Amphomycin: Effect of the Lipopeptide Antibiotic on the Glycosylation and Extraction of Dolichyl Monophosphate in Calf Brain Membranes†

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ABSTRACT: The effect of the lipopeptide antibiotic amphomycin on the biosynthesis of mannosylphosphoryldolichol (Man-P-Dol), glucosylphosphoryldolichol (Glc-P-Dol), and *N*-acetylglucosaminylpyrophosphoryldolichol (GlcNAc-P-P-Dol) by calf brain membranes has been studied. When calf brain microsomes are incubated with GDP-[^{14}C]mannose in the presence of amphomycin, the transfer of [^{14}C]mannose into Man-P-Dol is blocked, and consequently the labeling of a dolichol-linked oligosaccharide and glycoprotein is reduced. Under conditions that totally inhibited the formation of Man-P-Dol, [^{14}C]mannose was still incorporated into an endoglycosidase H resistant, lipid-linked oligosaccharide that appears to be identical with $Man_5GlcNAc_2$, a dolichol-linked oligosaccharide synthesized by class E *thy*-1$^-$ mutant lymphoma cells [Chapman, A., Trowbridge, I. S., Hyman, R., & Kornfeld, S. (1979) *Cell (Cambridge, Mass.) 17*, 509–515]. All of the [^{14}C]mannosyl residues in the calf brain heptasaccharide are released by digestion with jack bean α-mannosidase. When membranes containing endogenous, prelabeled [^{14}C]$Man_5GlcNAc_2$-P-P-Dol are incubated with unlabeled GDP-mannose, amphomycin, and an amount of exogenous Dol-P that overcomes the inhibitory effect on Man-P-Dol biosynthesis, the lipid-linked oligosaccharide is elongated and becomes sensitive to endoglycosidase H. Amphomycin had no effect on the transfer of mannose from exogenous Man-P-Dol to endogenous oligosaccharide lipid or the transfer of mannose from GDP-mannose to exogenous $GlcNAc_2$-P-P-Dol. The lipopeptide antibiotic inhibits the transfer of glucose from UDP-glucose to Dol-P but not to ceramide or a particle-bound glucan with the structural features of glycogen. The enzymatic transfer of glucose from exogenous Glc-P-Dol into endogenous oligosaccharide lipid was also unaffected by the antibiotic. The transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P catalyzed by calf brain membranes was inhibited by amphomycin. By blocking the biosynthesis of GlcNAc-P-P-Dol, amphomycin indirectly prevented the formation of $GlcNAc_2$-P-P-Dol and the subsequent transfer of the disaccharide to a polypeptide acceptor associated with gray matter membranes. The antibiotic had no direct effect on the conversion of exogenous GlcNAc-P-P-Dol to $GlcNAc_2$-P-P-Dol or the transfer of GlcNAc from UDP-GlcNAc to peripheral sites on endogenous N-linked oligosaccharides. In addition to inhibiting three glycosylation reactions involving Dol-P as substrate, the antibiotic interfered with the extraction of endogenous, prelabeled Dol-^{32}P by $CHCl_3$–CH_3OH (2:1). Amphomycin had no effect on the extraction of endogenous, prelabeled [^{3}H]Man-P-Dol or the bulk of the membrane phospholipids. The effect of the antibiotic on glycosylation reactions involving Dol-P, the observation that the inhibitory effects on the synthesis of all three glycolipids could be reversed by exogenous Dol-P, and the apparently selective effect on the extraction of Dol-P could be due to the lipopeptide forming a complex with Dol-P, possibly at the specific reactive sites for the acceptor lipid in glycosyltransferases bound to calf brain membranes, and thereby obstructing glycosylation.

Asparagine-linked carbohydrate chains are initially synthesized as precursor oligosaccharides containing glucose, mannose, and *N*,*N*′-diacetylchitobiose while joined to dolichyl pyrophosphate. Many aspects of this lipid-mediated process have been reviewed (Lucas & Waechter, 1976; Waechter & Lennarz, 1976; Hemming, 1977; Parodi & Leloir, 1979). Since tunicamycin blocks the addition of the first *N*-acetylglucosamine (GlcNAc)[1] residue in the dolichol-linked oligosaccharide by inhibiting the transfer of GlcNAc-1-P to Dol-P (Tkacz & Lampen, 1975; Takatasuki et al., 1975; Struck & Lennarz, 1977; Waechter & Harford, 1977; Ericson et al., 1977), the biosynthesis of the dolichol-linked oligosaccharides is blocked completely. Thus, tunicamycin is useful for studying the biosynthesis, metabolic fate, and activity of the unglycosylated forms of glycoproteins normally containing N-glycosidically bound oligosaccharides. However, because the antibiotic has very little effect on the synthesis of Man-P-Dol or Glc-P-Dol, it has limited utility for studying intermediate stages in the assembly of dolichol-linked oligosaccharides. Inhibitors of the synthesis of other lipid intermediates would provide valuable tools for clarifying various steps in the intricate assembly process.

The antibiotic amphomycin produced by *Streptomyces canus* was first isolated in 1953 (Heinemann et al., 1953). The structure of the lipopeptide was elucidated by Bodanszky and his co-workers (Bodanszky et al., 1973) and shown to be an undecapeptide containing either 3-isododecenoic or 3-anteisotridecenoic acid, attached to the N-terminal aspartic acid residue by an amide linkage. Recent studies by Tanaka et al. (1977, 1979) have shown that amphomycin inhibits the synthesis of peptidoglycan in Gram-positive bacteria by blocking the transfer of phospho-*N*-acetylmuramyl pentapeptide from UMP to undecaprenyl monophosphate, the prokaryotic glycosyl carrier lipid. Considering the structural relationship between undecaprenyl monophosphate and Dol-P, it is reasonable to expect that the antibiotic would also affect glycosylation of the eukaryotic carrier lipid. In vitro studies reported by Elbein and his colleagues (Kang et al., 1978a,b; Ericson et al., 1978; Kang & Elbein, 1979) have, indeed, demonstrated that glycosylation reactions involving Dol-P in microsomal preparations from aorta and plants are inhibited by amphomycin.

This paper presents enzymatic evidence that amphomycin inhibits the transfer of mannose, glucose, and GlcNAc-1-P

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[1] Abbreviations used: Man-P-Dol, mannosylphosphoryldolichol; Glc-P-Dol, glucosylphosphoryldolichol; GlcNAc-P-P-Dol, *N*-acetylglucosaminylpyrophosphoryldolichol; GlcNAc, *N*-acetylglucosamine.

