A homogenizer, operating at maximum speed for 90 sec. The latter instrument gives a preparation with a slightly higher activity and is also more thoroughly homogenized.

The crude homogenate was filtered through 4 layers of cheesecloth and centrifuged at 750 × g for 10 min in a Sorvall RC-2B centrifuge. The supernatant was filtered through cheesecloth wetted with the homogenizing buffer to remove the thick layer of floating lipid and centrifuged at 9,000 × g for 15 min. The supernatant from the second spin was filtered through cheesecloth and centrifuged at 48,000 × for 30 min. The 48,000 × g pellet was resuspended in homogenizing buffer to a protein concentration of 20–30 mg/ml and designated P-3. The particulate preparation loses ~ 50% of the mannosyltransferase activity overnight upon storage at −20°C, but is stable with only a slight loss in activity thereafter for 7 weeks. All the experiments reported below were conducted with fresh P-3 preparations.

The transfer of mannose from GDP-[^{14}C]mannose into endogenous acceptors was assayed in standard incubations that contained 2.5 μl of 50 mM Tris-HCl, pH 7.2, 2.5 μl of 400 mM Mn^{2+}, 1 μl of 50 mM EDTA, 5 μl of 200 mM DTT, 20 μl of GDP-[^{14}C]mannose (417 cpm/pmole) and 65 μl of P-3 in a total volume of 100 μl. The reaction was initiated with the addition of GDP-[^{14}C]mannose and carried out at 37°C. At appropriate times, incubations were stopped by the addition of 20 volumes of C/M (2:1) and vortexed. Further processing to obtain [^{14}C]mannolipid, [Man-^{14}C]oligosaccharide-lipid and [Man-^{14}C] endogenous acceptors was conducted according to Waechter et al. (12).

For preparation and purification of [^{14}C]mannolipid, the standard incubation mixture was scaled up 100-fold. After incubation for 2 min at 37°C, the reaction was stopped with the addition of 50 volumes of C/M (2:1). Further processing was carried out through the completion of the C/M (2:1) extractions and washings as in the standard assay. After concentrating in a rotary evaporator, the organic phase containing the crude [^{14}C]-mannolipid (9.0×10^5 cpm) was chromatographed on a column of DEAE-cellulose [DE-52, Whatman, converted to the acetate form according to Rouser et al. (13)] as described by Forsee and Elbein (14). The radioactive fractions were pooled, desalted, and finally the partially purified [^{14}C]mannolipid (7.7×10^5 cpm) was taken up in C/M (2:1) and used in all subsequent experiments.

To study the transfer of mannose from [^{14}C]mannolipid into [Man-^{14}C]oligosaccharide-lipid and [Man-^{14}C] endogenous acceptors, incubations were performed exactly as

described by Waechter et al. (15) except that 10 mM DTT was also included. Subsequent processing to separate the unreacted substrate, [Man-^{14}C] oligosaccharide-lipid and [Man-^{14}C] endogenous acceptors was performed as in the standard assay.

The partially purified [^{14}C] mannolipid was characterized for its susceptibility to acid and base hydrolysis under the conditions given in the figure legends. It was also chromatographed on both silica gel G TLC plates in: A) $CHCl_3$-CH_3OH-H_2O, 60:25:4; and B) $CHCl_3$-CH_3OH-50% NH_4OH, 60:35:8, and EDTA impregnated Whatman SG-81 paper (16) in the developing solvent systems: C) 2,6-dimethyl-4-heptanone-glacial acetic acid-H_2O, 60:45:6; D) $CHCl_3$-CH_3OH-concentrated NH_4OH, 36:13:3; and E) $CHCl_3$-CH_3OH-H_2O, 65:25:4. Chemically synthesized β-mannosyl phosphoryl dolichol (kindly provided by Dr. C. D. Warren, Harvard Medical School) and calf brain [^{14}C] mannosyl phosphoryl polyisoprenol (generous gift of Dr. C. J. Waechter, University of Maryland, School of Medicine) were used as reference standards. After the chromatographic run, 0.5-cm segments of the gel were scraped along the chromatogram and counted in Scinti-Verse (Fisher Scientific Company, Silver Spring, Maryland). Similarly, 0.5-cm segments of the SG-81 paper chromatogram were cut out and counted. Unlabeled β-mannosyl phosphoryl dolichol was detected by the phosphate (17) and polyisoprenol (18) specific spray reagents.

The labeled sugar in the oligosaccharide-lipid and endogenous acceptors was examined by paper chromatography on the acid hydrolysates of these products (14). After development, the chromatograms were dried and cut into 1-cm segments for counting in ScintiVerse. The unlabeled reference sugars were identified by an alkaline $AgNO_3$ reagent (19).

An aliquot (24,000 cpm, 50 mg protein) of the labeled endogenous acceptors prepared from GDP-[^{14}C] mannose was subjected to proteolytic digestion as described by Baynes et al. (20). Gel filtration on 50 mg (24,000 cpm) of the labeled endogenous acceptors and the proteolytic digest (given above), was carried out according to Gold and Hahn (21).

