CTP-Dependent Dolichol Phosphorylation by Mammalian Cell Homogenates†

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ABSTRACT: A sensitive and specific assay method for measuring the phosphorylation of long chain polyprenols has been developed utilizing DEAE-Sephadex LH20 chromatography. Using this method, we have shown that crude cell homogenates from bovine liver, mouse plasmacytoma (MOPC-315), and Chinese hamster ovary (CHO) cells are capable of phosphorylating [1-^{3}H]dolichol (Dol) to form dolichyl phosphate (Dol-P) in the presence of CTP. ATP, GTP, and UTP were only slightly more effective than controls without nucleotide. CDP was 25% as effective as CTP. The apparent K_m for CTP is about 3 mM but was reduced to 0.3 mM in the presence of 15 mM ATP. The bovine product chromatographed identically with synthetic Dol-P and the product formed using *Lactobacillus plantarum* undecaprenol kinase, Dol and ATP. In the case of the CHO and MOPC-315 cells there is a second component present in the product mixture in addition to Dol-P. This component's mobility on thin-layer chromatography and on DEAE-Sephadex LH20 columns suggests it is not Dol-PP or a Dol fatty acid ester. Utilizing bovine liver homogenates, ^{32}P from [γ-^{32}P]CTP was incorporated into Dol-P, whereas label from [γ-^{32}P]ATP was not incorporated. The simplest explanation for the mechanism of Dol-P formation is a direct phosphoryl transfer from CTP to Dol.

Dolichols are long chain polyprenols having 16 to 22 isoprene units and are found in a wide variety of eucaryotic cells. Dolichol mono- and pyrophosphorylated esters have been implicated as essential glycosyl carriers for glycoprotein synthesis in many different cell types (Waechter & Lennarz, 1976). Analogous polyprenyl (undecaprenyl) phosphates having 11 isoprene units serve as glycosyl carriers in envelope polysaccharide biosynthesis in procaryotic cells (Hemming, 1974). The de novo biosynthesis of these carriers by prenyl transferases is thought to produce the pyrophosphate esters of polyprenols. The action of a variety of phosphatases in procaryotes results in the sequential dephosphorylation of these products to free undecaprenol (Goldman & Strominger, 1972; Willoughby et al., 1972). The free polyprenol may in turn be rephosphorylated by a phosphokinase (Higashi et al., 1970) to give undecaprenyl monophosphate. It has been suggested that the availability of undecaprenyl monophosphate is regulated by such phosphatases and kinases in procaryotes and is important in the control of bacterial cell envelope polysaccharide biosynthesis (Higashi et al., 1970). Similar potential control mechanisms have been postulated for eucaryotic glycoprotein synthesis using analogous phosphatases and phosphokinases to regulate the availability of dolichyl monophosphate (Hemming, 1977). In addition, dolichyl fatty acid esters may also serve as a source of dolichol for phosphorylation. We present evidence here for the presence of a dolichol phosphokinase in three types of mammalian cells. The reaction has a specific and unusual requirement for CTP as the phosphoryl donor.

Materials and Methods

[1-^{3}H]Dolichol (10 mCi/μmol) and [γ-^{32}P]ATP (1 mCi/μmol) were purchased from New England Nuclear. [1-^{3}H]-Dolichyl monophosphate was prepared biosynthetically using ATP,[1] [1-^{3}H]dolichol, and a crude membrane-associated undecaprenol phosphokinase isolated from *Lactobacillus plantarum* using a modification (Kalin & Allen, 1978) of the assay conditions described earlier for the analogous enzyme from *Staphylococcus aureus* (Higashi et al., 1970). The [1-^{3}H]dolichyl monophosphate was isolated as described below by DEAE-LH20 chromatography. DEAE-LH20 was prepared according to the method described by Peterson & Sober (1961) for the preparation of DEAE-cellulose. The binding capacity was 1 mequiv/g of wet resin. Nucleotides, synthetic dolichyl phosphate, and baker's yeast nucleoside-5′-diphosphate kinase were obtained from Sigma Chemical Co. Dolichol was isolated and purified from pig liver as previously described (Burgos et al., 1963).

Thin-layer chromatography was carried out on Sil G (Macherey-Nagel) or Silica Gel 60 F-254 (E. Merck) coated on plastic sheets using the following solvents: (I) diisobutyl ketone–acetic acid–H_2O (8:5:1, v/v); (II) diisobutyl ketone–acetic acid–H_2O (20:15:2, v/v); (III) benzene–ethyl acetate (9:1, v/v); (IV) $CHCl_3$–CH_3OH–H_2O (65:25:4, v/v). Reverse phase TLC was carried out as previously described on Kieselguhr G (E. Merck) plates in solvent V, acetone–H_2O (49:1, v/v) (Allen et al., 1976). Descending paper chromatography was carried out on Whatman no. 1 paper using solvent VI, H_2O-saturated butanol. Nucleotides were analyzed on PEI-cellulose F plates (E. Merck) using 0.75 M potassium phosphate buffer (pH 3.4) (Raué & Cashel, 1973).

