Development of a Quantitative Assay for Tissue Levels of Dolichyl Phosphate†

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ABSTRACT: A sensitive assay is described for quantitating dolichyl phosphate (Dol-P), the polyprenyl phospholipid which participates in N-linked glycosylation. The assay is based on a novel reaction of alkyl phosphates with phenyl chloroformate in which a monosubstituted mixed anhydride of phosphoric and carbonic acid is formed. Evidence in support of the proposed structure for the derivative includes a phosphate to phenyl ratio of 1, infrared spectra, elemental analysis, behavior during ion-exchange chromatography, and reactivity with primary and secondary amines. A simple, rapid procedure is described for the preparation of [^{14}C]phenyl chloroformate from [^{14}C]phenol; use of the radiolabeled reagent allows assay of Dol-P in the subnanomolar range. The assay was applied to the quantitation of total Dol-P levels in rat liver. Dol-P and Dol-PP and their glycosylated derivatives were extracted with organic solvents and degraded to free Dol-P by acid hydrolysis. Following saponification to hydrolyze contaminating phospholipids, Dol-P was purified by using diethylaminoethylcellulose chromatography and derivatized with [^{14}C]phenyl chloroformate. Tracer quantities of [^{3}H]Dol-P were added to the tissue before extraction in order to monitor purification and yield. Double-label counting of the isolated derivative allowed calculation of the level of total Dol-P in the original tissue sample. This procedure yielded values of 2.9 ± 0.9 nmol of Dol-P/g of rat liver.

Many tissues (e.g., liver, mammary gland, and oviduct) exhibit the capacity for rapid induction of N-linked glycoprotein synthesis, and thus a mechanism must exist which allows the cell to deal with an increased demand for dolichol-mediated oligosaccharide transfer. Several studies in recent years have suggested that the cell responds by augmenting the level of available dolichyl phosphate. The work of Lucas & Levin (1977) and Lucas (1979) with hen oviduct suggests that dolichyl phosphate levels rise during estrogen-stimulated development. In addition, elevated levels of dolichyl phosphate in developing rat brain were suggested by Harford et al. (1977, 1980) as an explanation for the observed increase in mannosyltransferase activity. Such changes in dolichyl phosphate levels may be due to increased de novo synthesis mediated by long-chain prenyltransferase activity (Grange & Adair, 1977; Wellner & Lucas, 1979) or by phosphorylation of free dolichol by dolichol kinase (Allen et al., 1978; Rip & Carroll, 1980; Burton et al., 1979). In support of this concept, evidence for elevated levels of long-chain prenyltransferase (Adair & Keller, 1978) and dolichol kinase (Burton et al., 1981) has been reported in developing hen oviduct.

Although these reports are suggestive of a role for dolichyl phosphate in the regulation of glycoprotein synthesis, it is clear that definitive proof requires the development of an assay which directly quantitates this phospholipid. Further, since the levels of dolichyl phosphate are expected to be low relative to other tissue phospholipids, the assay must meet the requirements of high sensitivity and specificity. An enzymatic assay previously described by Behrens & Tabora (1978) is neither specific with respect to the prenyl phosphate substrate nor very sensitive. A high-pressure liquid chromatographic assay reported by us (Keller et al., 1979) suffers from low yields and relies on detection at 214 nm which is poorly discriminating.

In the present report, we describe a method which allows direct quantitation of dolichyl phosphate. The assay is based on the reaction of dolichyl phosphate with a derivatizing reagent which, when prepared as a radiolabeled compound, allows quantitation in the subnanomolar range. In addition, we describe procedures which allow isolation of dolichyl phosphate from rat liver and its quantitation with the above procedure.

