Estrogen Influences Dolichyl Phosphate Distribution among Glycolipid Pools in Mouse Uteri†

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ABSTRACT: The steroid hormone 17β-estradiol dramatically induces uterine N-linked glycoprotein assembly [Dutt, A., Tang, J.-P., Welply, J. K., & Carson, D. D. (1986) *Endocrinology (Baltimore) 118*, 661-673]. To determine the role that dolichyl phosphate availability plays in this induction, we studied the effects of estrogen priming on the content of dolichyl phosphate and the distribution of dolichyl phosphate among various glycolipids in uteri. Dolichol-linked saccharides were metabolically labeled to equilibrium with either [^{3}H]glucosamine or [^{3}H]mannose and extracted from primary explants of uterine tissue. The amount of dolichol-linked saccharide was calculated from the specific radioactivity determined for the corresponding sugar nucleotides extracted from the tissues. The major dolichol-linked saccharides identified were mannosylphosphoryldolichol (MPD), oligosaccharylpyrophosphoryldolichol (OSL), and *N,N'*-diacetylchitobiosylpyrophosphoryldolichol (CBL). Estrogen increased the levels of MPD and OSL 4-fold; however, CBL levels did not change. After 3 days of treatment, the levels of these glycolipids were very similar to those in uteri from pregnant mice. Remarkably, MPD constituted 90-95% of dolichol-linked saccharides detected under all conditions. The tissue contents of total dolichyl phosphate and alkali-labile dolichyl phosphate, presumably MPD, were estimated by liquid chromatography. The levels of alkali-labile dolichyl phosphate determined in this way were in good agreement with the values estimated for MPD by metabolic labeling; moreover, alkali-labile dolichyl phosphate constituted 50-98% of the total dolichyl phosphate pool. The variations in MPD content depended upon the steroid hormone influence, most notably that of estrogen. Owing to our inability to metabolically label glucosylphosphoryldolichol (GPD) effectively, it was not possible to determine the tissue levels of GPD; however, conditions were optimized for the in vitro assay of GPD synthase in crude microsomal fractions. The specific activity of GPD synthase was similar under all conditions studied. Consequently, it was concluded that fluctuation in GPD synthase activity was an unlikely mode of regulation of oligosaccharide assembly or glycoprotein synthesis. These studies provide the first determination of the levels of dolichol-linked saccharides in tissues and how these levels change during hormonal induction of glycoprotein assembly. Coupled with our earlier studies, the present work demonstrates that among a number of key points of N-linked oligosaccharide assembly and transfer only synthesis of MPD increases coordinately with the increase observed in lipid- and protein-linked oligosaccharide assembly that occurs in vivo in response to estrogen. We suggest that control of MPD levels is an important regulatory aspect of N-linked glycoprotein assembly in this system.

Induction of glycoprotein biosynthesis seems to be required for a variety of differentiation events (Lennarz, 1983; Surani, 1979; Grabel & Martin, 1983). In order to understand how glycoprotein assembly is coordinated with differentiation, it is necessary to understand how the individual steps in the sequence of glycoprotein assembly are modulated. Indeed, studies in a number of systems have addressed how the activities of various enzymes (Lennarz, 1983; Lucas & Levin, 1977; Lucas, 1979; Dutt et al., 1986a; Burton et al., 1981), the levels of sugar nucleotides (Dutt et al., 1986a; Kaplan et al., 1984), the levels of mRNA coding for glycosylatable proteins (Lau & Lennarz, 1983), the levels of dolichyl phosphate (Lennarz, 1983; Lucas & Waechter, 1976), and the apparent rates of synthesis of dolichol-linked saccharides (Lennarz, 1983; Grant et al., 1985) change in response to induction of glycoprotein synthesis. In some systems (Lennarz, 1983), it appears that a number of these factors change coordinately. In many cases, the availability of dolichyl phosphate is a limiting factor in the rate of glycoprotein assembly (Lucas & Waechter, 1976; Carson et al., 1981). Consequently, it seems likely that dolichol-linked saccharide availability also is a limiting factor in this process; however, no quantitative estimates of the levels of these glycolipids are available.

Lucas and Levin (1977) have shown that estrogen treatment of chick oviduct is accompanied by an increased transfer of mannose from GDP-mannose to endogenous lipid acceptors; however, similar levels of mannosylphosphoryldolichol synthase activity were seen when exogenous dolichyl phosphate was added to these assays (Lucas & Levin, 1977). On the bases of these observations, it was suggested that increased dolichyl phosphate availability accounted for the increase in glycosyltransferase activity observed in response to estrogen treatment. Consistent with this proposal was the later observation that dolichol kinase activity was stimulated in response to estrogen treatment of chick oviducts (Burton et al., 1981). Furthermore, it has been shown that supplementation of hen oviduct tissues with dolichyl phosphate can stimulate N-linked glycoprotein biosynthesis in vivo (Carson et al., 1981). It is of interest to know if glycoprotein assembly in mammals is regulated the way it is in hen oviduct, and so we have been

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investigating these processes in a corresponding mammalian tissue, the uterus. Recently, we have found that estrogen is a potent inducer of glycoconjugate biosynthesis in mouse uteri (Dutt et al., 1986a,b). In the uterus, this induction is not accompanied by changes in either sugar nucleotide levels or the activities of certain enzymes involved in dolichol-linked oligosaccharide assembly and transfer; however, in contrast to the situation in chick oviduct, estrogen treatment results in a large increase in the in vitro activity of mannosylphosphoryldolichol synthase (Dutt et al., 1986a). In this case, the increase in in vitro activity cannot be explained by increased dolichyl phosphate availability. In addition, dolichyl phosphate supplementation does not stimulate uterine glycoprotein synthesis in tissue slice experiments. In this report, we show that the levels not only of total dolichyl phosphate but also of dolichol-linked saccharides change in response to estrogen treatment. This provides the first quantitative determination of dolichol-linked saccharide levels in tissues. The data indicate that uteri contain very high levels of dolichyl phosphate and dolichol-linked saccharides. Moreover, the observations are consistent with the notion that changes in the rate of mannosylphosphoryldolichol synthesis may be an important factor regulating dolichol-linked oligosaccharide assembly in this system.

Materials and Methods

Materials. CF1 mice were purchased from TIMCo (Houston, TX). Solvents used for liquid chromatography were purchased from Bodman Chemicals, Inc. (Media, PA). Dolichyl phosphate, dolichol-11, 17β-estradiol, and tunicamycin were obtained from Sigma (St. Louis, MO). Tissue culture media were purchased from Irvine Scientific (Santa Ana, CA). DE52 was obtained from Whatman (Clifton, NJ), and silica gel 60 thin-layer chromatography plates were from E. Merck (Darmstadt, FRG). Phosphorous oxychloride was obtained from Eastman-Kodak (Rochester, NY). Dolichyl-11-phosphate was synthesized as described by Keller et al. (1985). [2-^{3}H]Mannose (20 Ci/mmol) and [6-^{3}H]glucosamine (25 Ci/mmol) were obtained from ICN Pharmaceuticals (Irvine, CA). UDP-[6-^{3}H]glucose (4.7 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). All chemicals used were reagent grade.