Aliquots of untreated and protease-treated endogenous acceptor(s) were applied to Whatman No. 3MM paper and subjected to high voltage electrophoresis in a Savant FP 30B unit using 1.5 M formic acid (pH 1.8) as the electrolyte in the electrode vessels. After electrophoresis at 30 volts/cm for 2 h, the paper was dried, cut up into 0.5-cm segments, and counted.

GDP-[^{14}C] mannose (specific activity 210 μCi/μmole) was purchased from New England Nuclear Corporation (Boston, Massachusetts) and unlabeled GDP-mannose from Sigma Chemical Company (St. Louis, Missouri). All other reagents were from commercial sources and of the highest purity available.

Radioactivity was measured in a Packard Tri Carb 3375 liquid scintillation counter. Protein was determined by the Lowry procedure (22) with bovine serum as the standard.

RESULTS

The kinetics of mannosyl transfer into 3 products when crude mammary membranes are incubated with GDP-[^{14}C] mannose is shown in Fig. 1. On the basis of incorporation and turnover of radioactivity into products soluble in C/M (2:1), C/M/W (10:10:3), and the insoluble residue fraction, mannosyl transfer from GDP-[^{14}C] mannose into the 3 products is consistent with the following precursor-product sequence of reactions:

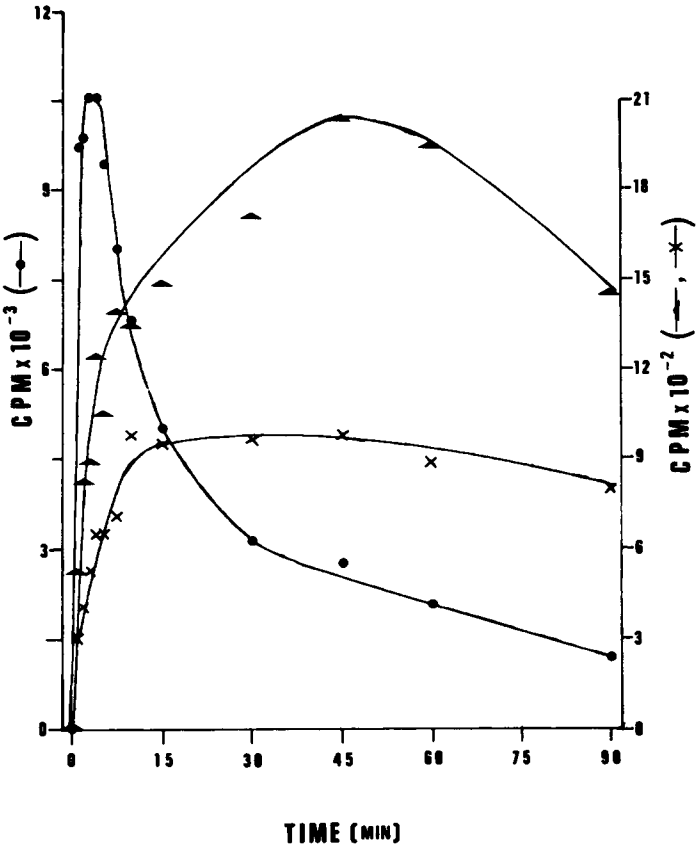

Fig. 1. Incorporation of radioactivity into C/M (2:1) soluble (–●–), C/M/W (10:10:3) soluble (–▲–), and into endogenous acceptors(s) fraction (–x–) upon incubation of GDP-[^{14}C] mannose with bovine mammary membrane preparation. Standard incubations were run in individual tubes and processed as given under Materials and Methods.

GDP-[^{14}C] mannose → [^{14}C] Mannolipid → [Man-^{14}C] Oligosaccharide-lipid
→ [Man-^{14}C] Endogenous "Acceptors" (Protein).

Analogous studies with other systems have made similar observations (14, 15, 20, 23–26). These results do not, however, rule out the possibility that mannose may be transferred directly from GDP-[^{14}C] mannose to endogenous acceptors in the mammary membrane preparation.

REVERSIBILITY OF MANNOLIPID SYNTHESIS

An experiment was conducted to examine the reversibility of the mannolipid synthesis, starting with GDP-[^{14}C] mannose (details are given in the legend to Fig. 2). There is a significant transfer of mannose from mannolipid to give GDP-[^{14}C] mannose indicating that mannose is bound to the lipid by an activated phosphodiester linkage and GDP, rather than GMP is the other product of this reaction.