Experimental Procedures

Chemicals. [U-^{14}C]Phenol was obtained from either New England Nuclear (5.6 mCi/mmol), Amersham (87 mCi/mmol), or ICN (33 mCi/mmol). All of the lots tested proved to be satisfactory. Dodecyl phosphate was from Fairfield Chemical Co. and dolichyl phosphate (80–90% pure) was from Sigma. Triethylamine (TEA) was from Aldrich. Phenyl chloroformate was obtained from Tridom-Fluka and purified by high-pressure liquid chromatography (HPLC) (see below). Phosgene (12% in toluene) was from Matheson Coleman and Bell. Phenol, used in pilot experiments to prepare unlabeled phenyl chloroformate, was ultrapure (preservative free) from Bethesda Research Laboratories. [1-^{3}H]Dolichyl phosphate was prepared from [1-^{3}H]dolichol by using the chemical phosphorylation procedure of Wedgwood et al. (1974). [1-^{3}H]Dolichol was prepared by $NaB^{3}H_4$ reduction of dolichal obtained by the oxidation of pig liver dolichol. Briefly, dolichol (100 mg, 90% pure, Sigma) was purified by semipreparative HPLC using a Magnum-9 Partisil-10 column (Whatman) and 6% ether in hexane as the solvent. The purified dolichol was oxidized to dolichal by treatment with pyridinium chlorochromate (Corey & Suggs, 1975) and isolated by using HPLC as above. The purified dolichal (~50 mg) was then reduced with 100 mCi of $NaB^{3}H_4$ (Amersham) in alkaline ethanol (Keenan & Kruczek, 1975). Thin-layer chromatography (TLC) of a small aliquot [chloroform as solvent; anisaldehyde stain (McSweeny, 1965)] indicated that essentially all of the dolichal was reduced to [^{3}H]dolichol. The radioactive product was isolated by HPLC; the yield was 50 mCi. The sample was then phosphorylated and purified by TLC. The final overall yield after elution from TLC was 20 mCi (5 μmol).

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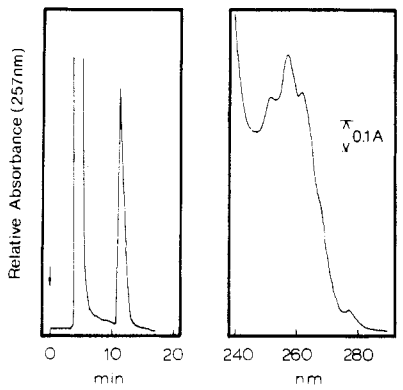


FIGURE 1: (Left) High-pressure liquid chromatography of [^{14}C]phenyl chloroformate. 0.1 mL of the reaction mixture from the [^{14}C]phenyl chloroformate synthesis was subjected to high-pressure liquid chromatography as described in the text. Effluent detection was at 257 nm. (Right) UV profile of phenyl chloroformate. The material purified by high-pressure liquid chromatography was scanned at 20 nm/min on a Beckman Model 25 spectrophotometer.

Solvents. Acetone was ACS quality from Fisher and contained 0.5% water as supplied. All other solvents were HPLC grade from Fisher or Burdick & Jackson.

Solvent Systems. The following solvent systems were employed: solvent A, $CHCl_3/CH_3OH$ (5:1); solvent B, hexane/2-propanol/H_2O (2:6:1.5); solvent C, $CHCl_3/CH_3OH$ (2:1); solvent D, $CHCl_3/CH_3OH$/15 M NH_3/H_2O (65:35:2:2); solvent E, $CHCl_3/CH_3OH$ (3:2); solvent F, $CHCl_3/CH_3OH/H_2O$ (10:10:3); solvent G, $CHCl_3/CH_3COOH$ (3:1).

Preparation of [^{14}C]Phenyl Chloroformate. The procedure used to synthesize [U-^{14}C]phenyl chloroformate was adapted from a patent awarded to the A. G. Bayer Co. (1962). To a screw-capped tube, immersed in ice, containing 250 μCi of [U-^{14}C]phenol (the mass depending on the specific activity) was added 0.2 mL of 12% phosgene in toluene. Eighty-five microliters of 22% NaOH (aq) was then added, the tube was capped, and the reaction was stirred vigorously on ice. Because of the toxic nature of phosgene, the reaction was carried out in a well-ventilated hood. After 15 min, the toluene layer was removed and reduced to ~0.1 mL with a gentle stream of nitrogen at ice bath temperature in the hood. The solution was then diluted to ~0.5 mL with hexane and reduced in volume again. This procedure was repeated 2 times more. Finally, the sample was subjected to HPLC in hexane with a 25-cm Partisil-5 column (Whatman) and a flow rate of 2 mL/min. Phenyl chloroformate, which elutes at ~10 min, was detected by its absorption at 257 nm (Figure 1, left). In some cases, due to the variable nature of the column characteristics, it was necessary to add 5 or 10% dichloromethane to the hexane to effect elution of the phenyl chloroformate. Such modifications did not alter the apparent purity (by UV spectral analysis) or reactivity of the product. Following HPLC, the sample was concentrated by evaporation of the solvent with a stream of nitrogen at room temperature. UV analysis of the purified [^{14}C]phenyl chloroformate is shown in Figure 1, right. From the absorbance curve, the disintegrations per minute (dpm) and the specific activity of the phenol, it was possible to calculate an extinction coefficient for phenyl chloroformate. A value of 2.4×10^2 at 257.5 nm was obtained with hexane as a solvent. An identical value was obtained with commercially available phenyl chloroformate purified by preparative HPLC. This value allows the preparation of [^{14}C]phenyl chloroformate solutions of known specific activity by using the ^{14}C-labeled material and unlabeled, commercially available phenyl chloroformate.

