We examined changes in the levels of the dolichol forms in Chinese hamster ovary cells containing alterations in the levels of activity of two enzymes in the oligosaccharyl-P-P-dolichol biosynthetic pathway, namely UDP-GlcNAc:dolichyl phosphate:GlcNAc-phosphotransferase (GlcNAc-1-phosphotransferase) and mannosylphosphoryldolichol (Man-P-Dol) synthase. Under normal conditions in wild type cells, Glc₆Man₆GlcNAc₆-pyrophosphoryldolichol was the most abundant form. Of the other anionic forms of dolichols, dolichyl phosphate, Man-P-Dol, glucosylphosphoryldolichol, and Man₆GlcNAc₆-pyrophosphoryldolichol were approximately equally abundant. When 3E11 cells (a tunicamycin-resistant Chinese hamster ovary line containing 15 times more GlcNAc-1-phosphotransferase activity than wild type), B4-2-1 cells (a mutant lacking Man-P-Dol synthase activity), and wild type cells incubated with or without tunicamycin were utilized, significant changes in the levels of most of the anionic dolichol derivatives, with the exception of dolichyl phosphate, were found. Since changes in dolichyl phosphate levels were not detected under a variety of conditions where the levels of enzyme activity utilizing this substrate were varied, all three enzymes appear to have access to the same pool of dolichyl phosphate, and further, to have similar $K_m$ values for dolichyl phosphate.

$N$-Linked glycosylation of proteins is a complex multistep process. Oligosaccharides to be attached to asparagine residues are first assembled on a long-chain polyisoprenoid lipid called dolichyl phosphate. The amount of dolichyl phosphate in cells and tissues is thought to be an important factor in determining the amount of flux through the glycosylation pathway (Lucas and Levin, 1977; Carson and Lennarz, 1981; Carson et al., 1983; Ekstrom et al., 1982; Kousvelari et al., 1983; Spiro and Spiro, 1986; Carson et al., 1987). Also, the level of glycosylated intermediates seems to be important in determining the level of glycosylation (Carson and Lennarz, 1981). Therefore, to understand the regulation of glycosylation, it is necessary to determine the steady state levels of the various dolichol derivatives in cells under a variety of conditions.

Most studies of the dolichol intermediates to date have used sugar precursors to follow the metabolism of these lipids (Lucas and Levin, 1977; Krag, 1979; Carson et al., 1987). Using this approach, levels of the unglycosylated dolichyl intermediates cannot be determined. Also, using this approach, one cannot directly compare the levels of the glycosylated intermediates such as mannosylphosphoryldolichol (Man-P-Dol), glucosylphosphoryldolichol (Glc-P-Dol), and N-acetylgalactosaminylphosphoryldolichol (GlcNAc-P-P-Dol), since they are not all labeled when one uses a single sugar precursor.

In order to address these issues, it is preferable to follow these molecules by labeling the lipid moiety rather than the saccharide moiety. A metabolic precursor of the lipid moiety in euukaryotic cells is mevalonate (Brown and Goldstein, 1990). One problem inherent in the use of mevalonate to label dolichol derivatives is the separation of these molecules from the major metabolites of mevalonate in cells, cholesterol and ubiquinone. In addition, it is necessary to use a variety of techniques to separate the various forms of dolichol-related molecules, acylated, phosphorylated, and glycosylated from one another. However, the use of labeled mevalonate as a precursor permits the simultaneous analysis of all the forms of dolichol in cells, in contrast to the use of labeled sugars.

In this study, we determined the steady state levels of all dolichol intermediates in Chinese hamster ovary (CHO) cells under conditions where the levels of activity of two key glycosylation enzymes, namely UDP-GlcNAc:dolichyl phosphate:GlcNAc-phosphotransferase (GlcNAc-1-phosphotransferase) and Man-P-Dol synthase were varied. Implications for the role of dolichyl phosphate in the regulation of glycosylation in cells is discussed.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—The mevalonate incubation conditions were established with a proline-auxotrophic clone of CHO cells, Pro⁻⁵, which was obtained from Dr. Pamela Stanley (Albert Einstein College of Medicine, Bronx, NY). WT-E6 (another proline-auxotrophic clone) and Pro⁺⁵ were used to analyze dolichol-containing lipids in

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†Current address: Carnegie Institution of Washington, Dept. of Embryology, 110 W. University Parkway, Baltimore, MD 21210.

‡Current address: Laboratory of Neurosciences, National Institute on Aging, NIH, Bethesda, MD 20892.