Metabolic Labeling of Tissues and Glycolipid Extractions. Primary explants of uterine tissues were prepared and were metabolically labeled with [^{3}H]mannose or [^{3}H]glucosamine exactly as described (Dutt et al., 1986a,b). For extractions of ^{3}H-labeled glycolipids, uterine tissue from one animal was rinsed 3 times with 5 mL of ice-cold phosphate-buffered saline (PBS)[1] and homogenized in 1 mL of PBS containing 1 mM sodium pyrophosphate. An aliquot was removed for protein determination, and the remaining homogenate was quick-frozen in a dry ice/acetone bath and lyophilized. The dried residue was rehydrated for 10–20 min in 0.25 mL of water and then sequentially extracted with chloroform/methanol (2:1 v/v), water, and then chloroform/methanol/water (10:10:3) v/v) as described by Lucas et al. (1976). [^{3}H]Mannose-labeled material in chloroform/methanol (2:1) extracts was further analyzed routinely by thin-layer chromatography on silica gel 60 using chloroform/methanol/water (65:25:4 v/v) as the developing solvent. Authentic dolichyl phosphate was run in a parallel lane as a standard. Typically, mannosylphosphoryldolichol represented 25–35% of the [^{3}H]glycolipids present in these extracts. When [^{3}H]mannosylphosphoryldolichol generated by uterine microsomes as described (Dutt et al., 1986a) was added to tissues and carried through parallel extractions, recoveries exceeded 90%. [^{3}H]Mannose-labeled material in the chloroform/methanol/water (10:10:3 v/v) extracts was routinely analyzed as the oligosaccharide released by mild acid hydrolysis (Lucas et al., 1976) by chromatography over 1 cm × 110 cm columns of Sephadex G-50 (fine) developed with 0.1 M ammonium formate/5% (v/v) ethanol/0.02% (w/v) sodium azide. The elution position of authentic $Glc_{1-3}Man_9GlcNAc_2$ oligosaccharide was determined by using a standard generated by hen oviduct (Carson et al., 1981). Recovery of radioactivity in the aqueous phase following acid hydrolyses ranged from 80% to 90%. Typically, large oligosaccharides constituted 50–75% of these extracts. When [^{3}H]oligosaccharide lipid generated in hen oviduct was added to tissues and carried through parallel extractions, acid hydrolyses, and column chromatographic procedures, recoveries ranged from 70% to 90%. When tissues were metabolically labeled with [^{3}H]mannose in the presence of tunicamycin (1 μg/mL), incorporation into the chloroform/methanol/water (10:10:3 v/v) extracts was inhibited by 60%, whereas incorporation into the chloroform/methanol (2:1 v/v) extracts was unaffected. Higher concentrations of tunicamycin, e.g., 5 μg/mL, inhibited [^{3}H]mannose incorporation into the chloroform/methanol/water (10:10:3 v/v) extract by 90% or greater; however, at these concentrations, secondary effects on protein synthesis were more severe.

[^{3}H]Glucosamine-labeled glycolipids in chloroform/methanol (2:1 v/v) extracts were routinely analyzed by ion-exchange chromatography using Whatman DE52 resin equilibrated in chloroform/methanol (2:1 v/v). ^{3}H-Labeled extracts were applied to approximately 1 mL of resin in chloroform/methanol (2:1 v/v), and the resin was eluted with an additional 10 mL of chloroform/methanol (2:1 v/v). The resin was then eluted with 10 mL of chloroform/methanol (2:1 v/v) containing 30 mM ammonium acetate. The dolichyl pyrophosphate linked oligosaccharides were then eluted with 10 mL of chloroform/methanol (2:1 v/v) containing 100 mM ammonium acetate (Parodi & Leloir, 1979; Hanover & Lennarz, 1979). The ^{3}H that eluted with 100 mM ammonium formate typically constituted 10–20% of the total ^{3}H in the chloroform/methanol extract. When [^{3}H]chitobiosyl lipid generated in hen oviduct microsomes (Carson et al., 1981) was added to tissues and carried through similar extraction and chromatographic procedure, recoveries ranged from 30% to 50%. In order to correct for these procedural losses, values for material recovered in the 100 mM ammonium acetate fraction were multiplied by 2.5. [^{3}H]Glucosamine incorporation into the 100 mM ammonium acetate eluate was inhibited by 70% when tissues were incubated in the presence of 1 μg/mL tunicamycin, and by more than 90% by 5 μg/mL tunicamycin, whereas incorporation into the DEAE run through and 30 mM ammonium acetate eluates was unaffected by the drug. The ^{3}H in the 100 mM ammonium acetate eluate was quantitatively converted into a water-soluble form by mild acid hydrolysis (Lucas et al., 1976; Parodi & Leloir, 1979), and the acid-released material displayed the paper chromatographic characteristics (Carson et al., 1981; Lucas et al., 1976) of *N*-acetylglucosamine (10%) and *N,N'*-diacetylchitobiose (90%). [^{3}H]Glucosamine-labeled lipids in the chloroform/methanol/water (10:10:3 v/v) phase were ana-

[1] Abbreviations: MPD, mannosylphosphoryldolichol; OSL, oligosaccharylpyrophosphoryldolichol; CBL, *N,N'*-diacetylchitobiosylpyrophosphoryldolichol; GPD, glucosylphosphoryldolichol; PBS, phosphate-buffered saline; Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine; DEAE, diethylaminoethyl; GlcN, glucosamine; E_2, 17β-estradiol; dol-P, dolichyl phosphate; dol-11-P, dolichyl-11-phosphate; Tris, tris(hydroxymethyl)aminomethane.

lyzed by ion-exchange chromatography as described above. In this case, 2–10% of the ^{3}H-labeled lipids eluted with 100 mM ammonium acetate. When [^{3}H]oligosaccharide lipid generated in hen oviduct was added to tissues and carried through parallel extractions and ion-exchange chromatographic steps, recoveries in the 100 mM ammonium acetate fraction ranged from 30% to 50%. In order to correct for these procedural losses, value for material recovered in the 100 mM ammonium acetate fraction were multiplied by 2.5. Synthesis of ^{3}H-labeled lipids in the 100 mM ammonium acetate eluate was inhibited by 70% by 1 μg/mL tunicamycin and by more than 90% by 5 μg/mL tunicamycin. In addition, most (80–90%) of the radioactivity eluted on Bio-Gel P-4 (−400 mesh) chromatography at a similar position to $Glc_{1-3}Man_9GlcNAc_2$ standard derived from hen oviduct.

Direct Determination of Tissue Content of Dolichyl Phosphate. Uteri from 5–10 mice were pooled and homogenized in 1 mL of water with a Polytron tissue homogenizer (Luzern, Switzerland) at setting 7 for 30 s. An aliquot was removed for protein determination, and 2–10 μg of dolichyl-11-phosphate was added prior to extraction or any hydrolysis steps as an internal standard to permit determination of procedural losses. To determine total tissue dolichyl phosphate content, a portion of the homogenate was incubated in tetrahydrofuran/0.5 N hydrochloric acid (4:1 v/v) at 50 °C for 90 min to release saccharides linked to dolichyl phosphate and dolichyl pyrophosphate (Carson et al., 1981; Lucas et al., 1976). The mixture then was neutralized with sodium hydroxide, and the tetrahydrofuran was evaporated under nitrogen. The solution then was brought to 50% (v/v) ethanol and 2 M potassium hydroxide and incubated in a boiling water bath for 90 min. These conditions have been shown to quantitatively convert dolichol-linked saccharides and dolichyl pyrophosphate to dolichyl phosphate (Keller et al., 1985). The nonsaponifiable lipid fraction containing dolichyl phosphate was extracted from the hydrolysate twice with 5 volumes of petroleum ether (Carson & Lennarz, 1981). The dolichyl phosphate fraction was selected from these extracts by ion-exchange chromatography as described by Keller et al. (1985). Dolichyl phosphate in the mannosylphosphoryldolichol fraction was converted to dolichol by performing strong alkaline hydrolysis on the homogenates without the acid pretreatment step (Parodi & Leloir, 1979). The portion of the total dolichyl phosphate in the mannosylphosphoryldolichol fraction was determined as the difference between the value obtained for total dolichyl phosphate content and the value obtained for dolichyl phosphate content in extracts not pretreated with acid.