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from their nucleotide derivatives to Dol-P catalyzed by membrane preparations from central nervous tissue. Other mannosyl-, glucosyl-, and *N*-acetylglucosaminyltransferases associated with the same microsomal preparations from calf brain are not affected by the lipopeptide antibiotic. In the calf brain in vitro system, the inhibitory effect on Man-P-Dol, Glc-P-Dol, and GlcNAc-P-P-Dol synthesis is reversed by the addition of exogenous Dol-P, but not by an excess of sugar nucleotide or divalent cations. The consequences of blocking the formation of monosaccharide derivatives of Dol-P on subsequent steps in the assembly of dolichol-linked oligosaccharides have been investigated. These studies indicate that at least one and possibly the first four α-mannosyl residues added to $ManGlcNAc_2$-P-P-Dol are derived from GDP-mannose. These results are in accord with recent work on aorta (Kang et al., 1978b; Spencer & Elbein, 1980), rabbit liver (Schutzbach et al., 1980), and class E *thy*-1$^-$ mutant mouse lymphoma cells (Chapman et al., 1980). Direct evidence for the addition of α-mannosyl residues to the calf brain dolichol-linked heptasaccharide from Man-P-Dol is also presented. Moreover, we have found that amphomycin interferes with the extraction of endogenous prelabeled Dol-^{32}P but not [^{3}H]Man-P-Dol or the major membrane phospholipids. The effect of the antibiotic on the extraction of Dol-P and the enzymatic results encourage future studies to investigate the possibility that the lipopeptide binds directly to the polyisoprenyl monophosphate, perhaps at the reactive sites occupied by the acceptor lipid in the membrane-associated glycosyltransferases.

Materials and Methods

Materials. UDP-[U-^{14}C]glucose (283 mCi/mmol), UDP-[6-^{3}H]glucose (3.7Ci/mmol), and UDP-*N*-acetyl[U-^{14}C]glucosamine (300 mCi/mmol) were purchased from Amersham Corp. GDP-[U-^{14}C]mannose (246 mCi/mmol) and GDP-[1-^{3}H]mannose were obtained from New England Nuclear. [γ-^{32}P]CTP was from ICN Pharmaceuticals, Inc. Unlabeled GDP-mannose and α-mannosidase (jack bean) were from Boehringer Mannheim. Pronase (protease type V), dolichyl monophosphate (grade III), unlabeled *N*-acetylglucosamine, and *N,N'*-diacetylchitobiose were purchased from Sigma Chemical Co. Endo-β-*N*-acetylglucosaminidase H was from Miles Laboratories, Inc. Amphomycin (calcium salt) was a gift from Bristol Laboratories and Dr. M. Bodanszky, Case Western Reserve University. For use in enzymatic studies, the lipopeptide was dissolved in 0.1 N acetic acid, and the solution was adjusted to 0.05 M sodium acetate (pH 7.0) with 0.2 N NaOH. All other chemicals and reagents were acquired from standard commercial sources. The [^{3}H]-$Man_5GlcNAc_2$ from class E *thy*-1$^-$ mutant mouse lymphoma cells was generously provided by Dr. Ian Trowbridge, of the Salk Institute.

Enzyme Preparation. Membranes prepared from whole calf brain or gray matter by the procedure described for white matter (Waechter & Harford, 1977) were used as enzyme. Protein was measured by the method of Lowry et al. (1951).

Assay for the Transfer of Labeled Sugars from Their Respective Nucleotide Derivatives into Calf Brain Endogenous Acceptors. The enzymatic transfer of *N*-acetyl[^{14}C]glucosamine (Waechter & Harford, 1977), [^{14}C]mannose (Waechter et al., 1976), and [^{14}C]glucose (Scher et al., 1977) from their nucleotide derivatives into membrane-associated endogenous acceptors was assayed by the procedures reported in the earlier references.

Assay Utilizing Partially Purified Exogenous [^{14}C]Glycolipids as Substrates. The preparation of the labeled substrates and the assays for the conversion of exogenous [^{14}C]-GlcNAc-P-P-Dol to [^{14}C]$GlcNAc_2$-P-P-Dol, and *N,N'*-diacetylchitobiosyl lipid to $ManGlcNAc_2$-P-P-Dol were performed as described previously (Waechter & Harford, 1979). The preparation of [^{14}C]Man-P-Dol (Harford & Waechter, 1979) and [^{14}C]Glc-P-Dol (Waechter & Scher, 1978) and the assays for the transfer of [^{14}C]mannose and [^{14}C]glucose from dolichyl monophosphate into endogenous acceptors were performed by the procedures described in the references denoted.

Preparation of Calf Brain Membranes Containing Endogenous, Prelabeled Dolichyl [^{32}P]Monophosphate and GDP-[^{3}H]mannose. Membranes containing endogenous, prelabled dolichyl [^{32}P]monophosphate were prepared as described elsewhere (Burton et al., 1979). Endogenous [^{3}H]-mannosylphosphoryldolichol was prelabeled by incubating calf brain membranes (10 mg of protein), 0.05 M Tris-HCl (pH 7.0), 0.125 M sucrose, 0.5 mM EDTA, 1 mM $MnCl_2$, and 2.5 μm GDP-[^{3}H]mannose (4764 cpm/pmol) in a total volume of 1 mL for 10 min at 37 °C. At the end of the incubation period, the membranes were washed twice with 10 mL of ice cold 0.1 M Tris-HCl (pH 7.0), 0.25 M sucrose, and 0.1 mM EDTA. The prelabeled membranes were then resuspended in the same buffer to study the effect of amphomycin on the extraction of the various lipids.

Preparation of Dolichol-Linked Oligosaccharides for Chromatographic Analysis. Dolichol-linked oligosaccharides labeled under various conditions were extracted with $CHCl_3$–CH_3OH–H_2O (10:10:3) and hydrolyzed with 0.1 N HCl in 80% tetrahydrofuran at 50 °C for 30 min as described earlier (Scher et al., 1977).

Enzymatic Treatment of Labeled Glycoproteins and Free Oligosaccharides. Glycopeptides were produced by treating the labeled glycoprotein fractions with Pronase under the conditions used previously (Waechter & Harford, 1977). The free [Man-^{14}C]oligosaccharides released from the carrier lipid by mild acid hydrolysis were incubated with endo-β-*N*-acetylglucosaminidase H (0.01 unit), 0.03 M NaCl, 0.03% bovine serum albumin, and 0.05 M citrate–phosphate buffer (pH 5.0) in a total volume of 0.125 mL, under a toluene atmosphere at 37 °C for 24 h. Mannose-labeled oligosaccharides were also incubated with jack bean α-mannosidase (1.6 units) and 0.05 M sodium acetate (pH 4.75) in a total volume of 0.05 mL at 37 °C for 24 h. The reaction was terminated by heating (100 °C, 3 min), and following centrifugation the supernatant fluid was analyzed chromatographically.