Radioactive components on the chromatograms were detected with a Packard 7201 radiochromatogram scanner at a scanning rate of 0.5 cm/min. When insufficient radioactivity was present for radioscanning, the chromatograms were scraped (silica gel) or cut (paper) into 1-cm sections and counted in vials containing 10 mL of 0.57% Omnifluor (New

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[1] Abbreviations used: CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate, GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; NTP, nucleotide triphosphate; CHO, Chinese hamster ovary; MOPC, mineral oil induced plasmacytoma; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; DEAE-LH20, diethylaminoethyl-Sephadex LH20; PEI, polyethylenimine.

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England Nuclear) in toluene–Triton X-100 (2:1) scintillation fluid.

Tissue and Homogenate Preparation. Chinese hamster ovary (CHO) cells were grown in suspension at 37 °C in sealed culture bottles containing 500 mL of Nutrient Mixture F-10 (Ham) supplemented with 10% calf serum, 5% fetal calf serum (Gibco), 1000 units of penicillin/L and 1000 μg of streptomycin/L (Tjio & Puck, 1968). The cells were harvested at 37 °C at cell densities between 1×10^5 and 3×10^5 cells/mL by centrifugation at 500*g* for 5 min and washed three times at 4 °C in 45 mL of PBS buffer, pH 7.5 (8 g of NaCl, 0.2 g of KCl, 0.763 g of Na_2HPO_4, and 0.2 g of KH_2PO_4 per L). All remaining steps were carried out at 0–4 °C. Approximately 1 mL of packed cells was suspended in 1.5 mL of hypotonic homogenization buffer A (10 mM Tris (pH 7.5), 10 mM Na_2EDTA, and 10 mM KCl) and, after standing for 10–15 min, was subjected to 200 strokes with an "A" type pestle in a Dounce homogenizer. Phase contrast light microscopy revealed complete cell breakage. This homogenate and a 0.5-mL rinse of the homogenizer with buffer A were combined and dialyzed against 2.5 L of 10 mM Tris buffer (pH 7.5) for a total of 3–5 h with one change of buffer. This homogenate, containing 20–25 mg of protein/mL, is referred to below as the CHO homogenate.

Murine plasmacytoma, MOPC-315, was maintained by serial transplantation into BALB/c females (6–10 weeks) from Flow Laboratories. The tumors (0.5–2 g) were excised 2–3 weeks following subcutaneous transplantation. Necrotic tissue was dissected away and the remaining tissue was minced with scissors in 50% (w/v) ice cold homogenization buffer B consisting of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), and 1 mM Na_2EDTA. All subsequent procedures were carried out at 0–4 °C. The suspension was homogenized by 10 strokes with the "B" pestle, followed by 10 strokes with the "A" pestle of a Dounce homogenizer. This preparation, containing approximately 50 mg of protein/mL, was used as the MOPC homogenate.

Bovine liver was obtained from freshly slaughtered cows. The liver was then passed through a meat grinder. Any given preparation used on the same day or frozen for several weeks without thawing gave similar enzymic activity. There was, however, considerable variation from one preparation to another. A 20% (w/v) suspension of this minced liver in ice cold buffer B was homogenized by 20 stokes of a motor-driven glass–Teflon Potter-Elvehjem homogenizer, or by a procedure similar to that used with MOPC-315 cells. The homogenate contained 40–60 mg of protein/mL. This homogenate and the pellet resulting from centrifugation at 600*g* showed similar activity.

Protein was assayed by the method of Lowry et al. (1951) after solubilizing the protein by suspending the samples in 0.5 M NaOH and incubating for 15 min at 100 °C.

Phosphokinase Assay Procedure. The assay for dolichol phosphorylation was carried out under slightly different conditions from time to time for each of the tissues tested. The conditions generally used for the MOPC and bovine liver system contained, in a 0.2-mL reaction volume, 200 mM Tris buffer (pH 7.5), 0.5% Triton X-100, 60 mM $MgCl_2$, 15 mM CTP, 6.25 mM NaF, 0.2 mM *p*-chloromercuribenzoate, and 25 nM [1-^{3}H]dolichol (110 000 dpm, 10.1 mCi/μmol). For the CHO cells the assay mixture contained, in a 1.0-mL reaction volume, 50 mM Tris buffer (pH 7.5), 0.3% Triton X-100, 36 mM $MgCl_2$, 18 mM CTP, 0.2 mM *p*-chloromercuribenzoate, 6.25 mM NaF, and 50 nM [1-^{3}H]dolichol (110 000 dpm, 1.01 mCi/μmol). Any variations in these assays will be described in the legends of the figures and tables. Dolichol dissolved in absolute ethanol was always added first to the assay tube. The ethanol was removed under a N_2 stream, frequently while heating to 40–50 °C in a water bath. The other constituents and enzyme were then added, mixed well, and incubated at 37 °C for 60 min.

The reaction was stopped by the addition of 2 volumes of $CHCl_3$–CH_3OH (1:1, v/v), then mixed, and centrifuged. The $CHCl_3$ phase containing both dolichol and dolichyl phosphate was transferred to a clean tube. The H_2O–CH_3OH phase was reextracted with a volume of $CHCl_3$ equal to the original assay volume. An equal volume of CH_3OH was then added to the pooled $CHCl_3$ phases described above and mixed. This mixture was applied to a DEAE-LH20 column (0.5–0.75-mL bed volume).