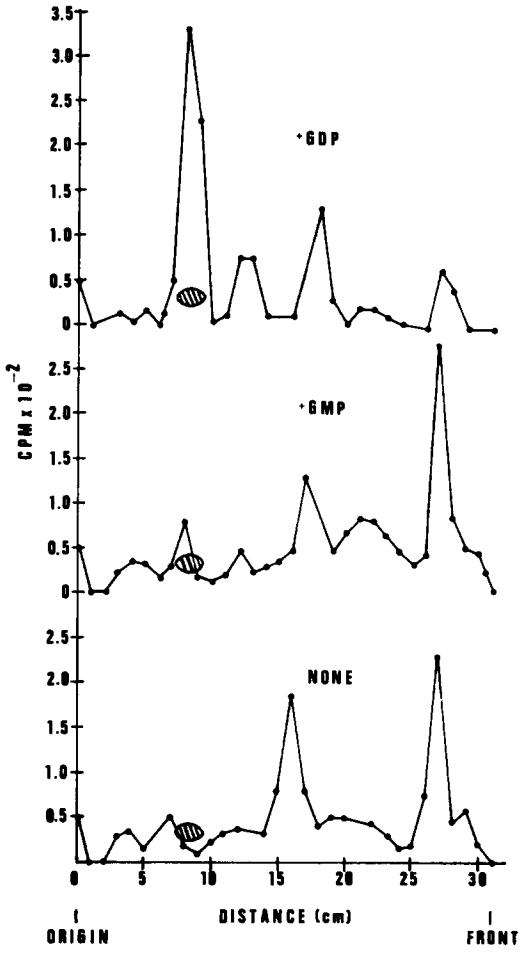

Fig. 2. Reversibility of mannolipid synthesis. Standard incubations containing GDP-[^{14}C]mannose and mammary membrane preparation were incubated for 2 min at 37°C, chilled on ice, and centrifuged in a cold room. Subsequent operations to wash and resuspend the membranes with guanine nucleotides were conducted according to Waechter et al. (15). After 10-min incubation at 37°C, 1 μmole of GDP-mannose was added to the incubation mixtures and the entire contents applied to Whatman 3MM paper as 1¼-inch-wide bands. In a parallel lane, 5 μmoles of GDP-mannose was also applied. After chromatography in solvent system F the GDP-mannose (hatched area) was located in the experimental lanes by examination under UV light and in the control lane by both UV absorption and alkaline $AgNO_3$ reagent (19). The experimental lanes were then cut up into 1-cm segments and counted in ScintiVerse.

In Fig. 2, minor peaks corresponding to mannose and mannose-1-phosphate are also observed. These are probably breakdown products of the hydrolysis of GDP-[^{14}C]-mannose and [^{14}C]mannolipid that have also been observed in other membrane systems (12, 15). These products may have resulted from enzymatic hydrolysis. Recently, it has been reported that divalent metal ions can also cause a nonenzymatic hydrolysis of sugar-nucleotides (27).

CONDITIONS FOR THE BIOSYNTHESIS OF [^{14}C] MANNOLIPID

The enzymatic transfer of mannose from GDP-[^{14}C] mannose to give [^{14}C] mannolipid was stimulated by a divalent metal ion and was inhibited by EDTA (data not shown). Mn^{2+} was the most efficient ion among the 7 ions tested (approximately twofold stimulation). Mammary membranes appear to have a reasonably high endogenous level of metal ions since there was a significant mannosyltransferase activity even in the absence of a $metal^{2+}$. In this regard, a considerable difficulty was encountered in keeping some of the mammary membrane preparations in a homogeneous suspension at 37°C and it was observed that precipitation could be avoided by the exclusion of $metal^{2+}$ from these incubations. This might parallel the change in ionic composition and balance of milk depending upon feeding of the animal and the stage of lactation (28). Also since milk is a rich source of Ca^{2+}, it is possible that mammary membranes have significant amounts of this ion. Mannolipid synthesis was linearly dependent on the amount of membrane protein up to 65 mg/ml (Fig. 3, inset) and the pH optimum was 7.2. The dependence of mannolipid formation on the concentration of GDP-mannose gave an apparent K_m of 4.8×10^{-6}M as calculated from a reciprocal plot.