Reaction of Alkyl Phosphates with Phenyl Chloroformate. The solution of alkyl phosphate (0.05–10 nmol) was taken to dryness at room temperature in a 1.4-mL disposable polypropylene centrifuge tube with a stream of dry nitrogen. To the residue were then added 0.1 mL of $CHCl_3$, 0.01 mL of 10 mM triethylamine in $CHCl_3$, and 0.02 mL of 1.0 mM phenyl chloroformate in hexane. The solution was vortexed and immediately evaporated to dryness with a stream of nitrogen in a hood at room temperature. The residue was taken up in 10–20 μL of solvent C. Analysis of the product was carried out by TLC in either solvent A or acetone for dolichyl phosphate reactions and solvent C for other phosphate ester reactions (dodecyl phosphate and phospholipids). Chromatograms of derivatized dolichyl phosphate were developed with iodine vapor or anisaldehyde spray while chromatograms of other phosphate esters were developed with a molybdenum blue spray (Applied Science).

Analytical Techniques. HPLC was carried out by using a Lab Data Control Constametric IIG pumping system and a variable wavelength detector. Sample injection was achieved by using a Rheodyne on-line sample injector fitted with a 0.1-mL sample loop. UV profiles were integrated on line with a Perkin-Elmer M-2 minigrator. TLC was carried out on plastic-backed precoated silica gel 60 plates (Merck). Tanks (27 × 27 × 7.6 cm) were lined with filter paper and equilibrated for at least 1 h prior to use. Radioactivity on thin-layer plates was detected by using a Packard Model 7200 radiochromatogram scanner. Quantitation of radioactivity on thin layers was achieved by using a Packard Model 306 sample oxidizer. Plates to be analyzed were first wetted with hexadecane and covered with a strip of cellophane tape. The plates were then cut into 0.5-cm sections, and each section was placed between a Combusto-cone and a Combusto-pad (both from Packard). Oxidation was carried out by using a burn time of 1 min. Dispensor settings were 13 mL for Oxifluor, 9 mL for Oxisorb, and 13 mL for Oxiprep (all reagents are from New England Nuclear). Counting efficiencies were 22% for ^{3}H and 67% for ^{14}C. Alternatively, plates could be scraped and eluted for radiochemical analysis with solvent B. This solvent elutes the dolichyl phosphate derivative with approximately the same efficiency (~75%) as chloroform-containing solvents but does not cause quenching in scintillation counting. For elution, the plate was wetted with water, and 0.5-cm fractions were scraped into 1.4-mL polypropylene centrifuge tubes. One milliliter of solvent B was added, and the tube was vortexed for 10 s and centrifuged in a microfuge (Beckman) for 20 s. The supernatants were poured directly into 20-mL glass scintillation vials to which was added 10 mL of ACS (Amersham). When samples containing both ^{3}H- and ^{14}C-labeled nuclides were analyzed, a double-label program was employed in which the efficiency of counting ^{3}H and ^{14}C was 15% and 57%, respectively. Spillover of ^{14}C cpm was 10%, while ^{3}H spillover was negligible.

Elemental analysis was carried out by Galbraith Laboratories, Knoxville, TN. Infrared spectra were obtained in KBr disks by using a Perkin-Elmer Model 337 grating IR spectrometer. Solutions of phospholipids were assayed for phosphate content according to the method of Ames (1966).

Purification and Quantitation of Dolichyl Phosphate from Rat Liver. The early steps in the purification are based on the extraction procedures described by Spiro et al. (1976). [^{3}H]Dolichyl phosphate (600 000 dpm/g of original tissue) is added to the resuspended membrane fraction prior to organic solvent extraction. The Spiro procedure yields two fractions: a solvent E extract and a solvent F extract. Greater than 95%