§To whom correspondence should be addressed: Dept. of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205. Tel.: 301-955-3869.

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1 The abbreviations used are: Man-P-Dol, mannosylphosphoryldolichol; Glc-P-Dol, glucosylphosphoryldolichol; GlcNAc-P-P-Dol, N-acetylgalactosaminylphosphoryldolichol; CHO, Chinese hamster ovary; TM, tunicamycin; HPLC, high pressure liquid chromatography.

2 Portions of this paper (including part of "Experimental Procedures," part of "Results," Figs. 2-6, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
The presence and absence of the glycosylation inhibitor, tunicamycin (TM) (see below), a TM-resistant cell line selected from non-glycosylated CHO cells in the presence and absence of the glycosylation inhibitor, tunicamycin (*see above*), was cultured and passaged either with or without the addition of 27 μg/ml TM (from a 1000 x stock in 0.01 N NaOH). B-4-2-1, a Man-P-Dol synthase-deficient cell line and WTB, the prolactin-auxotrophic clone from which it was derived, were also used (Stoll et al., 1989). The cells were cultured, passaged, and counted as described previously (Krag, 1979; Rosenwald et al., 1988; Stoll et al., 1988). Cells were routinely checked for mycoplasma contamination (Becker and Levine, 1979) and found to be negative.

**Materials**—Cholesteryl ester hydrolase and cholesterol oxidase were from P. Worthington (Freehold, NJ). Mannheim lipase (E. Coli, p-n-tridecanoyl-sn-glycerol specific activity 40 units/mg), phospholipase A2 (C. sphenoides, 600 units/mg), phospholipase C (C. sphenoides, 100 units/mg), p-n-tridecanoyl-sn-glycerol phosphatase, p-hydroxyphenylacetic acid, and polyoxyethylene-9-lauryl ether were from Sigma. Chromatography standards (coenzyme Q₁₀, coenzyme Q₁₁, cholesterol, cholesteryl oleate, porcine liver dolichol (dolichol-32), dolichyl monophosphate, grade III, and cis-a-unsaturated polyenoyl,) were also obtained from Sigma. Polyenol (polyenol-12:0, 14:0) was isolated from leaves of Prunus serrata var. capsulif as described (Chojnacki and Vogtman, 1984). [³⁵S]Man-P-Dol, [¹⁴C]Glc-P-Dol, and N-acetyl[¹³C]glucosaminylphosphoryldolichol were synthesized as described previously (Waldman, Oliver, and Krag, 1987) using GDP-[¹³C]Man, UDP-[¹⁴C]Glc, or UDP-[³⁵S]GlcGlcNac, respectively, and dolichol phosphate in the presence of detergent-solubilized CHO membranes. These assays were used to determine the specific activities of the relevant enzymes in cells grown in the presence or absence of TM. Oligosaccharidyl-P-P-Dol standards were obtained by incubating cells with [²⁵⁴H]Man for 60 min and then extracting as described previously (Krag and Robbins, 1977). All solvents were from J. T. Baker Chemical Co. (Phillipsburg, NJ) except disobutyl ketone (2,6-dimethyl-4-heptanone) which was from Eastman Kodak. All solvents were reagent grade, except as noted. Mevinolin was the gift of Dr. April R. Robbins (National Institutes of Health). TM was a gift from Dr. J. B. Pouras, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. RS-2-[⁸²⁴H]Mevalonate (1.28 Ci/mmol) was from Amersham Corp. Mevalonate phosphate (24-26 Ci/mmol) was from Du Pont/New England Nuclear. Mevalonate labeled at the two different positions gave identical results.

**Incubation of Cells with [¹⁴C]Mevalonate and Extraction of Labeled Lipids**—Cells were plated one day prior to addition of label to 10-cm dishes in a minimum medium containing 10% fetal bovine serum. Cells were incubated with either 10 μl of labeling medium or mock-labeling medium for up to 72 hr. Labeling medium was a minimum medium essential with 10% fetal bovine serum, 20 μCi/ml [²⁵⁴H]- or [⁸²⁴H]mevalonate (0.30 mM final concentration), and 12 μg/ml mevinolin. Mevinolin was added from a 4 mg/ml stock in either dimethyl sulfoxide or 0.1 N NaOH. In some experiments, Pro-5, WT-CHO cells were incubated with 1 μg/ml TM for the last 12 hr of the 72-h incubation period. Cells were then washed with water to remove water-soluble radioactivity, and finally, the lipids soluble in chloroform/methanol/water (10/10/3) were extracted. Dishes incubated with mock-labeling medium were used for determining the amount of total cholesterol (free cholesterol plus cholesteryl esters). The lipids solubilized in chloroform/methanol/0.1 N HCl were extracted from the cells with water to remove water-soluble radioactivity, and finally, the lipids soluble in chloroform/methanol/water (10/10/3, v/v/v) were extracted. Dishes incubated with chloroform/methanol/0.1 N HCl were extracted. Dishes incubated with mock-labeling medium were used for determining the amount of total cholesterol, mevalonate, mevinolin, and TM (or NaOH alone), but no radioactivity. Normalization of data to cell number, cell protein, or total cell cholesterol content gave similar results. Routinely, normalization of data to cell number was performed.