The DEAE-LH20 column was prepared by treating the hydrochloride or free base form of the resin with 1 M HCOOH–0.5 M NH_4OH in $CHCl_3$–CH_3OH (1:1) followed by equilibration of the resin in $CHCl_3$–CH_3OH (1:1). The DEAE-LH20 could be recycled in the column with 1.25 mL of 1 M HCOOH–0.5 M NH_4OH in $CHCl_3$–CH_3OH (1:1). The columns were ready for use following immediate equilibration with 10 mL of $CHCl_3$–CH_3OH (1:1).

The dolichyl phosphate was separated from unreacted dolichol on these columns by a modification of a previously described preparative procedure (Samuel et al., 1974). Dolichol was eluted with 8 mL of $CHCl_3$–CH_3OH (1:1). Dolichyl phosphate was eluted with 2.0 mL of 0.25 M ammonium formate in $CHCl_3$–CH_3OH (1:1). The dolichyl phosphate fraction was desalted by washing with 1.0 mL of H_2O and the resulting $CHCl_3$ phase was transferred to a scintillation vial. The aqueous phase was routinely reextracted with an additional 0.75 mL of $CHCl_3$ to ensure quantitative recovery of product. The pooled $CHCl_3$ extracts were either allowed to air-dry overnight or a carborundum chip was added and the solvent removed by gently boiling on a hot plate. The dried samples were counted with 10 mL of tolune-based scintillation fluid containing 0.4% Omnifluor. The total recovery of radioactivity was routinely greater than 90%.

Conditions for the analysis of phosphatase activity in the homogenates were identical with those used in the phosphokinase assays, except that approximately 4500 dpm of [1-^{3}H]dolichyl phosphate was added to the assay mixture in place of [1-^{3}H]dolichol.

Preparation of Polyprenol for Characterization. The dolichyl phosphate product was hydrolyzed to free alcohol enzymically by a crude phosphatase from baker's yeast. This crude phosphatase preparation was made by suspending 4 g of baker's yeast cake in 40 mL of 0.05 M Tris buffer (pH 7.5) and passing the suspension twice through a French press at 18 000 psi at 0 °C. The mixture was centrifuged at 800*g* for 10 min and the supernatant fraction was used as the crude phosphatase. The [^{3}H]polyprenyl phosphate product was hydrolyzed with this crude phosphatase according to a procedure described earlier (Allen et al., 1976). The hydrolysis mixture was extracted with $CHCl_3$–CH_3OH (1:1) or H_2O-saturated butanol and the organic phase was taken to dryness, resuspended in $CHCl_3$, and chromatographed on a silicic acid column in $CHCl_3$ to separate the free alcohol from polyprenyl phosphates. This alcohol cochromatographed with authentic dolichol from pig liver on reverse phase plates in solvent V. Dolichol and dolichyl phosphate were visualized using an anisaldehyde spray reagent (McSweeney, 1965) or a phospholipid spray reagent (prepared by 1/3.6 dilution of the reagent described by Murphy & Riley (1962)).

^{32}P-Labeling of Dolichyl Phosphate. [γ-^{32}P]CTP was prepared by a modification of the procedure of Keenan et al.

(1972) utilizing baker's yeast nucleotide-5′-diphosphate kinase. The enzyme (1.6 units) was incubated with 1.1 μmol of CDP, 50 nmol of [γ-^{32}P]ATP (specific activity 1 mCi/μmol), 330 nmol of $MgCl_2$, and 5.57 μmol of Tris buffer (pH 8.0), in a 50-μL reaction volume at 26 °C for 1 h. The nearly complete phosphoryl transfer of ^{32}P from ATP to form [γ-^{32}P]CTP was confirmed by TLC of the reaction mixture on PEI-cellulose. The products were identified by autoradiography and UV absorbance of cochromatographed standards of CTP, CDP, ADP, and ATP. The reaction was stopped by boiling for 5 min. The reaction mixture was then transferred to a tube containing 0.5 nmol of [1-^{3}H]dolichol (5 μCi, specific activity 10.1 mCi/μmol), 20 nmol of *p*-chloromercuribenzoate, 625 nmol of NaF, 5.67 μmol of $MgCl_2$, 700 nmol of ATP, Triton X-100 (final concentration 0.5%), and 30 μL of bovine liver homogenate 30% (w/v) in a total combined reaction volume of 100 μL. This mixture was incubated at 37 °C for 60 min after which 0.1 mL of H_2O was added and the product extracted with $CHCl_3$-CH_3OH according to the procedure described above. Controls carried out in the absence of unlabeled ATP, CTP, CDP, and nucleoside-5′-diphosphate kinase are described in the Results.

Results

Control of Phosphatase Activity. The development of an assay method for dolichol kinase required a mechanism for minimizing the hydrolysis of the nucleoside triphosphate substrate and the dolichyl monophosphate product by phosphatases. In order to assess the level of such activity, phosphatase controls were routinely run in parallel to assays for phosphokinase activity, substituting [1-^{3}H]dolichyl phosphate for [1-^{3}H]dolichol as substrate. These phosphatase controls also contained CTP so that any equilibrium established between phosphatase hydrolysis and phosphokinase rephosphorylation could be evaluated under conditions of the usual phosphokinase assays. Initial attempts to demonstrate phosphokinase activity were thwarted by high levels of endogenous phosphatase activity which would have effectively hydrolyzed any product formed. Subsequent assays, therefore, employed 6.25 mM NaF and 0.2 mM *p*-chloromercuribenzoate as phosphatase inhibitors in order to minimize product hydrolysis by endogenous phosphatases. Under these conditions, phosphatase activity resulted in hydrolysis of less than 20% of the dolichyl monophosphate.