of the radioactivity originally added is recovered in the solvent E extracts. The two extracts, containing free and glycosylated dolichyl phosphate and pyrophosphate, are pooled by first drying the solvent E extract under a stream of nitrogen at 40 °C and then adding the solvent F extract to the residue. The pooled extracts are then subjected to acid and base hydrolysis. Acid hydrolysis is carried out first because some glycosylated derivatives (e.g., dolichyl phosphomannose) degrade to free dolichol during base hydrolysis (Warren et al., 1975). To the sample is added 12 N HCl to give a final concentration of 0.01 M. The tube is flushed with nitrogen, capped, and heated 60 min at 70 °C. Pilot experiments in which [^{3}H]dolichyl phosphomannose (New England Nuclear) was added to an aliquot of the sample indicated that complete hydrolysis to free mannose occurs within 30 min under these conditions. The sample is then cooled and 10 N KOH added to a final concentration of 0.1 N. Any cloudiness generated by the addition of base is eliminated by adding a few drops of methanol. Saponification, carried out as above for acid hydrolysis, was shown to be quantitative by using [^{14}C]phosphatidylcholine (New England Nuclear). Following saponification, the sample is treated with chloroform (0.4 volume per total volume) and water (0.2 volume), vortexed, and centrifuged. The upper phase is discarded and the lower phase treated with $^2/_3$ its volume of 50% methanol. Following centrifugation, the upper phase is again discarded, and the lower phase is evaporated to near dryness under a stream of nitrogen at 40 °C. The residue is dissolved in 2 volumes of solvent C/g of original tissue and applied directly to a column of DEAE-cellulose prepared according to Rouser et al. (1963) and equilibrated in solvent C. Two cubic centimeters of resin is sufficient for each gram of tissue processed. Following application of the sample, the column is washed sequentially with 5 column volumes each of solvents C and G. The dolichyl phosphate is then eluted by application of solvent C containing 0.1 M ammonium acetate. Fractions (1 mL) are collected, and the tubes containing the bulk of the radioactivity are pooled. The overall yield of tritium after chromatography is generally between 50 and 60%. The pooled sample is treated with 0.25 volume of water and centrifuged. The resulting upper phase containing the extracted ammonium acetate is removed. The lower phase is washed with 50% methanol, transferred to a 1.4-mL polypropylene centrifuge tube, and evaporated to dryness under a stream of nitrogen at room temperature. The residue is dissolved in 0.1 mL of $CHCl_3$ and reacted with [^{14}C]phenyl chloroformate in the presence of triethylamine as described above. Following TLC the derivative region is identified by iodine staining and/or radioscanning and subjected to sample oxidation. The amount of dolichyl phosphate present in the original tissue sample is then calculated as follows:

$$\text{nmol of Dol-P}\cdot g^{-1} = \frac{[^3\text{H}]\text{Dol-P added}\cdot g^{-1}\ (\text{dpm})}{^3\text{H in derivative (dpm)}} \times \frac{^{14}\text{C in derivative (dpm)}}{[^{14}\text{C}]\text{phenyl chloroformate sp act. (dpm/nmol)}} - \text{nmol of } [^3\text{H}]\text{Dol-P tracer added}\cdot g^{-1}$$

Results

Reaction of Phenyl Chloroformate with Primary Alkyl Phosphates. In preliminary studies, it was observed that microgram quantities of dolichyl phosphate reacted immediately with phenyl chloroformate to yield a product which migrated at an R_f of ~0.5 on silica gel TLC (10-cm plate) in solvent A. Dolichyl phosphate migrated only slightly under these conditions. The reaction could be demonstrated by using either [^{3}H]dolichyl phosphate or [^{14}C]phenyl chloroformate (Figure 2). Occasionally, very small amounts of two other ^{3}H-labeled derivatives (R_f = 0.2 and 1.0) were observed when <0.1 nmol was derivatized. Chromatography could also be carried out by using acetone as the developer. With this solvent, the dolichyl phosphate derivative migrated at R_f ~0.3 while unreacted dolichyl phosphate remained at the origin. It is to be emphasized that the mobility of the derivative was highly dependent on the relative activation of the silica gel layer (which in turn was dependent on the ambient humidity).

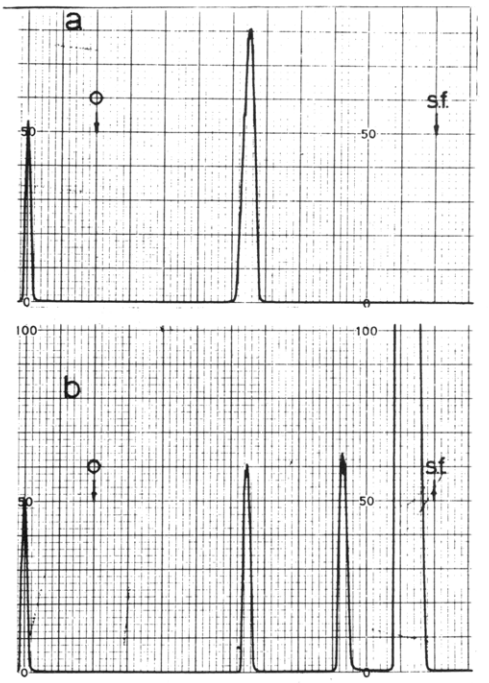
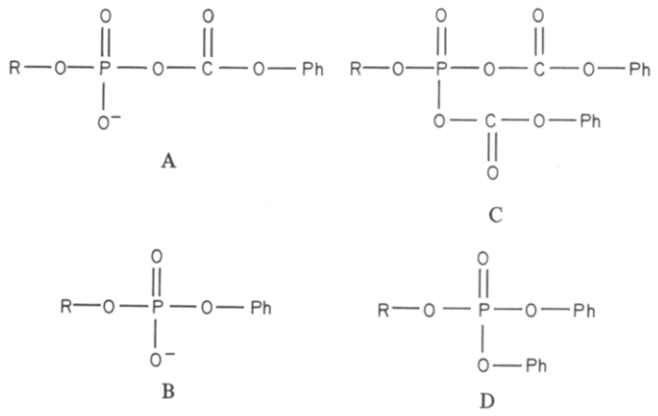