Measurement of Radioactivity. All radioactive samples were counted in Liquiscint (National Diagnostics). The delipidated membrane residues containing the labeled glycoprotein fractions were dissolved by treatment with 1% Na-$DodSO_4$ containing 0.1% mercaptoethanol at 100 °C for 5 min and then transferred to counting vials with three serial rinses of 5 mL of scintillation fluid.

Results

Effect of Amphomycin on the Transfer of Mannose from GDP-mannose into Calf Brain Endogenous Acceptors. Earlier work established that when calf brain microsomes were incubated with GDP-[^{14}C]mannose, [^{14}C]mannose was incorporated into Man-P-Dol, a dolichol-linked oligosaccharide and glycoprotein (Waechter et al., 1976). The data in Figure 1 (upper panel) show that the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose into Man-P-Dol is progressively inhibited by the addition of increasing amounts of amphomycin. As a consequence of the block in the labeling of Man-P-Dol, there was a marked reduction in the incorporation of label into a

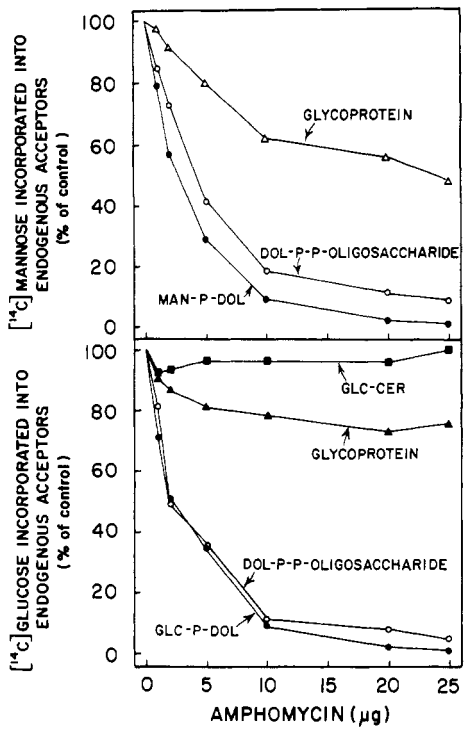

FIGURE 1: Effect of amphomycin on the transfer of mannose (upper panel) and glucose (lower panel) from their nucleotide derivatives into calf brain endogenous acceptors. (Upper panel) Incubation mixtures consisted of calf brain membranes (3.6 mg of protein), 50 mM Tris-HCl (pH 7.0), 0.125 M sucrose, 0.5 mM EDTA, 10 mM $CaCl_2$, 2.5 μM GDP-[^{14}C]mannose (219 cpm/pmol), 2.5 mM sodium acetate (pH 7.0), and the indicated amount of amphomycin in 0.2 mL. Following a 5-min incubation at 37 °C, the incorporation of [^{14}C]mannose into endogenous acceptors was assayed (Waechter et al., 1976). (Lower panel) Assay mixtures contained calf brain membranes (3.0 mg of protein), 50 mM Tris-HCl (pH 8.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 mM 5'AMP, 10 mM $CaCl_2$, 5 μM UDP-[^{14}C]glucose (518 cpm/pmol), 2.5 mM sodium acetate (pH 7.0), and the amount of amphomycin denoted in the figure in a total volume of 0.2 mL. The reaction mixtures were incubated for 10 min at 37 °C and the incorporation of [^{14}C]glucose into endogenous acceptors was assayed (Scher et al., 1977).

dolichol-linked oligosaccharide and glycoprotein. Since [^{14}C]mannose was still incorporated into a dolichol-linked oligosaccharide under conditions that totally arrested the synthesis of Man-P-Dol, it was of interest to compare the lipid-linked oligosaccharides enzymatically labeled in the presence and absence of the antibiotic. For this purpose, the [Man-^{14}C]oligosaccharides released from the carrier lipid by mild acid hydrolysis were analyzed on Bio-Gel P-4. The elution patterns shown in Figure 2 show that the [Man-^{14}C]oligosaccharide labeled in the presence of amphomycin (○) is shorter than the oligosaccharide labeled in the absence of the antibiotic (●), estimated to be $Man_{7-9}GlcNAc_2$. The [Man-^{14}C]oligosaccharide labeled under conditions that prevent [^{14}C]Man-P-Dol synthesis was insensitive to endoglycosidase H and is chromatographically identical with the endoglycosidase H resistant, $Man_5GlcNAc_2$ synthesized by class E *thy*-1$^-$ mutant mouse lymphoma cells (Chapman et al., 1979). All of the [^{14}C]mannose in the dolichol-linked heptasaccharide labeled by calf brain membranes in the presence of amphomycin was released by jack bean α-mannosidase. Furthermore, chromatographic analysis on Bio-Gel P-4 of the Pronase-digested [Man-^{14}C]glycoprotein fraction enzymatically labeled in the presence of amphomycin revealed a [Man-^{14}C]glycopeptide corresponding to $Man_5GlcNAc_2$. These results indicate that at least one and probably all of the α-mannosyl residues in $Man_5GlcNAc_2$-P-P-dol are acquired

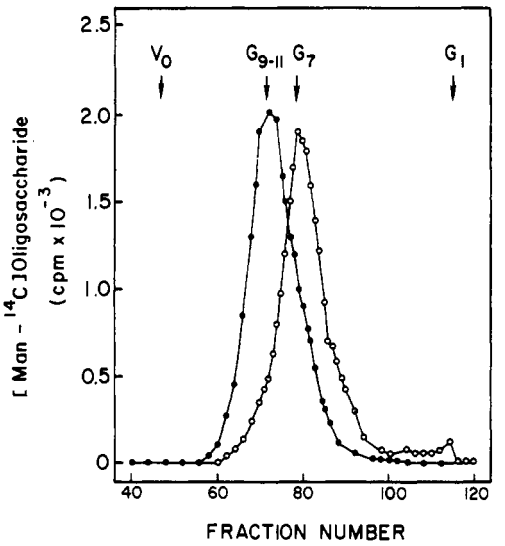

FIGURE 2: Gel filtration of lipid-linked [Man-^{14}C]oligosaccharides labeled in the presence (○) and absence (●) of amphomycin. The [Man-^{14}C]oligosaccharides enzymatically labeled by incubating calf brain membranes with 5 μM GDP-[^{14}C]mannose in the presence and absence of amphomycin (500 μg/mL) were released from the carrier lipid by mild acid hydrolysis and applied to a Bio-Gel P-4 column (0.9 × 107 cm). The column was equilibrated and washed with 0.1 M NaCl and 0.5-mL fractions were collected and counted to determine the elution positions of the labeled oligosaccharides. V_0 = blue dextran; G_7 = $Man_5GlcNAc_2$ from the class E *thy*-1$^-$ mutant mouse lymphoma cells, and G_1 = mannose.