DEAE-LH20 Column Chromatography. Chloroform extracts from incubation mixtures containing [1-^{3}H]dolichol, ATP, and *L. plantarum* membranes were chromatographed on DEAE-LH20 columns and fractions eluted as described in Materials and Methods. Two distinct components were cleanly separated as demonstrated by TLC of each respective fraction on silica gel in solvent III. The first fraction, eluted with $CHCl_3$-CH_3OH (1:1), gave a single radioactive peak with a R_f of 0.77 corresponding to dolichol (Table I). The second fraction, eluted with 0.25 M ammonium formate in $CHCl_3$-CH_3OH (1:1), gave a single radioactive peak with a R_f of 0.06 corresponding to dolichyl phosphate (Table I). In the absence of enzymic activity, the substrate, [1-^{3}H]dolichol, was recovered quantitatively in the first fraction and no radioactivity was found in the second fraction.

This method of separation gave good recoveries of radioactive dolichyl monophosphate with essentially no dolichol contamination in the dolichyl monophosphate fraction. It has advantages over the paper chromatographic method described by Higashi et al. (1970) for assaying undecaprenol phosphokinase because it is more rapid, more easily applicable when using radiolabeled polyprenols, and provides a convenient method of separating the phosphorylated polyprenols for subsequent analysis and characterization.

TABLE I: Chromatographic Mobilities of Enzyme Products.[a]

dolichyl phosphate synthesized by homogenates from	R_f solvent II[b]	III[c]	IV[b]	VI[d]
bovine liver	0.66	0.00	0.57	0.86
bovine liver (acid hydrolyzed)				0.85
MOPC 315	0.66, 0.55	0.00		
CHO	0.68, 0.50	0.00	0.64, 0.45	
L. plantarum	0.68	0.06	0.61	0.81
L. plantarum (acid hydrolyzed)				0.83
dolichol	0.94	0.77	0.97	0.93
dolichol monophosphate (synthetic)	0.68	0.00	0.61	0.88

[a] In each case, fraction 2 from DEAE-LH20 was chromatographed. [b] Silica Gel 60 F 254 (E. Merck). [c] Silica Gel G (Macherey-Nagel). [d] Whatman no. 1 paper (descending).

Product Characterization. The enzymic product produced from the reaction of [1-^{3}H]dolichol and CTP with each tissue homogenate studied was analyzed by TLC in several solvent systems. In each case the product was formed by a 1-h incubation followed by isolation of the phosphorylated product by DEAE-LH20 chromatography.

The product biosynthesized with bovine liver homogenates chromatographed (Table I, Figures 1A and 1D) as essentially a single component on TLC in three different solvent systems. It cochromatographed with synthetic dolichyl phosphate in solvents II and IV; cochromatography in solvent III was not tested. [1-^{3}H]Dolichyl phosphate, prepared as a standard using the membrane-associated undecaprenol phosphokinase from *L. plantarum*, had essentially the same R_f as the bovine product in solvents II, III, and IV. Undecaprenyl monophosphate prepared with the same *L. plantarum* enzyme also had a R_f of 0.66 in solvent II. Evidence that this membrane-associated ATP-dependent polyprenol phosphokinase from *L. plantarum* phosphorylates undecaprenol to give undecaprenyl monophosphate will be presented elsewhere (Kalin & Allen, 1978).

The phosphorylated products of CHO and MOPC-315 cell homogenates remained at the origin in solvent III but both appeared to give two principal radioactive components by TLC in solvents II and IV (Table I, Figure 1B, 1C, and 1E). In the case of the CHO product, for example, the faster chromatographing component corresponded to that of dolichyl phosphate, with a R_f of 0.64 (solvent IV), whereas the second, slower component, migrated with a R_f of 0.45 as would be expected for a more polar lipid. Dolichyl pyrophosphate has been reported to have a R_f of 0.14 in a similar TLC system ($CHCl_3$-CH_3OH-H_2O, 60:25:4, v/v), whereas dolichyl monophosphate chromatographed at a R_f of 0.63 (Warren & Jeanloz, 1975).

The *L. plantarum* and bovine phosphorylated product chromatographed on paper with a R_f of 0.86 in solvent VI. Dolichol in this system had a R_f of 0.93, whereas synthetic dolichyl monophosphate had a R_f of 0.88. The R_f's of both our enzymic product and synthetic dolichyl monophosphate were much greater than that reported in the literature for dolichyl monophosphate (R_f 0.0) using this solvent system (Daleo & Pont Lezica, 1977).