FIGURE 2: Thin-layer radiochromatogram scans of reactions of (a) [^{3}H]dolichyl phosphate (4 nmol, 5 × 10^5 dpm) with unlabeled phenyl chloroformate (20 nmol) and (b) unlabeled dolichyl phosphate (4 nmol) with [^{14}C]phenyl chloroformate (20 nmol, 1.5 × 10^6 dpm). Reactions were performed under standard conditions and chromatographed in solvent C. O, origin; s.f., solvent front. In (b), the large peak represents phenyl chloroformate and the middle peak represents the phenyl chloroformate–triethylamine complex. Dolichyl phosphate migrates at R_f = 0.05 in this system.

In order to characterize the phenyl chloroformate reaction and the nature of the major product formed, it was necessary to employ a model compound which could be obtained in quantities sufficient for physical and chemical analysis. For this reason, commercially available dodecyl phosphate was chosen. Figure 3 shows TLC of the product formed upon reaction of dodecyl phosphate with phenyl chloroformate according to the conditions given under Experimental Procedures. As was found with dolichyl phosphate, the major product migrated faster than the starting compound, suggesting a decrease in polarity. A review of the literature concerning phenyl chloroformate reactions (Matzner et al., 1965) indicated that the product observed could be one of the following: a monosubstituted derivative of a carbonic–phosphoric anhydride (A); a phosphodiester (B) (formed via spontaneous decarboxylation); a disubstituted derivative of the above-mentioned anhydride (C) or phosphotriester (D).

Several lines of evidence indicated that the product formed was the monosubstituted derivative (A). First, the uncharged derivatives (C and D) were ruled out when it was found that the product was retained on columns of DEAE-cellulose run in solvent F. DEAE-cellulose could also be used to purify the product, since the compound eluted at low salt concentrations



(R = dodecyl or dolichyl)

(0.1 M CH_3COONa) while the starting material, dodecyl phosphate, was retained under these conditions. Primary amines or ammonium acetate could not be employed in the eluate solutions, since the derivative was degraded by such compounds (see below). Second, when the derivative was prepared with [^{14}C]phenyl chloroformate, the product, after purification by preparative silica gel TLC, was found to have a phosphate/phenyl ratio of 1 (data not shown). Third, IR analysis gave major peaks at 1250 and 1750 cm^{-1}, consistent with a carbonic–phosphoric anhydride (Tarbell & Insalaco, 1967). Fourth, the product reacted with primary and secondary amines. As shown by Tarbell & Insalaco (1967) carbonic–phosphoric anhydrides react with amines to yield carbamates. In the present case, the product reacted with aniline to form a compound which, after crystallization from aqueous ethanol, melted at 123–125 °C [literature value for the phenyl carbanilate derivative, 126–127 °C (Crosby & Niemann, 1954)]. Fifth, elemental analysis of the product, prepared by elution from DEAE-cellulose with sodium acetate, was performed. Anal. Calcd for the sodium salt of compound A: C, 55.9; H, 7.35; P, 7.60; Na, 5.64. Found: C, 55.8; H, 8.14; P, 7.74; Na, 5.66. From the above studies, it was concluded that the product formed upon reaction of phenyl chloroformate with dodecyl phosphate according to the conditions described under Experimental Procedures was the carbonic–phosphoric anhydride (A).

Reaction of Phenyl Chloroformate with Dolichyl Phosphate. On the basis of the above studies with dodecyl phosphate, it was expected that the primary product of a reaction of dolichyl phosphate with phenyl chloroformate would be the carbonic–phosphoric anhydride. Several lines of evidence indicated that this was the case. When [^{3}H]dolichyl phosphate was treated with [^{14}C]phenyl chloroformate and the reaction mixture chromatographed on thin-layer plates in acetone, a major band was detected at R_f = 0.3 by iodine staining. Quantitative analysis of this band could be carried out by either sample oxidation or elution with solvent B as described under Experimental Procedures. Analysis of the ^{3}H dpm/^{14}C dpm ratio in the product by either of these methods gave essentially identical results and yielded a value consistent with a 1:1 stoichiometry of phenyl to phosphate groups (see below).