Table I: Effect of Various Divalent Cations on the Amount of Amphomycin Producing 50% Inhibition of Glycolipid Formation[a]

divalent cation added (10 mM)	amount (μg) of amphomycin producing 50% inhibition of the biosynthesis of	
	Man-P-Dol	Glc-P-Dol
$CaCl_2$	3	2
$MnCl_2$	22[b]	50
$MgCl_2$	61	39
$NiCl_2$	29	31
$CoCl_2$	21[c]	

[a] Mannolipid synthesis was assayed by incubating membranes (3.6 mg of protein), 50 mM Tris-HCl (pH 7.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 μM GDP-[^{14}C]mannose (219 cpm/pmol), 5 mM sodium acetate (pH 7.0), 0–100 μg of amphomycin, and the indicated divalent cation in a total volume of 0.2 mL at 37 °C for 5 min. The enzymatic synthesis of Glc-P-Dol was assayed by incubating membranes (3.0 mg of protein), 50 mM Tris-HCl (pH 8.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 mM 5'-AMP, 5 μM UDP-[^{14}C]glucose (518 cpm/pmol), 5 mM sodium acetate (pH 7.0), 0–100 μg of amphomycin, and the indicated cations in a total volume of 0.2 mL at 37 °C for 10 min. The amount of mannolipid and glucolipid synthesized was assayed and the data presented above were calculated. [b] $MnCl_2$ = 5 mM. [c] $CoCl_2$ = 5 mM.

directly from GDP-mannose or another intermediate other than Man-P-Dol and that the heptasaccharide can be transferred to polypeptide acceptors in calf brain membranes under these in vitro conditions.

The effect of amphomycin on the calf brain Man-P-Dol synthase activity was examined in more detail. The fatty acid moiety of the lipopeptide is evidently required for the inhibitory action on glycolipid synthesis because exposure of the antibiotic to mild acid (0.25 N acetic acid, 100 °C, 2 h), conditions that have been shown to liberate the fatty acylated aspartic acid residue at the N terminus (Bodanszky et al., 1973), completely abolished the inhibitory effect on Man-P-Dol formation. Heating the lipopeptide at neutral pH (100 °C, 2 h) did not reduce its inhibitory effect on glycolipid synthesis. The sen-

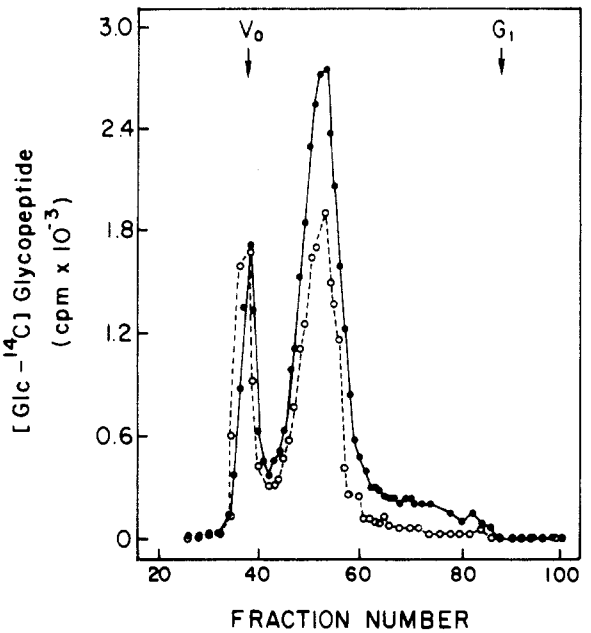

FIGURE 3: Gel filtration of Pronase-digested [Glc-^{14}C]glycoprotein fraction enzymatically labeled by 5 μM UDP-[^{14}C]glucose in the presence (○) and absence (●) of amphomycin (500 μg/mL). The Pronase digests were applied to a Bio-Gel P-4 column (1.7 × 73 cm). The column was equilibrated and washed with 0.1 M NaCl and 1-mL fractions were collected and counted. V_0 = blue dextran; G_1 = glucose.

sitivity of the mannosyltransferase to the antibiotic varied with the divalent cation added (Table I). The amount of antibiotic required to produce 50% inhibition of Man-P-Dol synthesis was considerably lower in the presence of Ca^{2+} than with Mn^{2+}, Mg^{2+}, Ni^{2+}, or Co^{2+}. The presence of bacitracin, another peptide antibiotic, did not affect calf brain Man-P-Dol synthase.

Additional studies showed that the lipopeptide antibiotic had no effect on the mannosyltransferases catalyzing the transfer of mannose from GDP-mannose to exogenous $GlcNAc_2$-P-P-Dol or from exogenous Man-P-Dol to endogenous dolichol-linked oligosaccharide. These results indicate that while amphomycin inhibits the transfer of mannose to Dol-P, it is not a general inhibitor of all the mannosyltransferases associated with the calf brain membranes used in these studies.

Effect of Amphomycin on the Transfer of Glucose from UDP-glucose into Calf Brain Endogenous Acceptors. Previous in vitro studies with a calf brain membrane system demonstrated that incubation of microsomes with UDP-[^{14}C]glucose resulted in the enzymatic labeling of Glc-P-Dol, oligosaccharide-P-P-Dol, and glucoprotein (Scher et al., 1977). The [^{14}C]glucopeptides produced by Pronase digestion of the [^{14}C]glucoprotein fraction have been found to be sensitive to endoglycosidase H. Under the same conditions, [^{14}C]glucose was also transferred directly from the sugar nucleotide into glucosyl ceramide and a particle-bound glucan, structurally resembling glycogen. The experiment shown in Figure 1 (lower panel) reveals that there is a sharp reduction in the synthesis of Glc-P-Dol upon the addition of increasing amounts of amphomycin. Inhibition of the synthesis of Glc-P-Dol resulted in a virtually complete block in the incorporation of [^{14}C]glucose into phospholipid-bound oligosaccharide and a diminished labeling of the glycoprotein fraction. This result suggests that under these in vitro conditions all of the labeled glucosyl residues in the dolichol-linked oligosaccharide are derived from Glc-P-Dol.