**Reverse-phase HPLC**—The labeled lipid molecules were subjected to ion-exchange chromatography on 3 ml DEAE-cellose (Schleicher and Schuell) (acetate form) equilibrated in either chloroform/methanol (2/1, v/v) or in chloroform/methanol/H₂O 10/10/3 (Behrens and Tabora, 1978). Neutral lipids were washed with the column with a total of 20 ml of the equilibrating solvent. Anionic lipids were eluted with 250 mM ammonium acetate in the equilibrating solvent unless indicated otherwise. Gel filtration chromatography of neutral lipids was achieved with a 1 x 45-cm gel filtration column (Froptogel HW 40F, E M Science, Cherry Hill, NJ) equilibrated in chloroform/methanol (1/1, v/v) containing 50 mM ammonium acetate. Four-tenths-ml fractions were collected. Internal standards were detected by anilin in solvent system A (see below).

Plastic-backed silica TLC plates (5 x 20 cm) were from J. T. Baker Chemical Co. All solvent mixtures were v/v. For separation of neutral molecules, solvent system A (hexane/diethyl ether/glacial acetic acid 70/30/1.5), B (toluene/ethyl acetate 95/5), and C (hexane/diethyl ether 9/1) were used. For separation of anionic lipids, solvent systems D (chloroform/methanol/water 80/45/5), E (chloroform/methanol/water 60/25/4), and F (chloroform/methanol/ammonium hydroxide 72/26/6) were used. Solvent system G (chloroform/methanol/ammonium hydroxide/water 60/25/2) was used for the separation of anionic lipids on SG-81 paper (Whatman, Clifton, NJ) pretreated once with 2.5% EDTA and twice with 0.4 M boric acid. For separation of oligosaccharidyl-P-P-Dols by silica TLC, solvent system H (chloroform/methanol/water 10/10/3) was used. Standards on tlc were detected either by spraying plates with a mixture of ninhydrin/ammonium/h₂so₄, 1/18/1) and heating to 110°C for 5-10 min (Dunphys et al., 1967) or by 5-10-min incubation in a chamber saturated with L₂ vapor. Standards on paper were detected by spraying the chromatogram with a 10% acetic acid solution of ninhydrin detected either by spraying the chromatogram with Enhance (Du Pont New England Nuclear) and exposing the chromatogram to x-ray film (X-O-Mat AR, Eastman-Kodak) or cutting the chromatogram into slices and eluting the counts from the matrix either directly with scintillation fluid (Liquidscint, National Diagnostics, East Palatine, FL) for neutral lipids (with prior soaking in 1% sodium dodecyl sulfate or methanol/water 1/1 for 1 h at 60°C for anionic lipids and oligosaccharidyl-P-P-Dols).

Reverse-phase HPLC was performed on C₁₈ column (4.6 mm x 25 cm; Partisil-10/25 PSX, Whatman) (Ekstrom et al., 1984). The column was run in solvent system I (methanol/n-propanol/hexane 80/10/10, v/v/v, 50 min, for 5 min, for 10 min, for 10 min) and solvent system J (hexane/diethyl ether 97/3) (Keller et al., 1982). Both HPLC separations were carried out on either a Varian 5000 HPLC or a Dionex BioLC at flow rates of 1 ml/min. All solvents were HPLC grade and were from J. T. Baker Chemical Co. with the exception of n-propanol which was from American Burdick-Jackson (Muskegon, MI). Internal cold standards were detected by absorbance at 214 nm with a Varian UV-50 detector or an internal Dionex UV detector.