Other controls were carried out to verify the nature of the phosphorylated product. The bovine product separated by

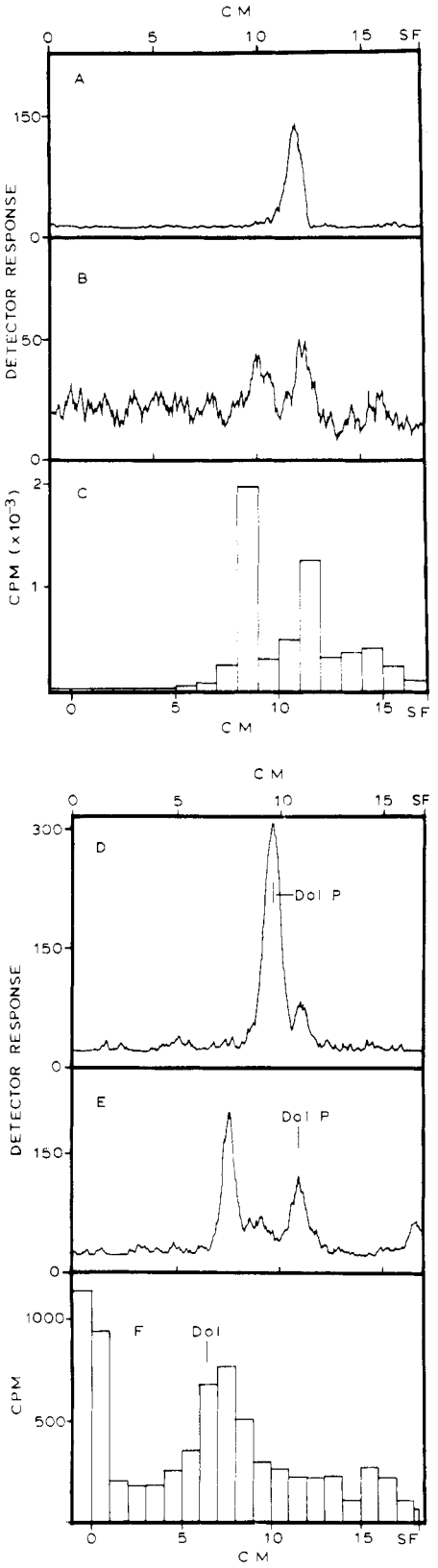

FIGURE 1: Chromatography of enzymic products. The enzymic products were synthesized in large scale incubation mixtures and extracted with $CHCl_3$-CH_3OH according to the procedures described in Materials and Methods. TLC was carried out on silica gel 60 F-254 sheets. TLC of (A) bovine product (29 000 dpm), (B) MOPC-315 product (18 200 dpm), and (C) CHO product (21 000 dpm) were carried out in solvent II. D and E represent TLC of bovine and CHO products, respectively, in solvent IV. In D and E synthetic dolichyl monophosphate (Dol P) cochromatographed as indicated. The bovine product was partially hydrolyzed to the free alcohol and chromatographed by reverse phase chromatography in solvent V (F) according to procedures described in Materials and Methods.

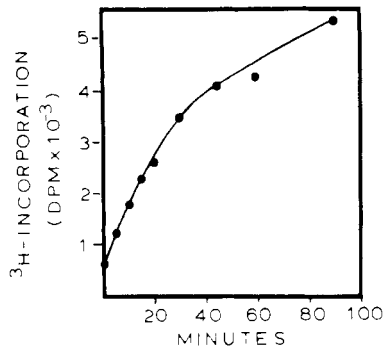

FIGURE 2: Time dependence of product formation. Bovine liver homogenate (8.25 mg/mL) was incubated in a 5-mL reaction mixture under conditions described in Materials and Methods. Aliquots (0.2 mL) were removed at the indicated times and assayed for product formation.

paper chromatography was eluted with $CHCl_3$-CH_3OH (1:1) and subjected to mild hydrolysis (0.01 N HCl) at 60 °C for 30 min. These conditions have been reported to completely hydrolyze dolichyl phosphomannose to dolichyl monophosphate without the hydrolysis of dolichyl monophosphate to free dolichol (Wedgewood et al., 1974; Pont Lezica et al., 1975). Paper chromatography of the acid-treated product in solvent VI showed no significant change in R_f from the unhydrolyzed dolichol product (Table I) and no radioactivity was observed at either the origin or at a position corresponding to dolichol.

Each of the phosphorylated products was partially dephosphorylated using a crude yeast phosphatase preparation first described by Kurokawa et al. (1971) and subsequently used to prepare free undecaprenol from undecaprenyl monophosphate (Allen et al., 1976). The resulting free alcohols were analyzed by reverse phase chromatography and all were found to cochromatograph with pig liver dolichol and to have the same R_f (0.41) as the unreacted substrate, [1-^{3}H]dolichol (Figure 1F).

Nucleotide Specificity. Each of the three tissues tested, MOPC-315, CHO, and bovine liver, was homogenized and used as described in Materials and Methods to study their ability to catalyze the phosphorylation of [1-^{3}H]dolichol in the presence or absence of CTP, ATP, GTP, and UTP. Table II clearly demonstrates that CTP is several times more active than any of the other nucleotides examined. In the case of MOPC-315 and bovine liver, ATP, GTP, and UTP were only slightly more effective than the control carried out in the absence of nucleotide. Boiled enzyme failed to catalyze product formation. The nature of the product formed in the absence of nucleotide is not known. Product synthesis does not appear to be due to dialyzable factors such as endogenous CTP since, in the case of the CHO homogenate, prolonged dialysis had little effect on the level of product formation. The percentage of total substrate converted to product varied with the tissue tested but, in the case of bovine liver, the product represented as much as 20% of the added dolichol when CTP was utilized as cosubstrate. Because of the presence of phosphatase activity in these preparations, CDP was also tested as a cosubstrate with the bovine liver homogenate. CDP was partially active but at a concentration of 15 mM gave only 25% of the activity of CTP under identical conditions.