To determine the dolichyl phosphate content in chloroform/methanol (2:1 v/v) extracts and chloroform/methanol/water (10:10:3 v/v) extracts, homogenates were first lyophilized, rehydrated with 0.5 mL of water, and then sequentially extracted as described above for ^{3}H-labeled lipids. These extracts were dried under nitrogen, incubated with tetrahydrofuran/0.5 N hydrochloric acid (4:1 v/v), subjected to strong alkaline hydrolysis, and extracted with ether as described above.

The dolichyl phosphate content of the final ether extracts in each case was determined by liquid chromatography as described by Keller et al. (1985). The sample (50–250 μL) was resuspended in the mobile phase and injected onto a 4.6 mm inside diameter × 25 cm Hypersil (5 μm) column (Rainin Instruments, Woburn, MA) equilibrated with a mobile phase of hexane/2-propanol/1.4 M phosphoric acid (954:46:06 v/v). The system was pumped at room temperature at a flow rate of 1 mL/min with back pressure of 560 psi. Dolichyl phosphate eluted at 7.8–8.5 min while dolichyl-11-phosphate eluted at 11.5–12.0 min. The liquid chromatographic apparatus consisted of two Beckman 100A pumps interfaced with a Beckman 421 controller. Elution profiles were monitored at 210 nm with a Hitachi Model 100-40 flow-through spectrophotometer and were recorded and integrated on an Altex C-R1A integrator. All these instruments were obtained from Beckman Instruments (Irvine, CA). The chemical amounts of dolichyl phosphate in each sample were calculated by comparison of the absorbance value obtained for the integrated dolichyl phosphate peaks with that of known amounts of standard pig liver dolichyl phosphate.

Glucosylphosphoryldolichol Synthase Assay. Crude uterine microsomes were prepared from 5–10 mice as described (Dutt et al., 1986a). Glucosylphosphoryldolichol synthase activity was assayed by using 20–500 μg of membrane protein in a 100-μL volume containing 0.4% (v/v) Triton X-100, 100 mM Tris–acetate, pH 7.0, 4 mM CDP-choline, 20 μM UDP-glucose, 10 mM $MgCl_2$, 1 μCi of UDP-[^{3}H]glucose, 2 mM dithiothreitol, and 5 μg of dolichyl phosphate. Incubations were performed for 1–30 min at 37 °C and stopped by the addition of 2 mL of chloroform/methanol (2:1 v/v). ^{3}H-Labeled lipids were extracted into the chloroform phase following the addition of 0.4 mL of 0.9% (w/v) sodium chloride. The aqueous layer was removed, and the organic layer was washed twice with 1.1 mL of methanol/water (1:1 v/v). A 0.5-mL sample of the chloroform layer was transferred to a scintillation vial and was air-dried. Radioactivity was determined by liquid scintillation spectrometry.

Where indicated, the divalent cation included, the concentration of UDP-glucose, the amount of dolichyl phosphate added, the detergent concentration, or the pH of the buffer was varied so that the optimal conditions for performing the assay could be determined. The chloroform-soluble reaction products were analyzed by ion-exchange chromatography on DE52 (Carson & Lennarz, 1981). The products eluting from the resin with 25 mM sodium acetate were further analyzed by thin-layer chromatography on silica gel 60 developed against either chloroform/methanol/water (65:25:4 v/v) or chloroform/2-propanol/95% ethanol/acetic acid (2:2:3:1 v/v) using authentic dolichyl phosphate as standard. One ^{3}H-labeled product with the chromatographic characteristics of glucosylphosphoryldolichol (Behrens & Leloir, 1970; Scher et al., 1977) was obtained in all instances. The ^{3}H-labeled, chloroform-soluble material also was subjected to mild acid hydrolysis as described above or strong base hydrolysis (2 M potassium hydroxide in 50% ethanol at 100 °C for 90 min). The released water-soluble products in both cases were then subjected to ion-exchange chromatography on DE52. In both cases, greater than 98% of the radioactivity eluted from the resin with water. These properties were consistent with the reported properties of the corresponding degradation products of glucosylphosphoryldolichol (Behrens & Leloir, 1970; Scher et al., 1977).

Other Procedures. Protein content was estimated by the procedure of Lowry et al. (1951) using bovine serum albumin as standard.

Results

Estimation of Dolichol-Linked Saccharide Levels by Metabolic Labeling to Equilibrium. As an initial approach in determining the levels of dolichol-linked saccharides in uterine tissues, we metabolically labeled tissue slices to equilibrium with either [^{3}H]mannose or [^{3}H]glucosamine. Preliminary experiments indicated that within 4 h incorporation into the dolichol-linked saccharide fraction had reached

maximal levels, i.e., had reached metabolic equilibrium; however, labeling was routinely conducted for 6 h to ensure complete labeling of glycolipid pools. A conventional scheme was used for the extraction of dolichol-linked saccharides (Lucas et al., 1976) with the modification that homogenates were quick-frozen, lyophilized, and briefly rehydrated prior to organic extraction. This procedure was employed to reduce the extraction volumes and because we found that omission of the lyophilization step resulted in lower, less reproducible yields of glycolipids (data not shown). The extracts were routinely analyzed by a combination of ion-exchange and thin-layer chromatographic methods. These procedures were absolutely essential because in some cases the bulk (75–95%) of the radioactivity in these extracts was in material other than dolichol-linked saccharides. [^{3}H]Glucosamine-labeled lipids in the chloroform/methanol (2:1) extracts were selected by ion-exchange chromatography, and the saccharides were released by mild acid hydrolysis (Lucas et al., 1976; Parodi & Leloir, 1979; Hanover & Lennarz, 1979). Paper chromatography of the acid-released products indicated that approximately 90% of this material comigrated with authentic chitobiose under all conditions studied (data not shown). Experiments in which tissue slices were incubated with tunicamycin (1.0 μg/mL) indicated that this agent inhibited the synthesis of 60–80% of the fractions designated as oligosaccharylpyrophosphoryldolichol or chitobiosylpyrophosphoryldolichol; however, the synthesis of material designated as mannosylphosphoryldolichol was unaffected by tunicamycin. Higher concentrations of tunicamycin, e.g., 5 μg/mL, inhibited synthesis of the putative dolichol-linked saccharides by 90% or more although secondary effects on protein synthesis were apparent. Analyses of selected samples by chromatography on Bio-Gel P-4 indicated that the bulk (85–90%) of the oligosaccharides derived from [^{3}H]-mannose-labeled or [^{3}H]glucosamine-labeled oligosaccharylpyrophosphoryldolichol displayed a molecular weight similar to that observed for the mature dolichol-linked oligosaccharide derived from hen oviduct (data not shown). These oligosaccharides appeared to be glucosylated because digestion with α-mannosidase converted the bulk of this material to components with molecular weights intermediate to $Glc_{1-3}Man_9GlcNAc_2$ and $Man_5GlcNAc_2$ standards.