**Determination of Mass of Cholesterol and Cholesteryl Esters in Cells**—The cholesterol and cholesteryl esters in cells were determined as given by Heider and Boyett (1978) with several modifications. Briefly, 10⁵-10⁶ cells were suspended in 20 μl of isopropanol (spectrophotometric grade). To determine the amount of free cholesterol, 0.4 ml of reagent A was added (0.05 M sodium phosphate, pH 7.0, cholesterol oxidase, 0.08 unit/ml, horseradish peroxidase, 30 units/ml, and p-hydroxyphenylacetic acid, 0.15 mg/ml). To determine the amount of total cholesterol (free cholesterol plus cholesteryl esters), 0.4 ml of reagent B was added (reagent A plus cholesterol ester hydrolase, 0.08 unit/ml, Triton X-100, 0.008%, and polyoxyethylene-9-lauryl ether, 0.0025%). All tubes were incubated for 30 min at 37°C. The reaction was terminated by the addition of 0.5 ml of 0.5 M NaOH. The pellet of cholesterol esters was determined by subtracting the value obtained for free cholesterol from that obtained for total cholesterol. The fluorescence of the samples was determined at an excitation wavelength of 320 nm and an emission wavelength of 415 nm. Standards were cholesterol and cholesteryl oleate.

**Preparation of [³⁵S]Mevalonate-labeled glycosylated and phosphorylated long-chain cholesterol and cholesteryl esters**—Cholesterol was treated with mild acid (freshly prepared 0.1 N HCl in 80% tetrahydrofuran (Gold Label, Aldrich, Milwaukee, WI) for 2 h at 50°C (Stoll et al., 1988) or by saponification for 1 h at 100°C (Adair and Keller, 1985) (as described except the methanolic KOH included one drop of concentrated hydrochloric acid). The labeled cholesterol was transformed to the neutral lipids by treatment with wheat germ acid phosphatase as described previously (Stoll et al., 1988), except the amount of enzyme was doubled. Protein concentration was determined by the
when the activities of one or two of these three enzymes were particular focus on dolichyl phosphate levels, in CHO cells phosphates as well as monosaccharyl phosphoryl prenols). All with [2-3H]mevalonate for varying lengths of time (4-72 h) transferase (Struck and Lennarz, 1980). In this study, we altered either by genetic means or by the inclusion of the amount of dolichyl phosphate and other dolichol-containing derivatives in cells.

RESULTS

Dolichyl phosphate is a substrate for three different enzymes in the assembly of oligosaccharide-lipid intermediates involved in the synthesis of N-linked glycans. These are Man-P-Dol synthase, Glc-P-Dol synthase, and GlcNAc-1-phosphotransferase (Struck and Lennarz, 1980). In this study, we determined the levels of the various dolichyl derivatives, with particular focus on dolichyl phosphate levels, in CHO cells when the activities of one or two of these three enzymes were altered either by genetic means or by the inclusion of the GlcNAc 1-phosphotransferase inhibitor, TM. In order to perform this study, we developed a procedure to determine the amounts of dolichyl phosphate and other dolichol-containing derivatives in cells.

Steady State Labeling of Polyisoprenoid Lipids and Determination of Dolichyl Phosphate Levels in Wild Type CHO Cells—We first established conditions for the steady state labeling of polyisoprenoid lipids in cells. Cells were incubated with [2-3H]mevalonate for varying lengths of time (4-72 h) (Fig. 1), and the lipids and oligosaccharyl-P-P-Dol were extracted from cells. The lipids were further fractionated by DEAE-cellulose chromatography as described under "Methods" into a neutral lipid fraction (which contained cholesterol, cholesteryl esters, ubiquinone, dolichol, and dolichyl esters) and an anionic lipid fraction (which contained prenyl phosphates as well as monosaccharyl phosphoryl prenols). All three fractions, neutral lipid, anionic lipid, and oligosaccharide lipid, had two rates of incorporation of [3H]mevalonate: a fast rate from 4-24 h and a slower rate from 24-72 h. Steady state labeling of all three pools was reached by 48-72 h. Five other experiments, although they differed in the absolute amount of incorporation in each fraction, showed similar kinetics. The same labeled compounds were detected in cells labeled for 24 and 72 h in approximately the same ratios (data not shown). An analysis of each of these fractions is described in the Miniprint Supplement (Figs. 2-6; Tables 1 and 2). Therefore, although some control experiments were performed by incubating cells with label. A shows the incorporation into neutral lipids (the flow-through from the DEAE-cellulose column); B, incorporation into oligosaccharyl-P-P-Dols (initially extracted into chloroform/methanol/H2O (10/10/3)); and C, incorporation into anionic lipids (the bound fraction from the DEAE-cellulose column).