Chromatographic comparison of the Pronase-digested [^{14}C]glycoprotein fractions labeled in the presence and absence

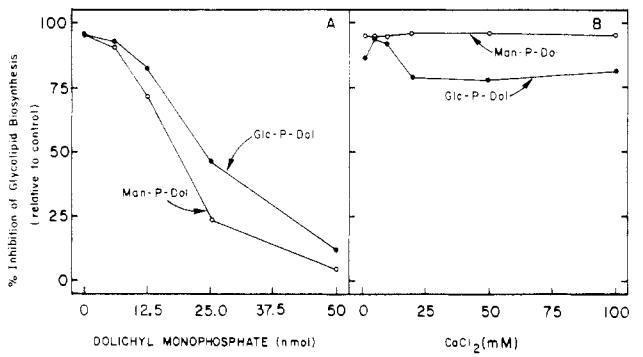

FIGURE 4: Effect of increasing amounts of exogenous dolichyl monophosphate (A) and $CaCl_2$ (B) on the inhibition of mannosylphosphoryldolichol (○) and glucosylphosphoryldolichol (●) biosynthesis by amphomycin. (Panel A) Mannolipid synthesis was assayed with membranes (2.1 mg of protein), 50 mM Tris-HCl (pH 7.0), 0.125 M sucrose, 0.5 mM EDTA, 10 mM $CaCl_2$, 0.5 mM sodium acetate (pH 7.0), 0.04% Triton X-100, the indicated amount of exogenous dolichyl monophosphate, and 1.3 μM GDP-[^{14}C]mannose (310 cpm/pmol) in the presence and absence of 10 μg of amphomycin in a total volume of 0.2 mL. The incubation period was 5 min at 37 °C. The enzymatic synthesis of glucosylphosphoryldolichol was assayed by incubating membranes (1.5 mg of protein), 50 mM Tris-HCl (pH 8.0), 0.125 M sucrose, 0.5 mM EDTA, 10 mM $CaCl_2$, 0.5 mM sodium acetate (pH 7.0), 2.5 mM 5′-AMP, 0.04% Triton X-100, the indicated amount of exogenous dolichyl monophosphate, and 2.5 μM UDP-[^{14}C]glucose (518 cpm/pmol) in the presence and absence of 10 μg of amphomycin in a total volume of 0.2 mL. The reaction mixture was incubated for 10 min at 37 °C and the amount of labeled glucolipid was assayed. (Panel B) To measure mannosylphosphoryldolichol synthesis membranes (2.0 mg of protein), 50 mM Tris-HCl (pH 7.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 μM GDP-[^{14}C]mannose (219 cpm/pmol), 0.5 mM sodium acetate (pH 7.0), and the indicated concentration of $CaCl_2$ were incubated in 0.2 mL at 37 °C for 5 min with and without 10 μg of amphomycin. Glucosylphosphoryldolichol synthesis was assayed by incubating membranes (2.1 mg of protein), 50 mM Tris-HCl (pH 8.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 mM 5′-AMP, 5 μM UDP-[^{14}C]glucose (406 cpm/pmol), 0.5 mM sodium acetate (pH 7.0), and the indicated concentration of $CaCl_2$ in the presence and absence of 10 μg of amphomycin in 0.2 mL for 10 min at 37 °C. The assay procedures used for mannolipid (Waechter et al., 1976) and glucolipid (Scher et al., 1977) synthesis described previously were followed.

of amphomycin shows that the labeling of the glycogen-like glucan excluded by Bio-Gel P-4 was insensitive to the presence of the antibiotic, but there was a reduction of approximately 50% in the label eluting in the position of the [Glc-^{14}C]-glycopeptide (Figure 3). No apparent differences in size are discernible between the [Glc-^{14}C]glycopeptides labeled in the presence or absence of amphomycin. The [^{14}C]glucosyl residues incorporated into glycopeptide in incubations where the labeling of lipid intermediates is totally inhibited may be added directly from UDP-[^{14}C]glucose to an N-linked oligosaccharide. It is also possible that an unrelated glycopeptide co-eluting with the N-linked oligosaccharide is labeled directly by UDP-[^{14}C]glucose in this in vitro system.

As observed for mannolipid synthesis, the sensitivity of Glc-P-Dol synthase activity varies with the divalent cation present (Table I). The glucosyltransferase is also more sensitive to amphomycin inhibition in the presence of Ca^{2+} as compared to Mn^{2+}, Mg^{2+}, or Ni^{2+}. Although the inhibition of Glc-P-Dol synthesis could not be reversed by an excess of Ca^{2+} or UDP-glucose, the inhibition was overcome by the addition of exogenous Dol-P (Figure 4). While the calf brain Glc-P-Dol synthase was potently inhibited by amphomycin, the antibiotic had no effect on the glucosylation of ceramide (Figure 1) or glycogen (Figure 3). Thus, under the in vitro conditions used in these studies, the only glucosyltransferase affected by amphomycin was Glc-P-Dol synthase.

Exogenous Dolichyl Monophosphate Overcomes the Inhibitory Effect of Amphomycin on Mannosylphosphoryldolichol and Glucosylphosphoryldolichol Biosynthesis by Calf Brain Membranes. The mechanism of action of amphomycin on the brain glycosyltransferases was probed by examining the effect of adding excess sugar nucleotide, acceptor lipid, and Ca^{2+} on the inhibition of Man-P-Dol and Glc-P-Dol biosynthesis. The inhibition of Man-P-Dol and Glc-P-Dol synthesis was consistently 50% in incubations containing 15 μg/mL of antibiotic and the appropriate sugar nucleotide substrate at concentrations ranging from 1 to 10 μM. In contrast to this result, the addition of increasing amounts of exogenous Dol-P to reaction mixtures containing 10 μg/0.2 mL of amphomycin gradually restored mannolipid and glucolipid formation relative to the controls, lacking the antibiotic (Figure 4, panel A). The inhibitory effect of the antibiotic on Man-P-Dol synthesis by hen oviduct and rat liver microsomes could also be overcome by the addition of high concentrations of exogenous Dol-P. In a similar study, exogenous Dol-P reversed the inhibition of GlcNAc-P-P-Dol synthesis by calf brain microsomes.

Because the lipopeptide contains a methyl aspartyl and three neighboring aspartyl residues (Bodanszky et al., 1973), it was suspected that the inhibitory effect may be caused by chelation of the divalent cations required by the brain glycosyltransferases. However, complexing Ca^{2+} is probably not responsible for the block in mannosylation or glucosylation of Dol-P, since adding Ca^{2+} in a 5- to 10-fold excess of the amount of antibiotic present did not reverse the effect of the antibiotic (Figure 4, panel B).