The amount of glycolipid in each fraction was calculated on the basis of previous measurements of the specific activity of sugar nucleotides extracted from tissues labeled under similar conditions (Dutt et al., 1986a,b). For these calculations, it was assumed that [^{3}H]mannose-labeled oligosaccharylpyrophosphoryldolichol contained a full complement of nine mannose residues. Our routine analyses by Sephadex G-50 chromatography would not have discriminated between oligosaccharides containing six to nine mannose residues. Therefore, the calculated estimates may be low in some cases although analyses of selected samples by Bio-Gel P-4 chromatography did not evidence any size differences among oligosaccharides derived from animals receiving 0 or 2 days of E_2 estrogen treatment (see above).

According to this method, the content of various dolichol-linked saccharides in uteri of mice as a function of the duration of estrogen treatment is presented in Figure 1. In all cases, mannosylphosphoryldolichol was the predominant dolichol-linked saccharide, constituting 90–95% of the total. The tissue content of mannosylphosphoryldolichol increased from 0.5 to 2.0 nmol/mg of tissue protein following estrogen treatment. In other experiments, the values obtained for mannosylphosphoryldolichol increased from approximately 1.0 to 3.0 nmol/mg of tissue during estrogen treatment.

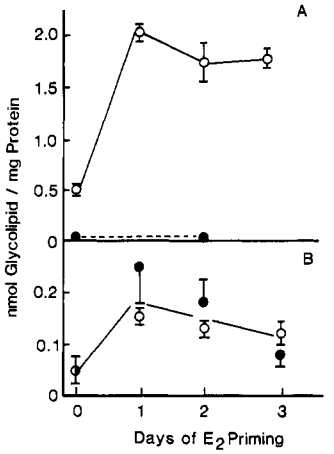

FIGURE 1: Levels of dolichol-linked saccharides in uteri during estrogen treatment. Injection schemes, metabolic labeling conditions, and extraction and analyses of dolichol-linked saccharides were as described under Materials and Methods. Open symbols represent results obtained from experiments using [^{3}H]mannose as a precursor, and closed symbols represent experiments using [^{3}H]glucosamine as a precursor. Panel A: (○) mannosylphosphoryldolichol; (●) chitobiosylpyrophosphoryldolichol. Panel B: oligosaccharylpyrophosphoryldolichol. E_2, 17β-estradiol. The values presented are the averages and ranges of duplicate determinations performed on uterine explants derived from two mice in a representative experiment.

Table I: Dolichol-Linked Saccharide Levels[a] in Uteri of Pregnant Mice[b]

dolichol-linked saccharide	nmol of glycolipid/mg of protein	dolichol-linked saccharide	nmol of glycolipid/mg of protein
$MPD_{(Man)}$	1.99 ± 0.08	$CBL_{(GlcN)}$	0.028 ± 0.003
$OSL_{(Man)}$	0.055 ± 0.005	$OSL_{(GlcN)}$	0.062 ± 0.010

[a] Dolichol-linked saccharides were metabolically labeled to equilibrium for 6 h in uterine tissue slices with either [^{3}H]glucosamine or [^{3}H]mannose and then were sequentially extracted from the tissue as described under Materials and Methods. The values presented reflect the averages and variation of results obtained from duplicate determinations performed on slices obtained from separate mice from at least two individual experiments. Nanomoles of glycolipid were calculated on the basis of specific activities determined for the sugar nucleotide precursors GDP-mannose (Dutt et al., 1986a) or UDP-*N*-acetylhexosamine (5.61×10^5 cpm/nmol; see text) extracted from the tissues under these conditions. Abbreviations: MPD, mannosylphosphoryldolichol; OSL, oligosaccharylpyrophosphoryldolichol; CBL, *N*,*N*′-diacetylchitobiosylpyrophosphoryldolichol; Man, mannose; GlcN, glucosamine. The abbreviations in the parentheses next to each glycolipid abbreviation indicate which radioactive sugar was used as a precursor in the determination. [b] Uterine slices were prepared from uteri obtained from mice 4 days after mating as described (Dutt et al., 1986a). Pregnancy was verified in these animals by observing the presence of blastocyst-stage embryos in the uterine flushings.

In general, the values obtained for oligosaccharylpyrophosphoryldolichol lipid levels using [^{3}H]mannose as a precursor were in good agreement with those estimated by using [^{3}H]glucosamine (Figure 1B and Table I). Both precursors indicated that oligosaccharylpyrophosphoryldolichol levels were 10–20-fold lower than mannosylphosphoryldolichol. The levels of oligosaccharylpyrophosphoryldolichol increased from approximately 30 to 120 pmol/mg of tissue protein in response to estrogen.

Estrogen treatment resulted in approximately a 4-fold increase in the tissue levels of both mannosylphosphoryldolichol and oligosaccharylpyrophosphoryldolichol; however, chitobiosylpyrophosphoryldolichol levels remained low in all cases and ranged between 30 and 75 pmol/mg of tissue protein. These values were not influenced by estrogen treatment. After

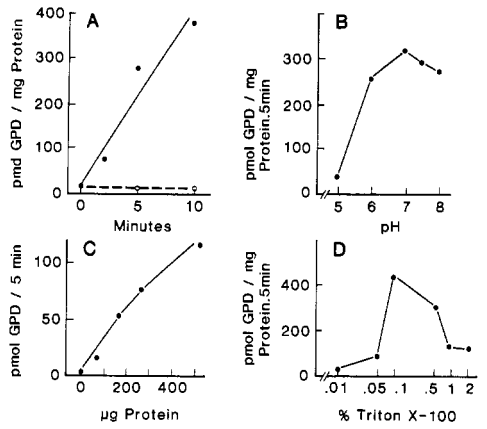

FIGURE 2: Optimization of glucosylphosphoryldolichol synthase assay. A crude microsomal fraction was prepared as described previously (Dutt et al., 1986a) and used for all assays. The assay mixture routinely contained 0.4% (v/v) Triton X-100, 100 mM Tris–acetate, pH 7.0, 4 mM CDP-choline, 20 μM UDP-glucose, 10 mM $MgCl_2$, 1 μCi of UDP-[^{3}H]glucose, 2 mM dithiothreitol, and 5 μg of dolichyl phosphate with 200–300 μg of membrane protein in a final volume of 100 μL. Assays routinely were performed for 5 min at 37 °C and stopped by the addition of 2 mL of chloroform/methanol (2:1 v/v) and further processed as described under Materials and Methods. Assay conditions were varied from the routine conditions to determine optima as described in each panel. (A) Time dependence in the presence (●) or absence (○) of exogenous dolichyl phosphate; (B) pH dependence; 50 mM Tris-HCl buffer was used at pH 7.4 and 8.0, and 50 mM Tris–acetate buffer was used at pH 5.0, 6.0, and 7.0; (C) protein dependence; and (D) Triton X-100 dependence; percent was calculated on a volume to volume basis. The amount of crude microsomal protein added to these assays was 250 μg.

2–3 days of estrogen treatment, the values obtained for the tissue levels of mannosylphosphoryldolichol and chitobiosylpyrophosphoryldolichol approximated the levels determined in uteri of pregnant mice (Table I). Oligosaccharylpyrophosphoryldolichol levels were higher in the estrogen-treated mice than in the pregnant mice; however, levels of oligosaccharylpyrophosphoryldolichol appeared to gradually decrease after the initial stage of estrogen treatment and approached the levels observed in pregnant mice by day 3 of estrogen treatment. The similarity in the levels of dolichol-linked saccharides determined in these cases indicates that the ovariectomized model system reproduces important aspects of the pathway of dolichol-linked oligosaccharide assembly seen in a normal, i.e., nonovariectomized, reproductive cycle. It therefore appears that even though a normal cycling animal would be under the influence of both progesterone and estrogen (Psychoyos & Casimiri, 1980), estrogen alone is capable of supporting these aspects of oligosaccharide assembly.