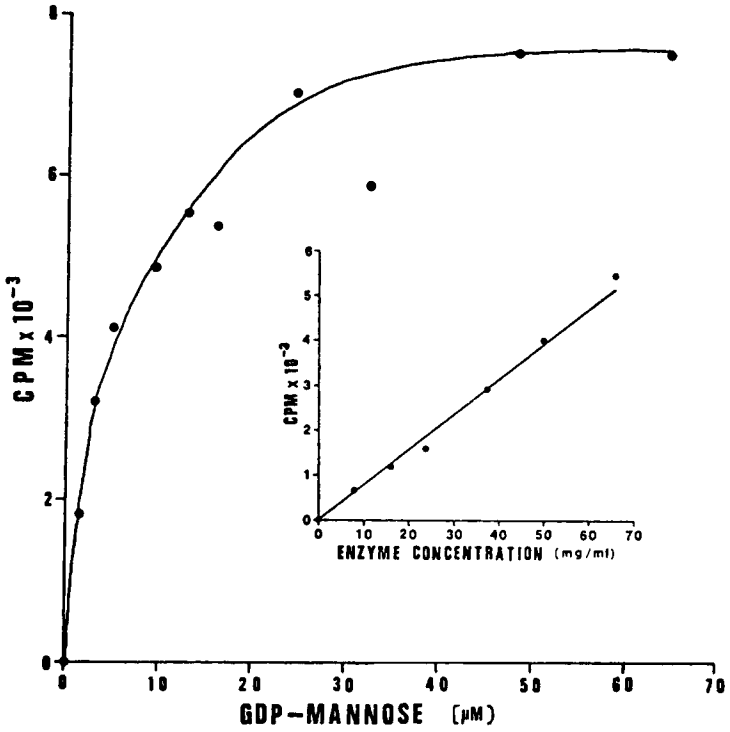

Fig. 3. Effect of GDP-[^{14}C] mannose concentration on the synthesis of [^{14}C] mannolipid. Standard incubations were run at 37°C for 2 min and processed to obtain [^{14}C] mannolipid as given under Materials and Methods. Inset: Formation of mannolipid as a function of mammary membrane concentration. Standard incubation mixtures, but with varying amounts of membrane preparation were incubated for 2 min at 37°C and processsed to obtain [^{14}C] mannolipid as given under Materials and Methods.

CHARACTERIZATION OF MANNOLIPID AS MANNOSYL PHOSPHORYL POLYISOPRENOL

When the mannolipid isolated from a scaled-up incubation mixture by extraction with C/M (2:1) was chromatographed on a column of DEAE-cellulose, more than 85% of the radioactivity was eluted as a sharp and fairly symmetrical single peak with a maximum at ~ 50 mM ammonium acetate.

The partially purified [^{14}C] mannolipid was chromatographed on both TLC plates of silica gel G and EDTA impregnated Whatman SG-81 paper in a number of solvent systems along with authentic β-mannosyl phosphoryl dolichol and calf brain mannosyl phosphoryl polyisoprenol. The results presented in Table I strongly indicate that the mammary mannolipid is a mannosyl phosphoryl polyisoprenol.

The half life of mannolipid under acidic and basic conditions of hydrolysis as given in Fig. 4 is compatible with similar results obtained for the hydrolysis of mannosyl phosphoryl polyisoprenol in several other systems (12, 15, 21).

Paper chromatography on the hydrolytic products revealed mannose and mannose phosphate (a product that cochromatographed with mannose-1-phosphate), respectively, as the products of acid and base hydrolysis. All of these data strongly imply that the mannolipid formed by mammary membranes is a mannosyl phosphoryl polyisoprenol.

STIMULATION OF MANNOLIPID BIOSYNTHESIS BY EXOGENOUS DOLICHOL PHOSPHATE

Since the [^{14}C] mannolipid synthesized in the mammary membrane system was chromatographically identical to synthetic β-mannosyl phosphoryl dolichol, it was of interest to see if exogenously added dolichol phosphate could serve as an acceptor of mannose from GDP-[^{14}C] mannose. Dolichol phosphate was found to stimulate the synthesis of [^{14}C] mannolipid at a linear rate up to 500 μM concentration (Fig. 5). As much as 8.5-fold stimulation in the synthesis of mannolipid was obtained at the highest concentration (500 μM) of exogenous dolichol phosphate. The radioactive mannolipid formed in the presence of exogenous dolichol phosphate had the mobility of mannosyl phosphoryl dolichol when chromatographed on thin layer plates of silica gel G in solvent systems A and B.

TABLE I. Mobility of [^{14}C] Mannolipid on TLC and Silica Gel Loaded Whatman SG 81 Paper Pretreated With EDTA (14)*

Glycolipids	Solvent system				
	A	B	C	D	E
Mammary endogenous lipid	0.31	0.60	0.59	0.31	0.52
Synthetic β-mannosyl phosphoryl dolichol	0.33	0.60	–	–	–
Calf brain mannosyl phosphoryl polyisoprenol	0.29	0.62	0.58	0.31	0.56

*Homemade TLC plates of silica gel G, approximately 0.5 mm gel thickness, were used. The plates and the SG 81 paper were activated at 110°C for 60 min immediately before use. Other details are given in the text.