As expected, the dolichyl phosphate derivative was retained on columns of DEAE-cellulose equilibrated with solvent F and eluted with the same solvent containing 0.01 M CH_3COONa, indicating a negative charge was still present after derivatization. Furthermore, the compound was labile to treatment with NaOH, NH_4OH, and piperidine (all at 2 mM in solvent F for 10 min at room temperature). These findings, coupled with the results obtained on the model compound dodecyl phosphate, suggested that the monosubstituted carbonic–phosphoric anhydride derivative of dolichyl phosphate was being synthesized.

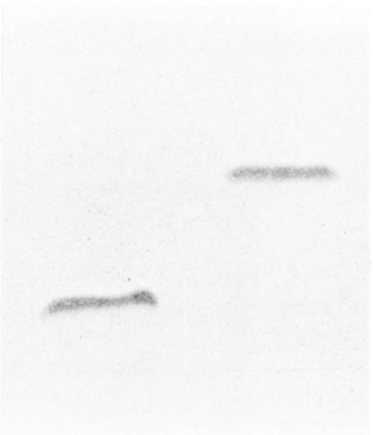


FIGURE 3: Thin-layer chromatography in solvent C of dodecyl phosphate (left) and product of reaction of dodecyl phosphate with phenyl chloroformate (right). See Experimental Procedures for details. Detection was with a molybdenum spray reagent.

Table I: Effect of Dolichyl Phosphate and Phenyl Chloroformate Concentration on Formation of Phenyl Carbonic Dolichyl Phosphoric Anhydride[a]

dolichyl phosphate (nmol)	phenyl chloroformate (nmol)	conversion (% of total ^{3}H)
0.06	1	10
0.06	10	85
0.06	100	85
0.6	1	22
0.6	10	98
0.6	100	99
10	1	1
10	10	35
10	100	99

[a] Varying amounts of dolichyl phosphate were mixed with 500 000 dpm of [^{3}H]dolichyl phosphate to give the amounts shown. Reactions were performed under standard conditions except that the concentration of phenyl chloroformate was varied. Following TLC, the plates were radioscanned and the areas under the peaks used to calculate percent conversion to product.

Effect of Reaction Conditions on the Formation of Anhydride Derivative of Dolichyl Phosphate. Several solvents and catalysts were examined in an effort to optimize the conditions for formation of the derivative. The major considerations in choosing a solvent were as follows: (1) solubility of reactants and catalyst; (2) sufficient polarity for rapid reaction [reactions involving chloroformates are known to be augmented by polar solvents (M. Matzner, personal communication)]; (3) nonreactivity with phenyl chloroformate. This last requirement ruled out the possibility of using alcohols or primary or secondary amines. Of the various solvents tested, several were found to be satisfactory, including chloroform, dichloromethane, dioxane, and acetonitrile. Chloroform was selected for all subsequent studies.

The addition of tertiary amines was found to enhance the reaction. Several amines examined were stimulatory, including pyridine, (dimethylamino)pyridine, and triethylamine. Triethylamine was arbitrarily chosen for all future experiments. The reaction was dependent on the concentration of triethylamine. A pilot study showed that maximal conversion of dolichyl phosphate to the anhydride derivative could be achieved at 0.5 mM triethylamine (data not shown).

Table I shows the effect of varying concentrations of dolichyl phosphate and phenyl chloroformate on the extent of con-

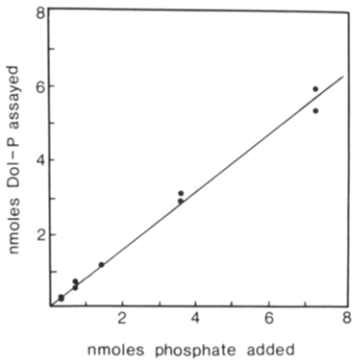


FIGURE 4: Quantitation of dolichyl phosphate using [^{14}C]phenyl chloroformate. Varying amounts of dolichyl phosphate were mixed with 600 000 dpm of [^{3}H]dolichyl phosphate and reacted with [^{14}C]phenyl chloroformate as described under Experimental Procedures. Following TLC in acetone, the derivative was subjected to sample oxidation and the dolichyl phosphate quantitated by using the formula given under Experimental Procedures.

version of dolichyl phosphate to the derivative. The data obtained suggest that conversion is augmented by increasing concentrations of dolichyl phosphate and by increasing ratios of phenyl chloroformate/dolichyl phosphate.