Assay of Glucosylphosphoryldolichol Synthesis. To complete the analysis of dolichyl phosphate distribution among glycolipid pools in vivo, it would have been desirable to perform similar types of metabolic labeling analyses for the determination of glucosylphosphoryldolichol levels. Experiments were performed in which tissue slices were metabolically labeled with high amounts, i.e., 1 mCi/mL, of [^{3}H]glucose or [^{3}H]-galactose. In all cases, very poor labeling of the tissues was achieved, even when the glucose concentration of the medium was lowered 10-fold (data not shown). As a consequence, we could not confidently determine the levels of glucosylphosphoryldolichol in tissue slice experiments. As an alternative, we optimized conditions for the in vitro assay of glucosylphosphoryldolichol synthase so that we could determine if this activity was influenced by estrogen treatment. An activity converting ^{3}H from UDP-[^{3}H]glucose to a chloroform-soluble product was found in a crude microsomal fraction

Table II: Glucosylphosphoryldolichol Synthase Activity in Uteri of Ovariectomized, Estrogen-Treated, and Pregnant Mice

condition[a]	pmol of GPD[b] formed (mg of protein)$^{-1}$ (5 min)$^{-1}$	
	plus dolichyl phosphate	minus dolichyl phosphate
ovariectomized		
no hormone	518 ± 11	14 ± 3
plus E_2 (1 day)	456 ± 24	ND[c]
plus E_2 (2 days)	497 ± 47	15 ± 3
plus E_2 (3 days)	642 ± 36	ND[c]
pregnant	360 ± 50	11 ± 4

[a] Mice were ovariectomized and injected with 0.1 μg of 17β-estradiol as described (Dutt et al., 1986a). Pregnant mice were obtained 4 days after copulation as described (Dutt et al., 1986a). Pregnancy was confirmed by observing the presence of blastocyst-stage embryos in the uterine flushings. [b] Crude microsomal fractions were prepared and glucosylphosphoryldolichol synthase was assayed as described under Materials and Methods. In the assays referred to as "plus dolichyl phosphate", 5 μg of dolichyl phosphate was added to each 100-μL incubation. The assays referred to as "minus dolichyl phosphate" did not receive any dolichyl phosphate addition. Abbreviations: E_2, 17β-estradiol; GPD, glucosylphosphoryldolichol. [c] Not determined.

prepared from uteri. Boiling reduced this activity by 95–98%. This activity was enriched 5–10-fold in the crude microsomal fraction, i.e., 100000*g* pellet, over either a 6000*g* pellet or the 100000*g* supernatant fractions that constituted the remainder of the uterine homogenates (data not shown). Less than 5% of maximal activity was observed if divalent cation was omitted from the incubation. Mg^{2+} was preferred over Mn^{2+}; in fact, Mn^{2+} addition inhibited activity in the presence of Mg^{2+} (data not shown). As shown in Figure 2, this activity was dependent on time and the addition of dolichyl phosphate to the assay (panel A), exhibited a pH optimum around pH 7 (panel B), was linearly dependent on the amount of protein added to the assay (panel C), and exhibited optimal activity with a Triton X-100 to protein ratio of 0.5–2.5 (v/w) (panel D). The $K_{m_{app}}$ values determined for dolichyl phosphate and UDP-glucose in this reaction were 5 μM and 10 μM, respectively (data not shown). The activity observed exceeded the values reported for glucosylphosphoryldolichol synthase in other systems (Clark et al., 1983; Welply et al., 1985) by 10–80-fold (Table II). Consequently, it was critical to verify that the assay measured glucosylphosphoryldolichol synthesis and not the synthesis of other chloroform-soluble compounds, e.g., glucosylceramide. The products of this reaction quantitatively bound and eluted from DEAE-cellulose with 25 mM ammonium acetate (Figure 3, panel A). This material migrated as a single peak with the chromatographic characteristics of glucosylphosphoryldolichol (Behrens & Leloir, 1970) upon thin-layer chromatography (Figure 3, panels B and C). Acid and base hydrolyses of this material released a water-soluble product that did not bind to DEAE-cellulose at neutral pH (data not shown). From these observations, it was concluded that the predominant product formed in the reaction was glucosylphosphoryldolichol. Therefore, these assay conditions were used to study the relative activities of glucosylphosphoryldolichol synthase in uterine membrane preparations.

These studies are summarized in Table II. It did not appear that uterine glucosylphosphoryldolichol synthase activity was substantially altered by estrogen treatment of ovariectomized mice. Microsomal fractions derived from ovariectomized mice receiving no hormone or 3 days of estrogen treatment were mixed in some experiments. The results obtained were equivalent to the sum of the expected individual activities (data not shown). Consequently, it did not appear that any diffusible

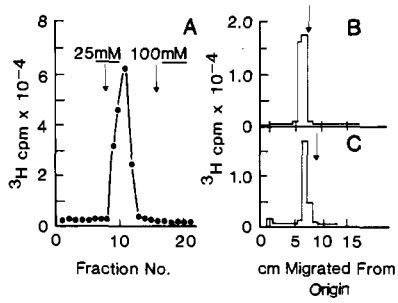

FIGURE 3: Characterization of products of glucosylphosphoryldolichol synthase assay. (A) Chloroform-soluble material generated by the glucosylphosphoryldolichol synthase assay was applied to a 2-mL column of DE52 that had been equilibrated with chloroform/methanol (2:1 v/v). The column was sequentially eluted with the 5 mL each of the same solvent containing 0, 25, or 100 mM ammonium acetate as indicated by the arrows. The peak of radioactivity obtained from the ion-exchange column was twice back-washed with methanol/water (1:1 v/v) to remove salt and was dried down under nitrogen, and the residue was spotted on thin-layer chromatography plates (silica gel 60, E. Merck, Darmstadt, FRG) along with 10 μg of a dolichyl phosphate standard. The plates were developed against (panel B) chloroform/methanol/water (65:25:4 v/v) or (panel C) chloroform/2-propanol/95% ethanol/acetic acid (2:2:3:1 v/v). The migration positions of the dolichyl phosphate standard are indicated by the arrows in panels B and C.

activators or inhibitors of glucosylphosphoryldolichol synthase were present in these preparations. This activity was about 30% lower in uterine membrane preparations derived from pregnant mice. Collectively, these results indicated that glucosylphosphoryldolichol synthase activity was fairly constant in uteri and seemed to be an unlikely point of regulation of oligosaccharide assembly. Only 2–3% of the activity observed in the presence of exogenous dolichyl phosphate was detected when dolichyl phosphate addition was omitted under each condition (Table II). These observations indicated that very little unglycosylated dolichyl phosphate was available in the membrane preparations under any conditions.