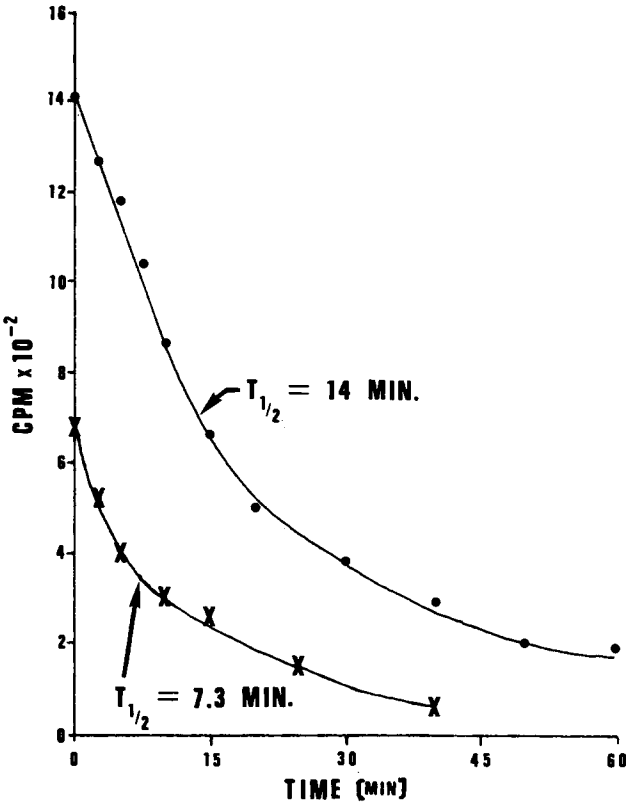

Fig. 4. Hydrolytic studies on [^{14}C] mannolipid. The radioactive glycolipid was dissolved in 1.0 ml of 0.1 N HCl in 50% 1-propanol and the reaction was carried out at 50°C (–•–). At indicated times, 0.1-ml aliquots were taken out and added to a mixture of 2 ml of C/M (2:1) and 0.5 ml of 20 mM NaOH. After vortexing, the 2 phases were separated by centrifugation and the radioactivity remaining in the organic phase was counted. For studying alkaline hydrolysis (–x–) reaction was carried out in 0.5 ml of 0.1 N NaOH in 90% ethanol at 85°C in screw cap tubes. At indicated times a 50-μl aliquot was transferred to a mixture of 4.0 ml of C/M (2:1) and 1.0 ml of 5 mM acetic acid. After vortex mixing, the 2 phases were separated by centrifugation and the radioactivity remaining in the organic phase was counted.

DIRECT TRANSFER OF MANNOSE FROM MANNOLIPID TO [MAN-^{14}C]-OLIGOSACCHARIDE-LIPID AND [MAN-^{14}C] ENDOGENOUS ACCEPTOR(S)

When a micellar suspension of [^{14}C] mannolipid was provided as a substrate in the mammary particulate system, there was a transfer of mannosyl moiety into a product soluble in C/M/W (10:10:3) and a product insoluble in C/M (2:1), C/M/W (10:10:3), water, or 10% TCA. The results in Fig. 6 clearly indicate that this transfer is virtually quantitative at short incubation times; with prolonged incubation there appears to be some loss of radioactivity, perhaps due to hydrolysis of either the [^{14}C] mannolipid or the [Man-^{14}C] oligosaccharide lipid. The amount of incorporation into [Man-^{14}C] endogenous acceptor(s) fraction is smaller than that obtained when GDP-mannose is the substrate and levels off after 20 min of incubation. These results do not rule out the possibility that initially there might have been transfer of [^{14}C] mannose to an endogenous pool of GDP

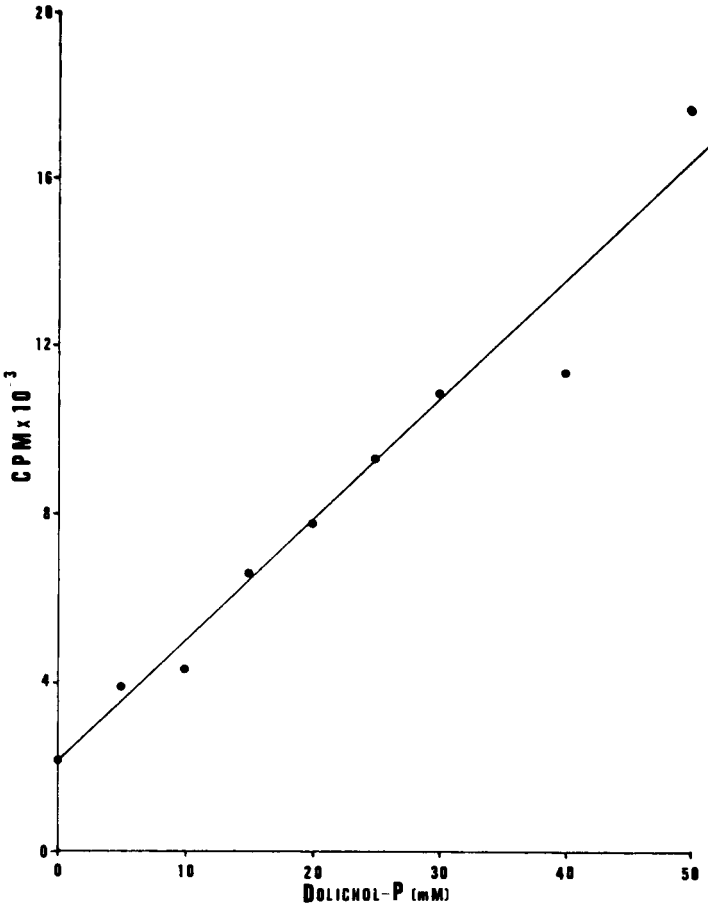

Fig. 5. Effect of dolichol phosphate on the incorporation of radioactivity from GDP-[^{14}C] mannose into the product soluble in C/M (2:1). Standard incubation mixtures that contained only 1 mg protein of the mammary membrane preparation were incubated for 2 min at 37°C with varying amounts of dolichol phosphate sonically dispersed in Ammonyx (at a final detergent concentration of 0.05%). [^{14}C] Mannolipid was isolated as given in the text.

to give rise to GDP-[^{14}C] mannose which might have then served as mannosyl donor for the glycosylation of the protein fraction. There is also the possibility that a part of mannose is incorporated into endogenous protein acceptor(s) directly from GDP-[^{14}C] mannose. When the [^{14}C] oligosaccharide-lipid was subjected to strong acidic hydrolysis and the products examined by paper chromatography, mannose was found to be the only labeled product.