Time, Substrate, and Protein Concentration Dependence. The time-dependent formation of product using the bovine liver preparation was linear up to 30 min as shown in Figure 2.

The dependence of product formation on the concentration of CTP is illustrated for bovine liver and CHO cells in Figure 3A, where the level of $MgCl_2$ was kept constant. The apparent

TABLE II: Nucleotide Specificity of Dolichol Phosphokinase.[a]

tissue	[^{3}H] dolichyl phosphate formation (dpm)					
	CTP	ATP	GTP	UTP	minus NTP	minus enzyme
bovine	20 737	2040	1889	1651	1523	144
CHO	5 424	861	1175	1248	341	34
MOPC-315	1 352	500	597	387	324	440

[a] The reaction conditions are the same as those described in Materials and Methods except the CHO assay also contained 10% dimethyl sulfoxide. The concentration of nucleotide triphosphate was 15 mM in each case and the final concentration of protein was 13, 13, and 23 mg/mL for bovine liver, CHO cells, and MOPC cells, respectively.

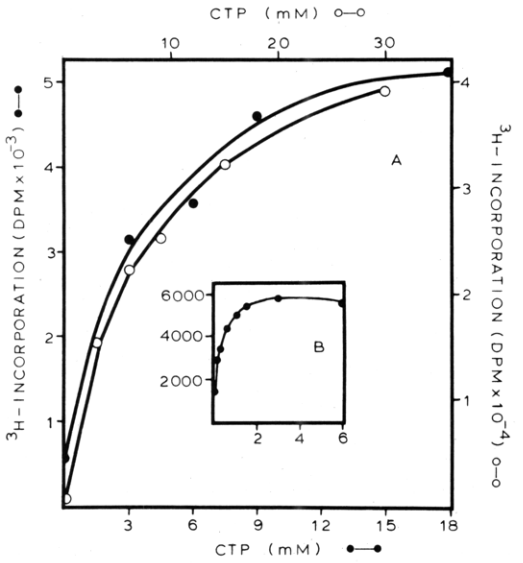

FIGURE 3: Dependence of product formation on CTP concentration. (A) Bovine liver (open circles) and CHO cell homogenates (closed circles) were incubated under the conditions described in Materials and Methods except that the CTP concentration was varied as indicated and the CHO reaction mixtures contained 10% dimethyl sulfoxide. The final protein concentrations for the bovine and CHO assays were 10.8 and 12.6 mg/mL, respectively. (B) Bovine liver homogenates were incubated under conditions described in Materials and Methods except that CTP concentration was varied and ATP added to a final concentration of 15 mM. The final protein concentration was 8 mg/mL. The values represent an average of several experiments.

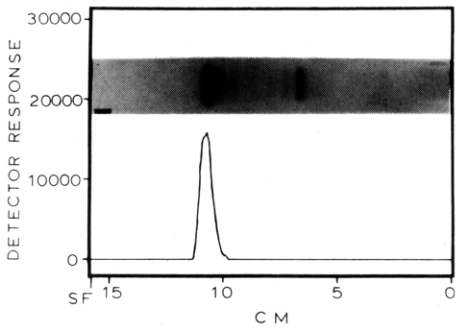

FIGURE 4: Labeling of dolichyl monophosphate with ^{32}P and ^{3}H. Bovine liver homogenate was incubated with [γ-^{32}P]CTP and [^{3}H]dolichol as described in Materials and Methods. A $CHCl_3$–CH_3OH (1:1) extract of the reaction mixture was chromatographed on silica gel 60 F-254 in solvent II. The sample contained approximately 280 000 dpm of ^{3}H and 800 dpm ^{32}P. The tracing represents only ^{3}H, since it was independently established that no response was detected from 800 dpm of ^{32}P at the radioscanner linear range used. An autoradiographic analysis of the same chromatogram, shown above the tracing, indicates the migration of ^{32}P, since the darkening of the X-ray film observed upon exposure to 250 000 dpm of ^{3}H alone was barely detectable.

1/2 maximal velocity was observed at approximately 3 mM CTP. However, if 15 mM ATP was included in the incubation mixture, the 1/2 maximal velocity was observed at 0.3 mM CTP (Figure 3B). ATP probably serves as a substrate for phosphatases present in the crude homogenate, thereby sparing CTP. It is clear, from the data shown in Figure 3A and Table II, that bovine liver was several times more active than the CHO cells at similar protein concentrations. Experiments carried out with a constant molar ratio of Mg^{2+}/CTP of 4/1, using CHO cells, gave essentially the same results as those described in Figure 3A. The enzyme showed a dependence, in CHO cells, on Mg^{2+} concentration at a constant CTP concentration. Activity increased as the ratio of Mg^{2+}/CTP was increased to 8/1. However, nearly maximum effect was observed at the ratio of 4/1 used in the routine assay conditions.

The dependence of product formation on dolichol concentration, tested with the bovine liver preparation, was linear up to the highest dolichol concentration tested (50 nM). The dependence of product formation on protein concentration using bovine liver showed a concave curvilinear effect which cannot be explained. CHO and MOPC cell homogenates were not tested. Although dimethyl sulfoxide was added in some experiments, it was subsequently shown to have no significant effect on enzyme activity.