For these studies we used 3Ell cells, a CHO cell line resistant chool was seen (data not shown), which suggests that phosphorylase activity did not occur, but rather utilization of dolichyl phosphate by the synthetic enzymes occurred during the longer harvesting procedure. We interpreted these data to mean that utilization of dolichyl phosphate occurred once the harvest procedure had begun. Since we were interested in accurately determining dolichyl phosphate levels, we used the rapid harvesting procedures for all subsequent experiments to prevent unwanted metabolism.

Effect of Increased Levels of GlcNAc-1-Phosphotransferase Activity on Levels of Dolichyl Derivatives—We determined the amounts of all the dolichyl forms, including dolichyl phosphate, during normal growth conditions in wild type cells. The analysis of these molecules is given in the Miniprint Supplement (Figs. 2-6; Tables 1 and 2). The single most abundant form of dolichol in cells under these conditions was GlcMan2ManGlcNAc-2-P-P-dolichol (Table 3). Although variation in incorporation into the dolichyl forms occurred among experiments, GlcMan2ManGlcNAc-2-P-P-dolichol was consistently the most abundant dolichol form in all experiments.

We were now able to determine how changes in the levels of different enzymes in the pathway affected the levels of dolichyl intermediates. First, we examined cells which contained different levels of the first enzyme in the synthesis of oligosaccharide-lipids, namely GlcNAc-1-phosphotransferase. For these studies we used 3Ell1 cells, a CHO cell line resistant
Fig. 7. Thin-layer chromatographic analysis of anionic lipids after two different methods of harvest. Cells (4.2 x 10^6/dish; two dishes for each treatment) were incubated for 24 h with [2-^3H] mevalonate as described under “Experimental Procedures.” The cells were harvested either by the methods described under “Experimental Procedures” or by an alternative method which was to remove the labeling medium from the cells, wash the monolayer four times with phosphate-buffered saline at ambient temperature, and remove the cells from the dish with 1 ml trypsin/EDTA for 10 min at 34 °C. The cells were diluted with 10 ml of solution A, transferred to a 16 ml glass conical tube, and pelleted by centrifugation at 1000 g for 5 min. After the supernatant fluid was removed, the cells were lysed by the addition of 1 ml of ice-cold methanol. After this point, the cells harvested by the two methods were treated identically. The lipids were extracted, and the anionic lipids were obtained as described under “Experimental Procedures.” The samples were desalted by extraction, concentrated under a stream of N2, resuspended in a small volume of chloroform/methanol (2/1, v/v), spotted onto a silica tlc plate, and separated in solvent system D. This solvent system separates dolichyl phosphate from monosaccharyl-D-P-Dols, but does not separate Glc-P-Dol from Man-P-Dol. The dried chromatograms were cut into 0.5 cm slices, soaked in 0.5 ml of 1% sodium dodecyl sulfate, and the slices were counted after the addition of scintillation fluid. The data shown are expressed as counts/min incorporated per million cells and has been corrected for recovery. Recoveries were 56% for A and 41% for B. The standards were: N = GlcNAc-P-P-Dol, M = Man-P-Dol, and D = dolichyl phosphate. O indicates the origin of the tlc, and F indicates the solvent front.

to TM, an inhibitor of the GlcNAc-1-phosphotransferase (Criscuolo and Krag, 1982; Waldman et al., 1987). 3Ell cells grown without TM have 15-fold higher levels of GlcNAc-1-phosphotransferase activity found in membranes prepared from these cells compared with membranes of wild type cells. Enzyme levels of 3Ell cells grown in the presence of TM cannot be measured (Waldman et al., 1987).

The relative amounts of the various dolichyl forms in wild type cells, 3Ell cells without TM, and 3Ell cells grown in the presence of drug are compared in Table 4. If the amount of GlcNAc-1-phosphotransferase activity was limiting under normal conditions, one might expect that in 3Ell cells grown without the TM, with 15 times the normal cellular activity, that GlcNAc-P-P-dolichol or other oligosaccharide-P-P-Dols might accumulate or the overall glycosylation rate might increase. In 3Ell cells grown without drug, the relative amount of oligosaccharyl-P-P-Dol increased at the expense of dolichyl phosphate, Glc-P-Dol and Man-P-Dol compared to the parent wild type cells (Table 4). Neither GlcNAc-P-P-dolichol nor GlcNAc-GlcNAc-P-P-dolichol was detected. The rate of mannose incorporation into protein and the rate of protein synthesis were similar in wild type and 3Ell cells (data not shown). Therefore, it appeared that a substantial increase in GlcNAc-1-phosphotransferase activity only slightly altered the steady state levels of the intermediates and did not increase overall glycosylation.