PULSE-CHASE KINETICS

When the GDP-[^{14}C] mannose was chased with an excess of unlabeled substrate prior to the maximum incorporation into the mannolipid fraction, there was a rapid loss of radioactivity from the C/M (2:1) soluble fraction. This loss was much faster than a

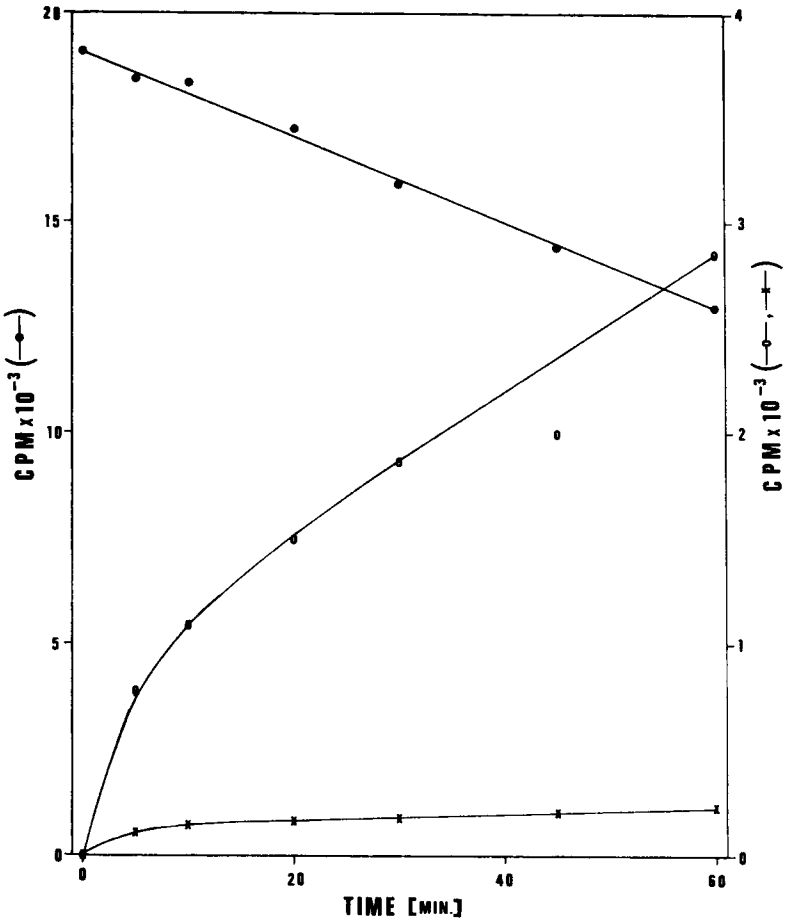

Fig. 6. Direct transfer of [^{14}C] mannose from [^{14}C] mannolipid into product soluble in C/M/W (10:10:3) and the endogenous acceptor protein(s). Details of experimental procedure are given in the text. –●–) Product soluble in C/M (2:1); –o–) product soluble in C/M/W (10:10:3); and –x–) insoluble delipidated membrane pellet {endogenous acceptor(s)}.

corresponding disappearance of the label from the control incubation (Fig. 7A). While the chase affected the incorporation of label into the oligosaccharide-lipid and the glycoprotein fractions as well (Figs. 7B and 7C), its effect on these reactions was not immediate and was much less pronounced. This might be expected from a pulse and chase in a precursor-product sequence of reactions. The unlabeled GDP-mannose would prevent any further appearance of label in the mannolipid fraction, but the radioactive mannolipid that would have been formed prior to the chase would continue to transfer [^{14}C] mannose into the oligosaccharide-lipid and endogenous acceptor(s) fractions for a time until all of the label from the mannolipid has been transferred to these products. The reduced incorporation of radioactivity into the oligosaccharide-lipid and endogenous acceptor(s) in the experimental fractions as compared to control incubation is also consistent with the above interpretation.