^{32}P-Labeling of Dolichyl Monophosphate. The mechanism of dolichol phosphorylation was tested using [γ-^{32}P]CTP and [1-^{3}H]dolichol. [γ-^{32}P]CTP, generated in vitro from CDP, [γ-^{32}P]ATP, and yeast nucleoside diphosphate kinase, was mixed with unlabeled ATP (in 15-fold molar excess to [γ-^{32}P]ATP to spare CTP from phosphatase breakdown), [1-^{3}H]dolichol, and bovine liver homogenate as described under Materials and Methods. A radioactive component containing both ^{3}H and ^{32}P was isolated by $CHCl_3$–CH_3OH extraction and subsequent DEAE-LH20 chromatography. Characterization of this component by TLC in solvent II showed coincidence of migration of ^{3}H and ^{32}P with dolichyl monophosphate (Figure 4). No labeled product was formed in controls where CDP was omitted. Therefore, ^{32}P from [γ-^{32}P]ATP was not a direct phosphoryl source for dolichol phosphorylation.

Assuming a simple phosphoryl transfer from [γ-^{32}P]CTP to [1-^{3}H]dolichol, the molar ratio of ^{3}H-labeled product to ^{32}P-labeled product should be 1.0 when correction is made for the specific activities of the respective ^{3}H-labeled and ^{32}P-labeled substrates.

In these experiments the appropriately calculated ratio of ^{3}H-labeled product to ^{32}P-labeled product was approximately 25. This disparity could be explained, in part, by an apparent decrease in the specific activity of [γ-^{32}P]CTP. If an active nucleotide diphosphate kinase in bovine liver homogenates was capable of generating unlabeled CTP from CDP and ATP present in the reaction mixture, the specific activity of the [γ-^{32}P]CTP would be predicted to decrease by 15-fold. The presence of such an activity was confirmed in control experi-

ments in which [γ-^{32}P]ATP and CDP were incubated (without a 15-fold molar excess of ATP) in the presence and absence of exogenous (baker's yeast) nucleoside diphosphate kinase, then incubated with dolichol and bovine liver homogenates. Under these conditions, the extent of dolichyl phosphate formation and molar ratio of ^{3}H-labeled product to ^{32}P-labeled product was the same whether exogenous (baker's yeast) nucleotide diphosphate kinase was added or not. [γ-^{32}P]CTP must have been generated by a nucleoside diphosphate kinase in the bovine homogenate since we have previously shown that γ-^{32}P from ATP was not a direct donor in dolichol phosphorylation. Furthermore, when the experiment was repeated and unlabeled CTP was added in 15-fold excess of the [γ-^{32}P]CTP, the ratio of ^{3}H-labeled product to ^{32}P-labeled product observed was similar to that seen when a 15-fold excess of ATP was used.

Discussion

We have presented evidence for an enzymic activity, present in bovine liver, MOPC 315, and CHO cells, capable of phosphorylating dolichol to form dolichyl phosphate in the presence of CTP. The demonstration of this activity for the first time in any mammalian system, its apparent presence in vastly different tissue types, and the unique requirement for CTP make this dolichol phosphokinase particularly interesting.

Three properties of polyprenyl phosphate have been invoked to characterize the product of our reaction as dolichyl monophosphate: (1) its anionic character on ion exchange chromatography; (2) its R_f values and cochromatography with synthetic dolichyl monophosphate in several solvent systems; and (3) the production of dolichol following treatment with a crude phosphatase from yeast.

The products of each tissue homogenate are polar anionic lipids as demonstrated by ion-exchange chromatography. This rules out dolichyl fatty acid esters or other neutral dolichol products as possible products.

The products cochromatograph or have R_f values characteristic of dolichyl phosphate by TLC in solvents II, III, and IV. These chromatographic systems can distinguish dolichyl monophosphate from other commonly reported dolichyl phosphate derivatives such as dolichyl phosphomannose, dolichyl pyrophosphomannose, and dolichyl pyrophosphate (Warren & Jeanloz, 1973, 1975; Wedgewood et al., 1974). The bovine product chromatographed identically with the product formed by *L. plantarum* polyprenyl alcohol phosphokinase, using dolichol and ATP, in all solvent systems tested. The R_f of the bovine product on paper in solvent VI was the same as that of synthetic dolichyl monophosphate. Acid hydrolysis of the product showed no change in its mobility on paper chromatography compared to the unhydrolyzed product. Under the conditions used in this work (60 °C at pH 2 for 20 min) other authors have reported the complete hydrolysis of dolichyl phosphomannose to dolichyl phosphate but resistance of dolichyl phosphate to hydrolysis to the free alcohol (Wedgewood et al., 1974; Pont Lezica et al., 1975).

Furthermore, treatment of the enzymic product with a crude yeast phosphatase capable of hydrolyzing polyprenyl phosphate to the corresponding alcohol produced a product which cochromatographed with authentic dolichol on reverse phase TLC.

Although the enzyme was active at particularly low concentrations of dolichol, it required a particularly high and unphysiological concentration (3 mM) of CTP in the absence of ATP. The addition of a large molar excess of ATP, however, reduced the apparent K_m for CTP to about 0.3 mM. The absence of a lag in the time dependence for product formation is supporting evidence for the absence of a CTP derived phosphoryl donor such as CDP analogues.