Chemical Determination of Total and Alkali-Labile Dolichyl Phosphate Levels. The dolichyl phosphate levels we estimated using the metabolic labeling procedure were much higher than those reported for total dolichyl phosphate in a variety of other tissues (Keller et al., 1985). Consequently, it was important to measure the dolichyl phosphate content of tissues independently to support the conclusions of the metabolic labeling studies. We utilized a liquid chromatographic analysis reported by Keller et al. (1985) to measure the dolichyl phosphate content of various uterine extracts directly. As described by these workers, we added a known amount of chemically synthesized dolichyl-11-phosphate at the outset of our extraction procedures. Dolichyl-11-phosphate is well separated from the tissue forms of dolichyl phosphate (see Figure 4) and so it allowed us to quantitate recoveries of dolichyl phosphate under all conditions. Recoveries ranged between 25% and 95% but in most cases were about 50%. In addition to determining the levels of total dolichyl phosphate, we determined the levels of dolichyl phosphate in alkali-labile forms, presumably mannosylphosphoryldolichol. Since base hydrolysis of mannosylphosphoryldolichol quantitatively releases mannosyl phosphate (Parodi & Leloir, 1979), the other product should be free dolichol. All other forms of dolichol-linked saccharides appear to be converted to the base-stable lipid dolichyl phosphate (Keller et al., 1985). Thus, direct saponification of tissues prior to organic extraction would convert any dolichyl phosphate in the form of mannosylphosphoryldolichol into free dolichol; however, if the tissue homogenates were preincubated with mild acid, a treatment

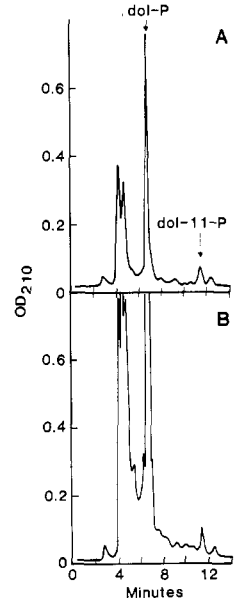

FIGURE 4: Liquid chromatography of dolichyl phosphate. Extracts were prepared from uteri as described under Materials and Methods. Portions of these extracts were applied to a 4.6 mm × 25 cm Hypersil (5 μm) column (Rainin Instruments, Woburn, MA) and eluted at 1 mL/min at ambient temperature with hexane/2-propanol/1.4 M phosphoric acid (954:46:0.6 v/v). A more polar solvent system than that used by Keller et al. (1985) was used to provide better separation between tissue-derived dolichyl phosphate (dol-P) and the dolichyl-11-phosphate (dol-11-P) internal standard. The elution profile was monitored at 210 nm. (A) Elution profile of tissue extracts not treated with mild acid prior to strong alkaline hydrolysis and extraction (the elution positions of tissue-derived dolichyl phosphate and the internal dolichyl-11-phosphate standard are indicated by the arrows); (B) elution profile of an identical fraction of tissue extract as described in panel A except that this fraction was acid treated prior to alkaline hydrolysis and extraction. Note that in both cases a similar amount of the dolichyl-11-phosphate standard is recovered although there is a large difference in the amount of tissue-derived dolichyl phosphate.

that converts mannosylphosphoryldolichol to mannose and dolichyl phosphate (Parodi & Leloir, 1979), and then saponified, all forms of dolichyl phosphate should be preserved. Consequently, we considered that values for total dolichyl phosphate could be best obtained by incubating extracts with mild acid prior to saponification and organic extraction. The values obtained when the acid pretreatment was omitted should reflect forms of dolichyl phosphate other than mannosylphosphoryldolichol, i.e., alkali-stable forms of dolichyl phosphate. The difference between the values obtained for total dolichyl phosphate and alkali-stable dolichyl phosphate should equal dolichyl phosphate in the form of mannosylphosphoryldolichol.

Routinely, uterine homogenates were divided, and half was pretreated with mild acid. Both halves were then subjected to strong alkaline hydrolysis and were extracted with ether. These extracts were further selected for dolichyl phosphate by ion-exchange chromatography as described (Keller et al., 1985) and analyzed by liquid chromatography. The results of such an analysis are presented in Figure 4. Dolichyl phosphate was well resolved from the dolichyl-11-phosphate internal standard as were a number of less polar, unidentified compounds. Acid pretreatment of these extracts (panel B) resulted in the recovery of substantially more dolichyl phosphate than when this step was omitted (panel A). These analyses were applied to uteri from a variety of conditions and the tissue levels of dolichyl phosphate derivatives quantitated. As shown in Table III, results were very reproducible. Moreover, the estimates we obtained for the tissue content of

Table III: Levels of Dolichyl Phosphate[a] in Mouse Uteri under Various Conditions

condition[b]	total nmol of dol-P/mg of protein	nmol of alkali-stable dol-P/mg of protein	nmol of alkali-labile dol-P/mg of protein
ovariectomized			
no hormone	2.17 ± 0.04	1.01 ± 0.02	1.16 (0.53)[c]
2 days E_2	3.83 ± 0.08	1.11 ± 0.03	2.71 (0.71)
pregnant	2.15 ± 0.05	0.07 ± 0.03	2.08 (0.97)

[a] Alkali-stable and total dolichyl phosphate was extracted and analyzed by liquid chromatography as described under Materials and Methods. Recoveries of internal standard dolichyl-11-phosphate (Keller et al., 1985) ranged from 25% to 95% in these experiments. The values presented reflect the averages and ranges of values obtained for duplicate determinations performed on extracts from two groups of four to six individual mice in each case. Abbreviations: dol-P, dolichyl phosphate; E_2, 17β-estradiol. [b] Mice were ovariectomized and injected with 0.1 μg of 17β-estradiol as described (Dutt et al., 1986a). Pregnant mice were obtained 4 days after copulation as described (Dutt et al., 1986a). Pregnancy was verified by observing the presence of blastocyst-stage embryos in the uterine flushings. [c] Values in parentheses indicate fraction of total dolichyl phosphate pool in the form of alkali-labile dolichyl phosphate, presumably mannosylphosphoryldolichol.

mannosylphosphoryldolichol (1–3 nmol/mg of protein) were in good agreement with the values we obtained using the metabolic labeling approach. The identity of this material as dolichyl phosphate was confirmed further by reverse-phase liquid chromatography (Adair & Keller, 1985) of extracts derived from uteri of pregnant mice. A characteristic family of five isoprenologue species was obtained that eluted at the same positions as the isoprenologues derived from standard pig liver dolichyl phosphate. In this case, recoveries were monitored through use of a [^{14}C]dolichyl phosphate internal standard. The values obtained for the tissue content of dolichyl phosphate using this technique agreed within 10% of the values estimated by the routine, straight-phase procedure used (data not shown).

Mannosylphosphoryldolichol increased from an initial value of 50% to a final value of 70% of the total dolichyl phosphate pool following estrogen treatment. In pregnant mice, mannosylphosphoryldolichol constituted greater than 95% of the total dolichyl phosphate pool. Similar analyses were performed on chloroform/methanol (2:1) and chloroform/methanol/water (10:10:3) extracts of tissues. It was found that greater than 95% of the dolichyl phosphate was extracted with chloroform/methanol (2:1) and exhibited the same base stability as mannosylphosphoryldolichol (data not shown). Collectively, these data supported the idea that uteri have relatively high levels of dolichyl phosphate derivatives, that estrogen treatment increases the levels of dolichyl phosphate derivatives, and that the major form of dolichyl phosphate in these tissues is mannosylphosphoryldolichol.

Discussion

There is much interest in determining how glycoprotein assembly is regulated. Given the myriad of oligosaccharide structures that can arise from a common lipid-linked oligosaccharide precursor and the relative consistency of structures present at a given glycosylation site in mature glycoproteins (Swiedler et al., 1985), it seems likely that regulation occurs at multiple steps. These steps would include not only the assembly of the precursor oligosaccharide but also the processing and further modifications of these structures (Hanover & Lennarz, 1981). To date, many studies of regulation of dolichol-linked oligosaccharide assembly have focused on measurements of glycosyltransferase activities (Lennarz, 1983; Lucas & Levin, 1977; Lucas, 1979; Dutt et al., 1986a; Parodi & Leloir, 1979; Scher et al., 1977; Clark et al., 1983; Welply et al., 1985). In addition, some work has addressed the potential for regulation at the level of dolichyl phosphate availability (Lucas & Waechter, 1976; Carson et al., 1981; Carson & Lennarz, 1981). Indeed, in some systems it is possible to stimulate oligosaccharide assembly and glycoprotein biosynthesis (Carson et al., 1981; Kousvelari et al., 1983) in vivo by supplementation of culture media with dolichyl phosphate. A number of studies have demonstrated that the major forms of dolichol-linked oligosaccharides found in vivo include mannosylphosphoryldolichol, dolichylpyrophosphoryl derivatives containing one to three saccharide residues, and mature oligosaccharylpyrophosphoryldolichol (Mohapatra et al., 1983; Grant & Lennarz, 1983; Pan & Elbein, 1982; Datema & Schwarz, 1981; Hubbard & Robins, 1980; Turco, 1980). The quantitation of these metabolic intermediates is basic to understanding regulation of this pathway.