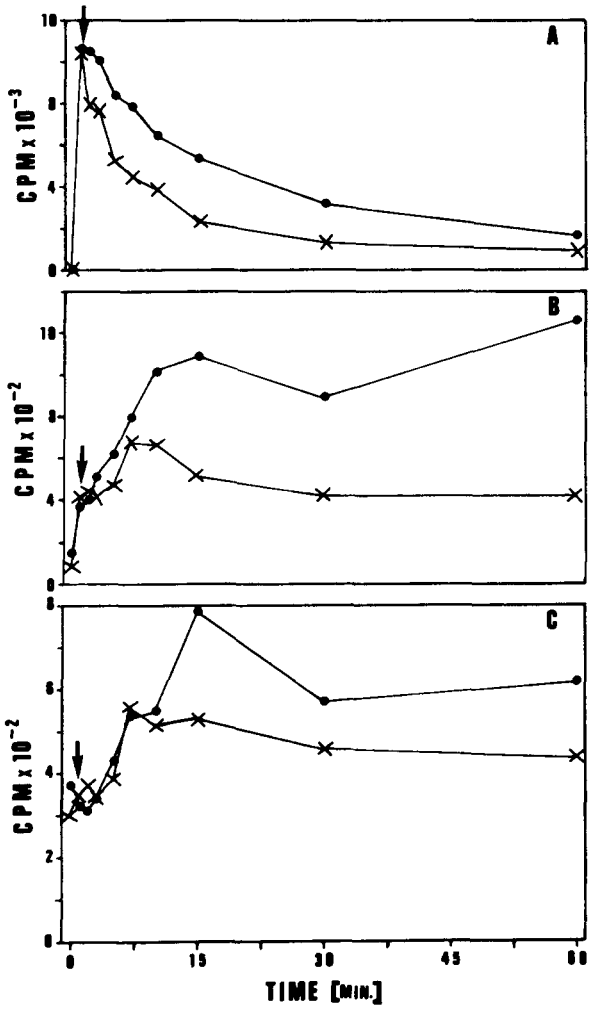

Fig. 7. Pulse-chase kinetics of mannosyl transfer into products soluble in C/M (2:1), C/M/W (10:10:3), and endogenous acceptor(s). Two tubes containing large scale incubation mixtures were prepared. Aliquots from each tube were withdrawn at different times and processed as for the standard assay. One min after the start of incubation (↓), a 100-fold excess of unlabeled GDP-mannose dissolved in 10^{-3} M Tris, pH 7.2, was added to the experimental tube. At the same time, an equal amount of 10^{-3} M Tris, pH 7.2, was added to the control tube (–•–).

NATURE OF METABOLICALLY STABLE ENDOGENOUS ACCEPTOR(S)

The radioactivity incorporated into the metabolically stable endogenous acceptor(s) was released as mannose after strong acid hydrolysis followed by paper chromatography in solvents F and G.

Of the several ionic and nonionic detergents tested for their ability to solubilize the radioactivity in the endogenous acceptor(s), only sodium dodecyl sulfate was effective. When the radioactivity in these products was solublized by sodium dodecyl sulfate and chromatographed on a column of Sephadex G-150 (Fig. 8), ~ 90% of the counts were recovered in 2 peaks eluting immediately after V_0 and a small amount was eluted in the V_i

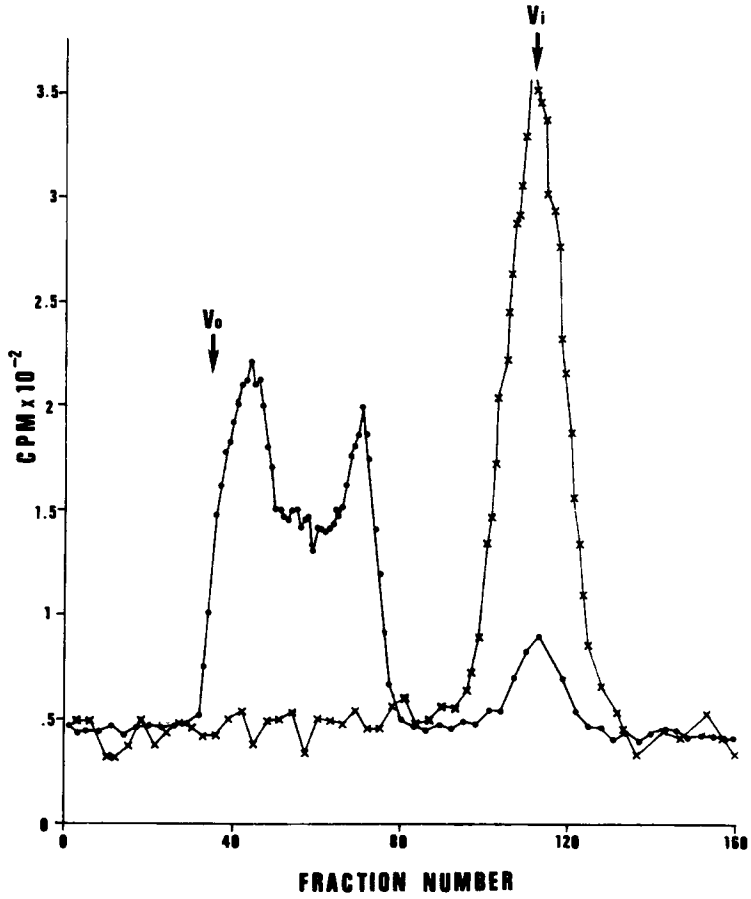

Fig. 8. Sephadex G-150 profile of protease treated (–x–) and untreated (–•–) radioactively labeled endogenous acceptor(s). After incubation with proteases as given in the text, solid sodium dodecyl sulfate was added to give a concentration of 1% and the tubes were heated at 50°C for 90 min. Conditions for gel filtration are given in the text.

region. After extensive digestion of these acceptors with proteolytic enzymes, all of the radioactivity was recovered as a single peak eluting near V_i. These results clearly imply that [^{14}C] mannose is linked to protein.