It is clear from the results that [γ-^{32}P]CTP acts as a donor of phosphate in the formation of dolichyl phosphate. This reaction does not proceed directly from ATP since [γ-^{32}P]ATP, alone, does not serve as a phosphoryl donor. Phosphoryl transfer could not proceed in a direct pathway through CDP since CDP is considerably less active than CTP as a substrate.

Interpretation of the results is complicated by the observations that the molar ratios of ^{3}H-labeled product/^{32}P-labeled product were larger than would be predicted from the specific activities of the labeled substrates being used, even if corrected for estimates of in vitro biosynthesis of unlabeled CTP by endogenous nucleoside diphosphate kinase. One explanation may lie in the fact that each reaction mixture contained a 20-fold excess of CDP, used to generate [γ-^{32}P]CTP. This excess CDP may have resulted in dilution of the γ-^{32}P label due to some yet unidentified exchange reaction, such as an adenylate kinase type reaction, 2CDP $\rightarrow$ CTP + CMP. Markland & Wadkins (1966), however, demonstrated the absence of such an activity in purified bovine liver mitochondrial adenylate kinase.

We favor the simplest explanation for the mechanism of dolichyl monophosphate formation, i.e., a simple phosphoryl transfer from CTP to dolichol. However, other possible enzymic pathways might be postulated for the ultimate formation of dolichyl monophosphate as measured in the assay. These schemes might include involvement of dolichyl pyrophosphate or cytidine diphosphate intermediates which are hydrolyzed or otherwise involved in the ultimate phosphorylation of dolichol. Although in the case of the CHO and MOPC-315 cells, there is a second component present in the product mixture, it is likely that this is a subsequent product of dolichyl phosphate metabolism rather than a precursor. If a comparison with previously reported R_f values of various dolichyl phosphate derivatives is made with the results presented here, it is a likely conclusion that the second, slower moving component is not dolichyl pyrophosphate. Although dolichyl pyrophosphate has a R_f value similar to the second component in solvent II (Warren & Jeanloz, 1975), its R_f value in a solvent system nearly identical with solvent IV, $CHCl_3$–CH_3OH–H_2O (60:25:4), is vastly slower (Warren & Jeanloz, 1973). Dolichyl pyrophosphomannose is also an unlikely choice for the same reasons. Dolichyl phosphomannose and dolichyl pyrophosphate *N*-acetylglucosamine are possibilities which cannot be excluded by comparison of the literature R_f values.

The majority of work on glycoprotein synthesis has heretofore been concentrated on glycosyl transferase activities with relatively little attention paid to the metabolism of the essential lipid acceptor, dolichyl monophosphate. The work presented in this paper firmly establishes an enzymic pathway leading to dolichol monophosphate.

The development of a highly sensitive and specific assay method as described here for this phosphokinase in liver, MOPC 315, and CHO cells should be of great value in evaluating the level of dolichol kinase activity in many tissues where dolichyl monophosphate has been implicated as a rate-limiting component in mammalian glycoprotein synthesis. For example, limiting dolichyl monophosphate has been suggested to have a role in regulating the rate of glycoprotein assembly during active myelination (Harford et al., 1977), diethylstilbesterol-induced differentiation in hen oviduct (Lucas & Levin, 1977), and reduced synthesis of mannosyl oligosaccharide-lipid by membranes from concanavalin A resistant CHO cells (Krag et al., 1977).

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Effect of Cholesterol on the Molecular Motion in the Hydrocarbon Region of Lecithin Bilayers Studied by Nanosecond Fluorescence Techniques†

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ABSTRACT: Effects of cholesterol on the dynamic structure of the hydrocarbon region of dipalmitoyllecithin vesicles were examined. Decays of the emission anisotropy and the fluorescence intensity of 1,6-diphenyl-1,3,5-hexatriene embedded in lecithin–cholesterol vesicles were measured over a temperature range of 10–60 °C. The emission anisotropy decreased rapidly with time and then leveled off. The rotational motion of the probe was analyzed by a model of wobbling diffusion confined in a cone. Cholesterol (10–50 mol %) decreased the cone angle in the liquid-crystalline phase and increased it in the gel phase. In the presence of 33 mol % cholesterol, the wobbling diffusion constant increased in the gel phase and changed little in the liquid-crystalline phase. The viscosity in the cone decreased in the gel phase and remained almost unchanged in the liquid-crystalline phase in the presence of 33 mol % cholesterol. The total fluorescence intensity followed a single exponential decay independently of the cholesterol content 0–50 mol %.

Since cholesterol is a major lipid component of many biomembranes, the understanding of its interaction with phospholipids is essential for the description of the hydrophobic environment in which various membrane processes occur.

Cholesterol has been reported to have a "dual effect on fluidity" of phospholipid bilayers. The ordered array of lipid acyl chains in the gel phase is fluidized by the addition of cholesterol, whereas in the liquid-crystalline phase cholesterol reduces the fluidity (Ladbrooke et al., 1968; Lippert & Peticolas, 1971; Oldfield & Chapman, 1971). At sufficiently high concentrations cholesterol abolishes the phase transition (Hinz & Sturtevant, 1972).

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