Our estimation of the levels of dolichol-linked oligosaccharides relied on our ability to reproducibly and efficiently extract these glycolipids. Furthermore, we utilized previously determined (Dutt et al., 1986a,b) values for the specific radioactivity of sugar nucleotide precursors extracted from tissues under identical conditions to calculate the mass of each glycolipid in our extracts. The values of 2 nmol/mg of protein were about 10 times higher than the highest level of dolichyl phosphate reported for mammalian tissues (Keller et al., 1985). The fact that uteri contain such relatively high levels of dolichyl phosphate may explain our inability to stimulate oligosaccharide biosynthesis in tissue slice experiments by supplementing uterine tissues with dolichyl phosphate (Dutt et al., 1986a); i.e., the system may already be "saturated" with dolichyl phosphate. Furthermore, the relatively high levels of dolichol-linked saccharides in these tissues are consistent with the unexpectedly long time required to label these pools to equilibrium. In other systems, this process occurs within minutes rather than hours (Hubbard & Robbins, 1980; Grant & Lennarz, 1983). The rates of equilibration should reflect how quickly these pools "turn over". Consequently, in systems that synthesize similar amounts of oligosaccharide, large pools of dolichol-linked saccharides would turn over more slowly than small pools.

Direct determination of the dolichyl phosphate content of uteri confirmed not only that this tissue contains a relatively high amount of dolichyl phosphate but also that, in fact, most of the dolichyl phosphate was glycosylated. Specifically, both our metabolic and chemical determinations indicated that mannosylphosphoryldolichol is the major (50–98%) form of dolichyl phosphate present under all conditions. We could not determine the tissue levels of glucosylphosphoryldolichol directly; however, we can put upper limits on these levels by subtracting the amount of dolichyl phosphate in the form of mannose- and glucosamine-containing glycolipids from total dolichyl phosphate. Consequently, glucosylphosphoryldolichol levels could be no higher than 1 nmol/mg of protein in uteri of ovariectomized mice, before or after 2 days of estrogen treatment; moreover, these values would have to be at least 50-fold lower in uteri of pregnant mice because nearly all of the dolichyl phosphate is in the form of other glycolipids. Because glucosylphosphoryldolichol synthase activity is similar under all conditions, we do not expect that the tissue levels of this compound vary greatly. Consequently, we suggest, but cannot prove at this point, that glucosylphosphoryldolichol levels are low relative to mannosylphosphoryldolichol. We further suggest that synthesis of glucosylphosphoryldolichol is an unlikely regulatory point for N-linked oligosaccharide

assembly in this system. It must be noted that we could detect very little transfer to endogenous dolichyl phosphate in uterine membranes derived from ovariectomized mice before or after estrogen treatment using three different dolichyl phosphate dependent glycosyltransferase assays (Dutt et al., 1986a, and this paper). These observations indicate that there is very little dolichyl phosphate available for glycosylation in those membrane preparations. This may be because the dolichyl phosphate that we could not account for in our metabolic labeling studies is, in fact, already glycosylated; alternatively, the dolichyl phosphate could be in subcellular fractions other than the one used to measure glycosyltransferase activity.

We previously determined that protein represents approximately 7% of the wet weight of mouse uteri (Dutt et al., 1986a); therefore, the tissue level of mannosylphosphoryldolichol ranges from 35 to 180 μM. Using the values reported for chitobiosylpyrophosphoryldolichol and oligosaccharylpyrophosphoryldolichol in this paper and the same reasoning, it is estimated that the tissue concentrations of these glycolipids are 1–2 μM and 2–8 μM, respectively. It seems likely that these intermediates are compartmentalized within the cell, and so local concentrations, i.e., within the endoplasmic reticulum, would be higher. It also seems likely that the tissue concentration of chitobiosylpyrophosphoryldolichol saccharides is comparable to the apparent K_m determined for the mannosyltransferase that utilizes this glycolipid (Herscovics et al., 1980; Sharma et al., 1982). Although mannosylphosphoryldolichol, oligosaccharylpyrophosphoryldolichol, and, to a lesser extent, total dolichyl phosphate levels rose in response to estrogen treatment, chitobiosylpyrophosphoryldolichol levels were the lowest and remained fairly constant. We have also found that the in vitro activity of chitobiosylpyrophosphoryldolichol synthase is low and fairly constant under these conditions (Dutt et al., 1986a). The lack of accumulation of chitobiosylpyrophosphoryldolichol suggests that this glycolipid is utilized efficiently for oligosaccharylpyrophosphoryldolichol synthesis under all conditions. Because we did not observe significant amounts of intermediates between the chitobiosyl and "mature" oligosaccharide, it seems that these intermediates are efficiently utilized as well. Consequently, the estrogen-dependent increase in glycoprotein synthesis is most closely paralleled by increases in mannosylphosphoryldolichol synthesis in vitro (Dutt et al., 1986a) and by the accumulation of mannosylphosphoryldolichol and its product, oligosaccharylpyrophosphoryldolichol, in tissue slice experiments.

It is striking that mannosylphosphoryldolichol is present at much higher levels than would be expected from the stoichiometry of oligosaccharide assembly (Parodi & Leloir, 1979; Hanover & Lennarz, 1981). As noted above, mannosylphosphoryldolichol synthase activity is the only one of four oligosaccharide biosynthetic activities studied in this system that is induced in response to estrogen treatment. It is possible that mannosylphosphoryldolichol performs a regulatory function in oligosaccharide assembly in addition to being a glycosyl donor. For example, some studies suggest that this glycolipid can allosterically activate *N*-acetylglucosaminylpyrophosphoryldolichol synthase (Kean, 1985; Kaushal & Elbein, 1985); however, the concentration required for this activation in other systems is much lower than the lowest level we determined for uteri. Another possibility is that mannosylphosphoryldolichol may serve to regulate dolichyl phosphate availability (Welply et al., 1985). Dolichyl phosphate may be diverted into the mannosylphosphoryldolichol pool to avoid overproduction of oligosaccharide while protecting dolichyl phosphate from degradation (Wedgwood & Strominger, 1980). In this case, release of dolichyl phosphate from the mannosylphosphoryldolichol pool could be coordinated with the need for oligosaccharide assembly. Such a release could be achieved either through utilization of the mannosyl moiety for oligosaccharide assembly or by reversing the mannosylphosphoryldolichol synthase reaction to form GDP-mannose and dolichyl phosphate (Parodi & Leloir, 1979). The GDP required for this reverse reaction conceivably could be derived by protein biosynthesis and thereby coordinate the process of oligosaccharide assembly with the synthesis of the protein acceptor. This possibility is consistent with the observed effects of inhibitors of protein synthesis on dolichol-linked oligosaccharide biosynthesis (Hubbard & Robbins, 1980; Grant & Lennarz, 1983).