Incorporation of Mannosyl Residues into Dolichol-Linked Heptasaccharide in the Presence of Amphomycin. The results described earlier in the text are compatible with the conclusion that Man-P-Dol is not involved in the series of mannosylation reactions leading to the synthesis of $Man_5GlcNAc_2$-P-P-Dol in calf brain. It was of interest to determine if the dolichol-linked heptasaccharide could be elongated when the restriction on Man-P-Dol formation was overcome by exogenous Dol-P. To answer this question, we prelabeled membranes by incubation with GDP-[^{14}C]mannose in the presence of an amount of amphomycin that prevented the labeling of Man-P-Dol. The size of the lipid-bound oligosaccharide was analyzed by gel filtration on Bio-Gel P-4 following mild acid hydrolysis. A single radiolabeled oligosaccharide, insensitive to endoglycosidase H and eluting in the position of $Man_5GlcNAc_2$, was observed (Figure 5, panel A). The membranes containing endogenous [^{14}C]$Man_5GlcNAc_2$-P-P-Dol were extensively washed and reincubated with unlabeled GDP-mannose, amphomycin, and an amount of exogenous Dol-P sufficient to restore Man-P-Dol formation. The labeled lipid-linked oligosaccharide was recovered and the [Man-^{14}C]oligosaccharide was liberated by mild acid hydrolysis. Chromatographic analysis showed that reversing the block in Man-P-Dol synthesis allowed $Man_5GlcNAc_2$ to be elongated, yielding an oligosaccharide that is cleaved by endoglycosidase H (Figure 5, panel B). The elongated oligosaccharide appears to be identical with the [Man-^{14}C]oligosaccharide estimated to be $Man_{7-9}GlcNAc_2$ labeled in the absence of the antibiotic. This result indicates that at least one and possibly four α-mannose units added to $Man_5GlcNAc_2$-P-P-Dol are donated by Man-P-Dol. Finding that elongation does not occur when the prelabeled membranes were reincubated under identical conditions without GDP-mannose (Figure 5, panel C) strengthens the conclusion that the additional glycosyl residues are acquired via Man-P-Dol.

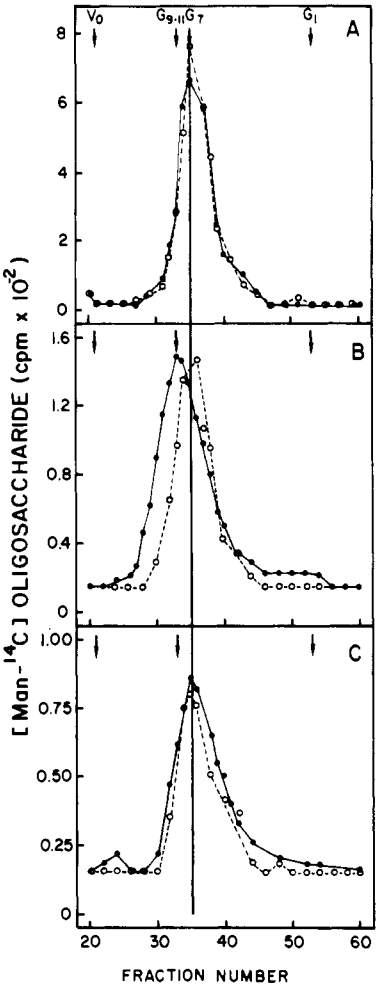

FIGURE 5: Elongation of dolichol-linked heptasaccharide in the presence of amphomycin when mannosylphosphoryldolichol synthesis is restored by exogenous dolichyl monophosphate. The lipid-linked [Man-^{14}C]oligosaccharides synthesized under the conditions described below were released by mild acid hydrolysis and chromatographed before (●) and after (○) treatment with endoglycosidase H on Bio-Gel P-4 (0.9 × 47 cm). The labeled oligosaccharides were eluted with 0.1 M NaCl and 0.5-mL fractions were counted. (Panel A) Calf brain membranes (72 mg of protein) were incubated in 80 mM Tris-HCl (pH 7.0), 0.2 M sucrose, 0.8 mM EDTA, 20 mM $CaCl_2$, 5 mM sodium acetate (pH 7.0), 0.5 mg of amphomycin, and 5 μM GDP-[^{14}C]mannose in a total volume of 3 mL for 30 min at 37 °C. (Panel B) The membranes containing [^{14}C]$Man_5GlcNAc_2$-P-P-Dol were extensively washed and reincubated with the same buffer as above, 500 μg of amphomycin, 5 mM sodium acetate (pH 7.0), 1 μmol of dolichyl monophosphate, 0.04% Triton X-100, and 5 μM unlabeled GDP-mannose in a total volume of 1 mL at 37 °C for 30 min. (Panel C) Same as panel B except that the unlabeled GDP-mannose was omitted. Calibration markers were the same as Figure 2.

Effect of Amphomycin on the Enzymatic Transfer of N-Acetylglucosamine from UDP-N-acetylglucosamine into Calf Brain Endogenous Acceptors. The effect of amphomycin on *N*-acetylglucosaminyl 1-phosphate and *N*-acetylglucosaminyltransferases associated with brain microsomes was also investigated. When gray matter membranes are incubated with UDP-[^{14}C]GlcNAc in the absence of amphomycin, label is incorporated into GlcNAc-P-P-Dol, $GlcNAc_2$-P-P-Dol, and two N-linked saccharide units (Harford & Waechter, 1979b). As seen in Figure 6, incremental additions of amphomycin block the transfer of [^{14}C]GlcNAc-1-P from UMP to Dol-P and consequently reduce the labeling of $GlcNAc_2$-P-P-Dol. The labeling of the glycoprotein fraction was also reduced by approximately 25%. As mentioned above, the inhibition of GlcNAc-P-P-Dol formation could be overcome by exogenous Dol-P. Under conditions defined previously (Waechter &

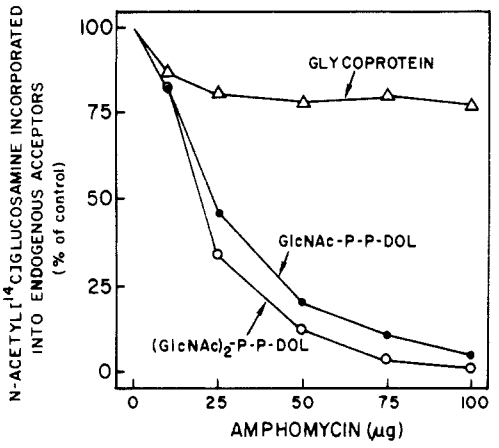

FIGURE 6: Effect of amphomycin on the incorporation of *N*-acetyl[^{14}C]glucosamine into gray matter endogenous acceptors. Reaction mixtures consisted of membranes (2 mg of protein), 50 mM Tris-HCl (pH 8.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 mM 5′-AMP, 10 mM $MgCl_2$, 5 mM sodium acetate (pH 7.0), 5 μM UDP-*N*-acetyl[^{14}C]glucosamine (564 cpm/pmol), and the indicated amount of amphomycin in 0.2 mL. Following a 10-min incubation at 37 °C the amount of *N*-acetyl[^{14}C]glucosamine incorporated into glycolipids and glycoprotein was measured (Waechter & Harford, 1977).