Another aliquot of the proteolytic digest was subjected to high voltage electrophoresis at pH 1.8. Two major and several minor peaks of radioactivity migrating towards the cathode, as expected of glycopeptides under these conditions of electrophoresis, were obtained. Since it was not possible to solubilize the labeled protein in a nonionic detergent, the sodium dodecyl sulfate-solubilized protein was therefore examined for its electrophoretic behavior in the control run. The solubilized labeled membrane products showed a slight anodic migration. Perhaps, in line with the Sephadex G-150 profile, the endogenous acceptors labeled in the mammary system are relatively low-molecular-weight proteins. These products might then exhibit an anionic behavior in the presence of sodium dodecyl sulfate and show some migration under these conditions of electrophoresis.

DISCUSSION

The studies presented in this report provide evidence that a 48,000 × g particulate fraction from the secreting mammary catalyzes the incorporation of mannose from GDP-[^{14}C]mannose into lipid soluble products and an insoluble glycoprotein fraction. The kinetics of mannosyl transfer are consistent with the following sequence of reactions:

$$\text{GDP-[}^{14}\text{C]mannose} \overset{Mn^{2+}}{\rightleftarrows} \text{[}^{14}\text{C]mannosyl phosphoryl polyisoprenol}$$

$$\rightarrow \text{[Man-}^{14}\text{C] oligosaccharide-lipid} \rightarrow \text{[Man-}^{14}\text{C] endogenous protein(s).}$$

The first reaction is freely reversible since in the presence of endogenously synthesized [^{14}C]mannosyl phosphoryl polyisoprenol and GDP, but not GMP, the particulate enzyme fraction catalyzes the formation of GDP-[^{14}C] mannose. Different divalent metal ions, especially Mn^{2+}, stimulate this reaction though there is a significant activity even in the absence of an added metal^{2+}. A number of different lines of evidence, as presented under Results, indicate that the product soluble in C/M (2:1) is mannosyl phosphoryl polyisoprenol. The observation that this compound cochromatographs with authentic β-mannosyl phosphoryl dolichol in a solvent system capable of separating such glycolipids on the basis of lipid moiety (29) indicates that the lipid component is probably dolichol.

The second intermediate in this pathway is assumed to be an oligosaccharide-lipid on the basis of its solubility in C/M/W (10:10:3). Glycolipid intermediates with similar solubility properties have been identified to be oligosaccharide-lipids in a number of different systems (3, 30). The rate of synthesis of this intermediate indicated that it might arise from the transfer of mannose from mannosyl phosphoryl polyisoprenol. Direct evidence for mannosyl transfer from [^{14}C]mannosyl phosphoryl polyisoprenol to an oligosaccharide-lipid and the glycoprotein fraction was obtained when mammary membranes were incubated with partially purified [^{14}C]mannosyl phosphoryl polyisoprenol in the presence of nonionic detergent Ammonyx. One might argue that mannosyl moiety could first be transferred to endogenous GDP to synthesize GDP-[^{14}C]mannose since this reaction is freely reversible; later, the GDP-[^{14}C]mannose might directly transfer mannose into the oligosaccharide-lipid and the glycoprotein. Our results have not ruled out this mode of mannosyl transfer; however, in light of the results of similar studies with other systems, it appears to be an unlikely possibility.

At present, we have no detailed structural information for the oligosaccharide-lipid soluble in C/M/W (10:10:3) apart from the fact that all of the water soluble radioactivity recovered after strong acid hydrolysis of this fraction cochromatographs with authentic mannose in systems F and G. The oligosaccharide-lipid migrates as a broad peak (R_f = 0.45) on Whatman 3MM in system F (S.R. Fram and I.K. Vijay, unpublished observations). Whether or not this is due to heterogeneity arising from several oligosaccharide-lipids due to oligosaccharide moieties of different sizes, is presently unknown. Such a heterogeneity has been reported for the oligosaccharide-lipid intermediates in a number of other systems (14, 24–26). Although analogous studies in other systems and the kinetic experiments as outlined here do implicate the oligosaccharide-lipid as an intermediate, more direct evidence is needed to definitively establish this glycolipid as an intermediate in the biosynthesis of mammary glycoproteins.

The endogenous acceptors for mannose incorporation have been shown to be glycoproteins on the basis of susceptibility to digestion with proteases followed by the appearance of mannosyl radioactivity in low-molecular-weight fragments as indicated by gel filtration and by electrophoresis.

The solubility properties of the endogenous acceptor(s) glycoproteins are indicative that these are tightly bound components of the membranes. Other than this, no information can be given on the nature of these products. In this regard, the lactating bovine mammary gland appears to be particularly attractive as a tissue of choice. It has been calculated that the entire apical membrane of the secretory cell must be replaced within 8–10 hours to replenish the material lost as fat globule membrane during secretion of fat droplets into milk (31). Further, a number of glycoproteins have been identified as components of the milk fat globule membrane (7–11).

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