In any case, it appears that the uterus has a tremendous capacity for glycoprotein assembly. In addition to the relatively high levels of dolichol-linked saccharides, the synthesis of a variety of glycoproteins is stimulated in response to estrogen (Dutt et al., 1986a,b). Furthermore, the enzymatic activities involved in oligosaccharide assembly appear to be high; e.g., glucosylphosphoryldolichol synthase activity is 10–80-fold higher in uteri than in other systems (Clark et al., 1983; Welply et al., 1985). Although both estrogen and progesterone jointly control many important aspects of uterine physiology (Psychoyos & Casimiri, 1980), the results presented in this paper, as well as in other reports (Dutt et al., 1986a,b), indicate that estrogen alone is capable of supporting many aspects of uterine glycoprotein biosynthesis. We are interested in determining the relationship that glycoprotein assembly bears to the vital biological functions of the uterus.

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Registry No. MPD, 55598-56-6; CBL, 59694-82-5; GPD synthase, 91116-95-9; estradiol, 50-28-2; dolichyl phosphate, 12698-55-4; pyrophosphoryldolichol, 37247-98-6.

REFERENCES

Adair, W. L., & Keller, R. K. (1985) *Methods Enzymol. 111*, 201–215.

Behrens, N. H., & Leloir, L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 153–159.

Burton, W. A., Lucas, J. J., & Waechter, J. C. (1981) *J. Biol. Chem. 256*, 632–635.

Carson, D. D., & Lennarz, W. J. (1981) *J. Biol. Chem. 256*, 4679–4686.

Carson, D. D., Earles, B. J., & Lennarz, W. J. (1981) *J. Biol. Chem. 256*, 11552–11557.

Clark, G. F., Miller, K. R., & Smith, P. B. (1983) *J. Biol. Chem. 258*, 14263–14270.

Datema, R., & Schwarz, R. T. (1981) *J. Biol. Chem. 256*, 11191–11198.

Dutt, A., Tang, J.-P., Welply, J. K., & Carson, D. D. (1986a) *Endocrinology (Baltimore) 118*, 661–673.

Dutt, A., Tang, J.-P., & Carson, D. D. (1986b) *J. Biol. Chem.* (submitted for publication).

Grabel, L. B., & Martin, G. R. (1983) *Dev. Biol. 95*, 115–125.

Grant, S. R., & Lennarz, W. J. (1983) *Eur. J. Biochem. 134*, 575–583.

Grant, S. R., Kousvelari, E. E., Banerjee, D. K., & Baum, B. J. (1985) *Biochem. J. 231*, 431–438.

Hanover, J. A., & Lennarz, W. J. (1979) *J. Biol. Chem. 254*, 9237–9246.
Hanover, J. A., & Lennarz, W. J. (1981) *Arch. Biochem. Biophys. 211*, 1–19.
Herscovics, A., Warren, C. D., Bugge, B., & Jeanloz, R. W. (1980) *FEBS Lett. 120*, 271–274.
Hubbard, S. C., & Robbins, P. W. (1980) *J. Biol. Chem. 255*, 11782–11793.
Kaplan, H. A., Woloski, B. M. R. N. J., & Jamieson, J. C. (1984) *Comp. Biochem. Physiol. A 77A*, 207–212.
Kaushal, G. P., & Elbein, A. D. (1985) *J. Biol. Chem. 260*, 16303–16309.
Kean, E. L. (1985) *J. Biol. Chem. 260*, 12561–12571.
Keller, R. K., Fuller, M. S., Rottler, G. D., & Connelly, L. W. (1985) *Anal. Biochem. 147*, 166–173.
Kousvelari, E. E., Grant, S. R., & Baum, B. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7146–7150.
Lau, J. T.-Y., & Lennarz, W. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1028–1032.
Lennarz, W. J. (1983) *CRC Crit. Rev. Biochem. 14*, 251–272.
Lowry, G. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lucas, J. J. (1979) *Biochim. Biophys. Acta 572*, 153–159.
Lucas, J. J., & Waechter, C. J. (1976) *Mol. Cell. Biochem. 11*, 67–78.
Lucas, J. J., & Levin, L. (1977) *J. Biol. Chem. 252*, 4330–4336.
Lucas, J. J., Waechter, C. J., & Lennarz, W. J. (1976) *J. Biol. Chem. 250*, 1992–2002.
Mohapatra, N., Lynn, W. S., & Bhattacharyya, S. N. (1983) *Biochem. J. 213*, 609–616.
Pan, Y.-T., & Elbein, A. D. (1982) *J. Biol. Chem. 257*, 2795–2801.
Parodi, A. J., & Leloir, L. F. (1979) *Biochim. Biophys. Acta 559*, 1–37.
Psychoyos, A., & Casimiri, V. (1980) *Prog. Reprod. Biol. 7*, 143–157.
Scher, M. G., Jochen, A., & Waechter, C. J. (1977) *Biochemistry 16*, 5037–5044.
Sharma, C. B., Lehle, L., & Tanner, W. (1982) *Eur. J. Biochem. 126*, 319–325.
Surani, M. A. H. (1979) *Cell (Cambridge, Mass.) 18*, 217–227.
Swiedler, S. J., Freed, J. H., Tarentino, A. L., Plummer, T. H., & Hart, G. W. (1985) *J. Biol. Chem. 260*, 4046–4054.
Turco, S. J. (1980) *Arch. Biochem. Biophys. 205*, 330–339.
Wedgwood, J. F., & Strominger, J. L. (1980) *J. Biol. Chem. 255*, 1120–1123.
Welply, J. K., Lau, J. T., & Lennarz, W. J. (1985) *Dev. Biol. 107*, 252–258.

Low-Temperature Solid-State ^{13}C NMR Studies of the Retinal Chromophore in Rhodopsin†

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ABSTRACT: Magic angle sample spinning (MASS) ^{13}C NMR spectra have been obtained of bovine rhodopsin regenerated with retinal prosthetic groups isotopically enriched with ^{13}C at C-5 and C-14. In order to observe the ^{13}C retinal chromophore resonances, it was necessary to employ low temperatures (−15 → −35 °C) to restrict rotational diffusion of the protein. The isotropic chemical shift and principal values of the chemical shift tensor of the ^{13}C-5 label indicate that the retinal chromophore is in the twisted 6-s-cis conformation in rhodopsin, in contrast to the planar 6-s-trans conformation found in bacteriorhodopsin. The ^{13}C-14 isotropic shift and shift tensor principal values show that the Schiff base C=N bond is anti. Furthermore, the ^{13}C-14 chemical shift (121.2 ppm) is within the range of values (120–123 ppm) exhibited by *protonated* (C=N anti) Schiff base model compounds, indicating that the C=N linkage is protonated. Our results are discussed with regard to the mechanism of wavelength regulation in rhodopsin.

The visual pigment rhodopsin found in vertebrate rod cells contains the protonated Schiff base (PSB)[1] of 11-*cis*-retinal (Figure 1) as its photoreactive chromophore [for reviews, see Ottolenghi (1980) and Birge (1981)]. Absorption of light produces an 11-cis → 11-trans isomerization of the retinal PSB that then dissociates (bleaches) from the protein as *all-trans*-retinal (Wald, 1968). This photochemical reaction channels light energy into the protein, setting into motion a

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[1] Abbreviations: bR, bacteriorhodopsin; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetate; MASS, magic angle sample spinning; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; ppm, parts per million; PSB, protonated Schiff base; Me_4Si, tetramethylsilane; λ_{max}, absorption maximum.

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