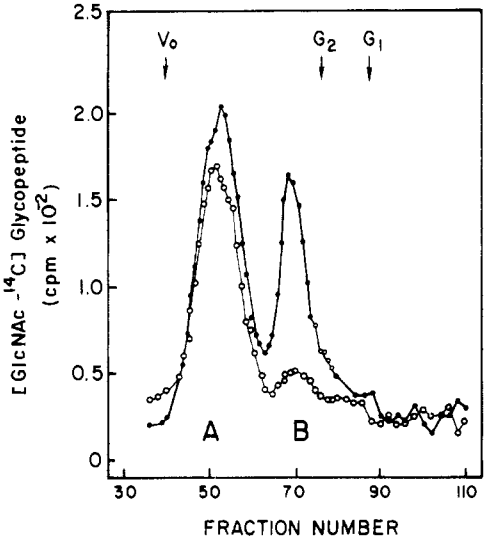

FIGURE 7: Chromatographic comparison of [GlcNAc-^{14}C]glycopeptides enzymatically labeled by gray matter membranes in the presence (○) and absence (●) of amphomycin. Gray matter membranes (16 mg of protein) were incubated with 80 mM Tris-HCl (pH 8.0), 0.2 M sucrose, 0.8 mM EDTA, 10 mM $MgCl_2$, 2.5 mM 5′-AMP, 5 mM sodium acetate (pH 7.0), and 7 μM UDP-*N*-acetyl[^{14}C]-glucosamine (564 cpm/pmol) with or without 500 μg of amphomycin in 1 mL. After an incubation of 45 min at 37 °C, the labeled glycoprotein fractions were isolated and treated with Pronase to produce the [GlcNAc-^{14}C]glycopeptides. The labeled glycopeptides were chromatographed on Bio-Gel P-4 (1.7 × 73 cm) by washing with 0.1 M NaCl and collecting 1 mL fractions. V_0 = blue dextran; G_2 = *N,N′*-diacetylchitobiose; G_1 = *N*-acetylglucosamine.

Harford, 1979), the transfer of GlcNAc from UDP-GlcNAc to exogenous GlcNAc-P-P-Dol was found to be insensitive to amphomycin.

To further assess the effect of the antibiotic on the labeling of endogenous glycoprotein, we analyzed the Pronase digests of the [^{14}C]glycoprotein fractions chromatographically on Bio-Gel P-4. From the gel filtraton pattern in Figure 7 (●), it can be observed that two glycopeptides (A and B) are radiolabeled during incubation with UDP-[^{14}C]GlcNAc in the absence of amphomycin. Glycopeptide A was previously shown to be an N-linked oligosaccharide, labeled by the direct addition of [^{14}C]GlcNAc residues from the sugar nucleotide to peripheral, nonreducing termini of endogenous glycoproteins. Glycopeptide B is labeled by the lipid-mediated transfer of [^{14}C]$GlcNAc_2$ from Dol-P-P to asparagine residues of endogenous polypeptide acceptors (Harford & Waechter, 1979b). The result depicted in Figure 7 (○) shows that by blocking the labeling of GlcNAc-P-P-Dol, and consequently $(GlcNAc)_2$-P-P-Dol, amphomycin indirectly inhibits the enzymatic labeling of the asparaginyl disaccharide (glycopeptide B).

Table II: Effect of Amphomycin on the Extraction of Dolichyl [^{32}P]Monophosphate from Calf Brain Membranes[a]

amphomycin added (μg)	amount of lipid recovered in $CHCl_3$–CH_3OH (2:1)		
	Dol-^{32}P (cpm)	[^{3}H]Man-P-Dol (cpm)	phospholipid (nmol)
none	1120	1230	660
2.5	899	1320	630
7.5	375	1410	660
20.0	176	1580	690

[a] Each incubation mixture consisted of calf brain membranes (2.9 mg of protein) containing Dol-^{32}P and [^{3}H]Man-P-Dol in 50 mM Tris-HCl (pH 7.0), 0.125 M sucrose, 0.5 mM EDTA, 10 mM $CaCl_2$, 15 mM sodium acetate, and the indicated amount of amphomycin in a total volume of 0.2 mL. After a 10-min incubation at 37 °C, 20 volumes of $CHCl_3$–CH_3OH (2:1) was added and the lipids extracted as described elsewhere (Burton et al., 1979). Lipid phosphorus was measured by the Bartlett procedure (1959).

These experiments establish that amphomycin obstructs the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P but has no effect on two other *N*-acetylglucosaminyltransferases associated with the same membrane preparations.

Effect of Amphomycin on the Extraction of Dolichyl Monophosphate from Calf Brain Membrane. Some of the enzymatic studies suggest that amphomycin could interfere with the synthesis of lipid intermediates by calf brain membranes by binding to Dol-P probably at the reactive sites of the acceptor lipid. Evidence for the direct association of the lipopeptide with endogenous Dol-P is presented in Table II. In this experiment, calf brain membranes containing endogenous, prelabeled Dol-^{32}P and [^{3}H]Man-P-Dol were mixed with different amounts of amphomycin, and then the lipids were extracted with $CHCl_3$–CH_3OH (2:1). As the concentration of the antibiotic present increased, lower amounts of Dol-^{32}P were recovered in the $CHCl_3$–CH_3OH extracts (Table II). The antibiotic had no effect on the extraction of [^{3}H]-Man-P-Dol or the majority of the membrane phospholipids. These data suggest that amphomycin may selectively form an inextractable complex with endogenous Dol-^{32}P that remains bound to the membrane residue. It appears that Ca^{2+} facilitates the binding of the lipopeptide to the polyisoprenyl monophosphate or the reactive site of the enzyme. The amount of endogenous and exogenous Dol-^{32}P remaining in the "delipidated" membrane residue was greater in the presence of Ca^{2+} compared to similar extractions in the absence of divalent cations or in the presence of Mn^{2+} or Mg^{2+}. This result may explain why the glycosylation of Dol-P is most susceptible to amphomycin inhibition in the presence of Ca^{2+}. The possibility that Dol-^{32}P was not recovered in the lipid extracts because amphomycin induced the enzymatic dephosphorylation of the polyisoprenylmonophosphate was eliminated by the finding that all of the labeled phospholipid was readily extracted after the "delipidated" residue was digested with Pronase. Further attempts to directly demonstrate the formation of an amphomycin-Dol-P (enzyme) complex are under way.