The reaction was found to proceed to a significantly greater degree when carried out in polypropylene tubes as compared to standard disposable borosilicate glass tubes. Experiments with [^{3}H]dolichyl phosphate indicated that the basis for this effect was due to the tendency of dolichyl phosphate not to redissolve when solutions containing the lipid were evaporated to dryness in the disposable borosilicate tubes. No such tendency was observed with polypropylene tubes.

Reaction with Other Phospholipids. Phenyl chloroformate was also tested for its ability to react with other phospholipids. Phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid (all from Sigma) were treated with phenyl chloroformate under the standard conditions described under Experimental Procedures. The formation of derivatives was assessed by TLC in solvent C followed by spraying for organic phosphorus. Of the phospholipids examined, phosphatidic acid and phosphatidylethanolamine showed substantial reaction (>50%) with phenyl chloroformate to yield compounds which migrated faster on TLC. Phosphatidylserine and phosphatidylglycerol showed slight reaction to faster moving components while phosphatidylcholine and phosphatidylinositol showed no reaction under the conditions employed. Because the nature of the products formed with these compounds was not studied in further detail, it is not possible to draw definite conclusions on the type of derivatives formed. However, the data are consistent with the possibility that phenyl chloroformate reacts readily with unhindered primary phosphates (as in dolichyl phosphate, dodecyl phosphate, and phosphatidic acid) and unhindered primary amines (phosphatidylethanolamine).

Quantitiation of Dolichyl Phosphate. Varying amounts of dolichyl phosphate were mixed with [^{3}H]dolichyl phosphate and reacted with [^{14}C]phenyl chloroformate in the presence of triethylamine in order to examine if phenyl chloroformate could be employed as a quantitating reagent. Following TLC in acetone, the phenyl carbonate derivative was identified by using iodine vapor and analyzed for ^{3}H/^{14}C by sample oxidation. As shown in Figure 4, the standard curve generated was linear, passed through the origin, and showed good reproducibility. The fact that the slope of the line is <45° is attributed to the fact that the dolichyl phosphate employed was only 80–90% pure with respect to phosphate.

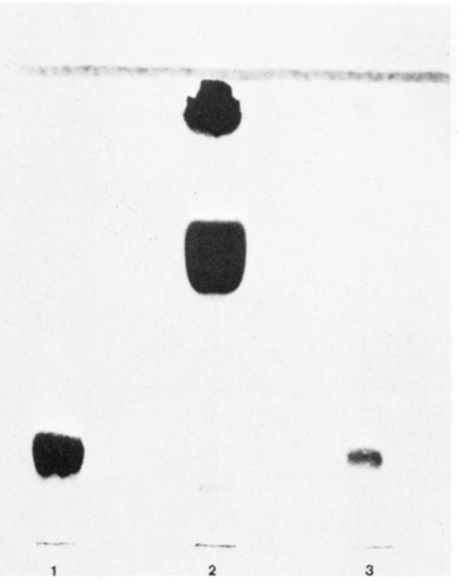


FIGURE 5: Monitoring of purification of dolichyl phosphate by thin-layer chromatography (solvent D). (Lane 1) Standard dolichyl phosphate (5 nmol). (Lane 2) Aliquot of acid-hydrolyzed, saponified extract prior to DEAE-cellulose chromatography. The material spotted contained ~1 mg of lipid. (Lane 3) Sample after elution from DEAE-cellulose. Detection was with anisaldehyde.

Determination of Dolichyl Phosphate Levels in Rat Liver. Multiple extraction techniques must be employed in order to recover all the naturally occurring derivatives of dolichyl phosphate. The procedure developed by Spiro et al. (1976) was used to extract free dolichyl phosphate, glycosylated dolichyl phosphate, and glycosylated dolichyl pyrophosphate from rat liver. These multiple extracts were pooled and subjected to acid hydrolysis to convert the derivatives to free dolichyl phosphate and base hydrolysis to saponify contaminating phospholipids.