Discussion

The antibiotic amphomycin inhibits peptidoglycan synthesis in Gram-positive bacteria by preventing the transfer of phospho-*N*-acetylmuramyl pentapeptide to undecaprenyl monophosphate (Tanaka et al., 1977, 1979). This paper describes detailed enzymatic studies on the inhibitory effect of the antibiotic on the synthesis of Man-P-Dol, Glc-P-Dol, and GlcNAc-P-P-Dol by membrane preparations from central nervous tissue. The consequences of blocking the formation of the three monosaccharide derivatives of Dol-P on subsequent glycosylation reactions and the possible mechanism of action of the lipopeptide have been investigated.

In the calf brain in vitro system used here, amphomycin inhibits the transfer of mannose, glucose, and GlcNAc-1-P from their nucleotide derivatives to Dol-P. Similar glycosylation reactions were previously shown to be amphomycin sensitive in aorta (Kang et al., 1978a,b; Kang & Elbein, 1979) and plants (Ericson et al., 1978). The effect of the antibiotic in the calf brain system appears to be selective for glycosylation reactions involving polyisoprenyl phosphomonoesters as substrates because other mannosyl-, glucosyl-, and *N*-acetylglucosaminyltransferases were not directly affected. The mannosyltransferase catalyzing the addition of a β-mannose residue from GDP-mannose to exogenous $GlcNAc_2$-P-P-Dol and the enzymes transferring mannose from exogenous Man-P-Dol to $Man_5GlcNAc_2$-P-P-Dol were not sensitive to the presence of the lipopeptide. Furthermore, the transfer of glucose from UDP-glucose to ceramide and a glycogen-like glucan as well as the enzyme catalyzing the transfer of glucose from exogenous Glc-P-Dol to oligosaccharide-P-P-Dol was not affected by an amount of antibiotic that totally blocked the glucosylation of Dol-P. While the antibiotic inhibited the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P, it did not impede the addition of the second GlcNAc residue to GlcNAc-P-P-Dol or the direct transfer of GlcNAc from UDP-GlcNAc to peripheral, nonreducing termini of endogenous membrane glycoproteins.

Since the inhibitory effect of amphomycin appears to be selective for the glycosylation of Dol-P, it was used to learn more about the role of Man-P-Dol and Glc-P-Dol in the assembly of dolichol-linked oligosaccharide intermediates. We have found that under conditions that prevent the labeling of Man-P-Dol by GDP-[^{14}C]mannose, [^{14}C]mannose is incorporated into an endoglycosidase H resistant, dolichol-linked oligosaccharide that is indistinguishable from $Man_5GlcNAc_2$, a dolichol-linked oligosaccharide synthesized by class E *thy*-1$^-$ mutant mouse lymphoma cells (Chapman et al., 1979). The labeled mannose units in the heptasaccharide are quantitatively liberated by jack bean α-mannosidase. Thus, at least one and probably all four of the α-mannosyl residues in the heptasaccharide are donated by GDP-mannose. Other laboratories have reached the same conclusion on the basis of enzymatic studies with aorta microsomes (Kang et al., 1978b; Spencer & Elbein, 1980), rabbit liver preparations (Schutzbach et al., 1980), and class E *thy*-1$^-$ mutant mouse lymphoma cells (Chapman et al., 1980). Moreover, in the calf brain membrane system, when the block on Man-P-Dol formation is overcome by exogenous Dol-P, the dolichol-linked heptasaccharide is further mannosylated and becomes sensitive to endoglycosidase H (Figure 5). Consistent with these results, earlier studies with brain microsomes (Harford & Waechter, 1979a) have shown that α-mannose units are added from exogenous Man-P-Dol to an endoglycosidase H sensitive lipid-linked oligosaccharide that is chromatographically identical with $Man_{7-9}GlcNAc_2$. These results provide good evidence for the role of Man-P-Dol as the mannosyl donor in the elongation of $Man_5GlcNAc_2$-P-P-Dol to a $Man_9GlcNAc_2$-P-P-Dol, having the detailed structure proposed by Li et al. (1978). The incorporation of [^{14}C]glucose into oligosaccharide lipid was barely detectable when calf brain membranes were incubated with UDP-[^{14}C]glucose and an amount of amphomycin sufficient to block Glc-P-Dol biosynthesis. This result indicates that the first glucosyl residue, and possibly all three glucose units, are donated by Glc-P-Dol. Our results do not exclude the possibility that the outermost glucosyl residue is acquired directly from UDP-glucose. Recent work with rat liver microsomes (Staneloni et al., 1980) and thyroid microsomes (Murphy & Spiro, 1980) suggests that all three glucosyl residues in the dolichol-linked oligosaccharides are donated by Glc-P-Dol in those tissues.

Studies were also conducted in an effort to gain some insight into the possible mechanism by which the lipopeptide inhibits the glycosylation of polyisoprenyl monophosphates. Attempts to overcome the inhibitory effect on glycolipid formation by adding high concentrations of the sugar nucleotide substrates and divalent cation (Figure 4B) were unsuccessful. These results are in agreement with similar studies with a solubilized enzyme preparation from aorta (Kang et al., 1978b). However, in contrast to the report on the aorta system the synthesis of Man-P-Dol, Glc-P-Dol (Figure 4A), and GlcNAc-P-P-Dol by calf brain membranes was effectively restored by the addition of exogenous Dol-P. These enzymatic data would be expected if the lipopeptide binds to Dol-P at the reactive sites on the glycosyltransferases and the complex or the antibiotic could be displaced by exogenous acceptor lipid. It would be of interest to see if the inhibition of the bacterial phospho-*N*-acetylmuramyl pentapeptide translocase can be reversed by the addition of a high concentration of undecaprenyl monophosphate. Finding that Dol-P is rendered inextractable in the presence of Ca^{2+} and amphomycin (Table II) may be another clue that the lipopeptide binds directly to the acceptor lipid. The observation that amphomycin does not affect the extraction of the major membrane phospholipids or Dol-P after it is mannosylated indicates that the affinity of the antibiotic for the polyisoprenyl monophosphate is fairly selective. The formation of a complex between amphomycin and Dol-P in the presence of a divalent cation would be analogous to the mechanism of action of bacitracin (Stone & Strominger, 1971; Storm & Strominger, 1973) in preventing the conversion of undecaprenyl pyrophosphate to undecaprenyl monophosphate.

Further studies on the interaction of the lipopeptide antibiotic with the membrane-bound glycosyltransferases may yield new information regarding the enzymatic mechanism for the glycosylation of Dol-P. Since Dol-P remains in the "delipidated" membrane residue after extraction with $CHCl_3$–CH_3OH (2:1) when amphomycin and Ca^{2+} are present but is readily recovered when the membrane residue is digested proteolytically, the antibiotic may also provide a means of obtaining Dol-P-enriched extracts from membranous preparations from calf brain and other animal tissues.

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