Following saponification and aqueous extraction, TLC in solvent D followed by anisaldehyde staining indicated two major fast moving bands and a single minor band ($R_f = 0.1$) which also stained positive for phosphate and comigrated with sphingomyelin (dolichyl phosphate stains very poorly with the molybdenum spray used to detect phospholipids). Ion-exchange chromatography removed the bulk of these contaminating lipids: TLC of the pooled sample eluted from DEAE-cellulose with 0.1 M CH_3COONH_4 showed a major band comigrating with [^{3}H]dolichyl phosphate ($R_f = 0.2$) and a few minor, faster moving bands (Figure 5). At this stage, the sample was of sufficient purity for derivatization with [^{14}C]phenyl chloroformate. Following reaction under the standard conditions, the double-labeled derivative was isolated by TLC, subjected to sample oxidation, and counted for ^{3}H and ^{14}C. The amount of dolichyl phosphate present in the original sample was then calculated according to the equation given under Experimental Procedures. The final overall yield of tritium was in the range of 10–20%. A mean value of 2.9 ± 0.9 nmol of dolichyl phosphate/g of rat liver was obtained from single determinations carried out on livers from six separate animals. When duplicate determinations were performed on a single liver, the values were found to agree within 6%.

In a separate experiment, an organic extract from 3 g of rat liver containing [^{3}H]dolichyl phosphate tracer was divided into three equal parts. One part received nothing while the second and third parts received 5 and 20 nmol of dolichyl phosphate, respectively. Dolichyl phosphate was then isolated and quantitated from each of the samples by using the present

Table II: Quantitation of Dolichyl Phosphate in Rat Liver[a]

sample	total dolichyl phosphate assayed (nmol·g^{-1})	exogenous dolichyl phosphate assayed (nmol)
liver extract	2.8	
liver extract + 5 nmol of dolichyl phosphate	8.2	5.4
liver extract + 20 nmol of dolichyl phosphate	21.6	18.8

[a] For details, see text.

procedure. As expected, the levels found in the samples which received exogenous dolichyl phosphate were the sum of the tissue level and the amount added (Table II), indicating that [^{3}H]dolichyl phosphate is valid as a recovery standard when employing tissue samples.

Discussion

The purpose of the present work was 2-fold. First, we wished to develop a specific quantitative assay for nanomolar amounts of dolichyl phosphate. Second, a purification procedure was required which would yield preparations of dolichyl phosphate of sufficient purity to be used in the assay.

The use of [^{14}C]phenyl chloroformate to assay dolichyl phosphate has the advantage of producing an easily isolable derivative which can be quantitated by liquid scintillation counting. The sensitivity of the assay depends on the specific activity of the [^{14}C]phenyl chloroformate and the extent of conversion of dolichyl phosphate to the derivative. By use of our highest specific activity material (87 mCi/mmol), 1 pmol of derivative would correspond to 128 cpm at the counting efficiency of the fluor used in the sample oxidizer (67%). If 500 ^{14}C cpm can be taken as a satisfactory counting level, the assay detects 4 pmol of derivative. Assuming a 50% conversion to derivative, 8 pmol must be present in the original reaction. This is ~100 times more sensitive than a nonspecific assay for organic phosphorus (Ames, 1966). It should be noted, however, that the use of high specific activity material necessarily increases the cost of the assay, since more radioactivity must be added to achieve a given mass of phenyl chloroformate.

With respect to purification, the procedure of Spiro et al. (1976) was chosen to effect initial extraction of dolichyl phosphate and its pyrophosphate derivatives. Although we cannot be certain that the extraction of endogenous dolichyl phosphate is quantitative by using this procedure, it is reassuring that the labeled tracer, [^{3}H]dolichyl phosphate, is recovered in >95% yield in the initial extracts. Following acid hydrolysis, saponification, and aqueous extraction, the major remaining lipids by TLC analysis appear to be sterols, free fatty acids (liberated from triglycerides and phospholipids), and sphingomyelin. The bulk of these lipids is removed during DEAE-cellulose chromatography. It should be noted that DEAE-cellulose has previously been employed for the purification of dolichyl phosphate (Behrens & Tabora, 1978). However, because of the elution sequence employed, the product as isolated contained several contaminants. We have found that these contaminants can be selectively removed by eluting the column with solvent G. The ability of DEAE-cellulose to purify dolichyl phosphate by using the elution procedure described herein is illustrated in Figure 5. Because of overloading, it is not possible to detect dolichyl phosphate in the extract applied to the DEAE-cellulose column by TLC analysis. However, when 2 mL of the extract, representing 1 g of liver and containing 30 mg of lipid, is applied to the column and chromatographed, the major band seen on TLC of the pooled fractions from the CH_3COONH_4 eluate is dolichyl phosphate.

The availability of a specific sensitive assay for dolichyl phosphate will allow several important studies to be carried out. For example, we plan to modify our extraction and purification procedures in order to quantitate the amount of free dolichyl phosphate and that to which is bound the various glycosyl residues. In addition, we plan to investigate directly whether the levels of dolichyl phosphate fluctuate during induction of dolichol-mediated glycosylation.

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