Growing 3Ell cells in the presence of tunicamycin, which does not alter the amount of mRNA but presumably decreases the amount of active transferase in the membranes, greatly alters the steady state levels of the dolichyl derivatives (Table 4). As stated above, 3Ell cells grown without drug have 15-fold higher levels of GlcNAc-1-phosphotransferase activity as do wild type cells. 3Ell cells grown in the presence of TM have a level of GlcNAc-1-phosphotransferase activity sufficient to grow normally and mannosylate proteins (Criscuolo and Krag, 1982). However, we cannot determine the precise amount of active enzyme using in vitro experiments (Waldman et al., 1987). As predicted from the mode of action of TM (Takasugi et al., 1975, Tanaka and Lau, 1975; Lehle and Tanner, 1976), the relative level of oligosaccharyl-P-P-Dol decreased significantly, presumably due to inhibition of the over expressed GlcNAc-1-phosphotransferase in 3Ell cells. However, the expected increase in the relative level of dolichyl phosphate, the substrate of the transferase, was not detected. Instead, the relative levels of Man-P-Dol and Glc-P-Dol increased 3.5 fold when 3Ell cells were cultured in TM, total amounts of dolichyl derivatives increased 2.5-fold when the data are normalized to cell number and the amounts of total lipid synthesized from mevalonate increased 1.5-fold. Protein synthesis and mannosylation rates were similar in

### Table 3

| Relative amounts of the polysaccharides in Chinese hamster ovary cells |
|-----------------------------|-----------------------------|
| Amounts of polysaccharides were determined as described under “Experimental Procedures” and the Miniprint Supplement from cells incubated with [3H]mevalonate for 72 h as described under “Experimental Procedures.” The data shown were normalized for cell number and the number of determinations for each is shown. Man-P-Dol is mannosylphosphoryldolichol and Glc-P-Dol is glucosylphosphoryldolichol. When the radioactivity extracted as oligosaccharyl-P-P-Dol was separated on tlc (Rosenwald and Krag, 1980), 60–70% of the material comigrated with known compounds and are identified in this table. The unidentified material (30–40%) in this fraction migrated near the solvent front. Complete analysis of this fraction is given in the Miniprint Supplement. |

<table>
<thead>
<tr>
<th>Relative amounts</th>
<th>Counts/min per million cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipid</td>
<td>15,800 ± 3,200 (n = 11)</td>
</tr>
<tr>
<td>Oligosaccharyl-P-P-Dol</td>
<td>1,270 ± 340 (n = 8)</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>14,400 ± 3,100 (n = 11)</td>
</tr>
<tr>
<td>Anionic lipid</td>
<td>1,570 ± 530 (n = 11)</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>690 ± 220 (n = 6)</td>
</tr>
<tr>
<td>Dolichyl phosphate</td>
<td>270 ± 110 (n = 3)</td>
</tr>
<tr>
<td>Man-P-Dol</td>
<td>260 ± 100 (n = 3)</td>
</tr>
<tr>
<td>Glc-P-Dol</td>
<td>390 ± 50 (n = 3)</td>
</tr>
<tr>
<td>Oligosaccharyl-P-P-Dol</td>
<td>190 ± 100 (n = 5)</td>
</tr>
<tr>
<td>GlcManGlcNAc-P-P-dolichol</td>
<td>580 ± 210 (n = 5)</td>
</tr>
</tbody>
</table>

3 J. R. Scocca and S. S. Krag, manuscript in preparation.
TABLE 4
Relative amounts of dolichol metabolites in cells expressing different amounts of active GlcNAC-1-phosphotransferase

Dolichols were labeled by incubating cells with [3H]mevalonate for 72 h either with or without 27 μg/ml TM for the entire labeling period (part A) or with or without 1 μg/ml TM for the last 12 h of the labeling period (part B) as described under “Experimental Procedures.” The addition of TM had no effect on the rate of incorporation of either [3H]methionine or [2-3H]mannose into protein in 3Ell cells. The addition of TM to wild type cells reduced the rate of incorporation of [2-3H]mannose into protein 10-fold with a minimal effect (20-40%) on the rate of incorporation of [3H]methionine into protein. Dolichol intermediates were extracted and analyzed as described under “Experimental Procedures” and the Miniprint Supplement. The total incorporation into dolichols was determined and set to 100%. The data shown is percent of the total for each intermediate. Part A, incorporation into total neutral lipids (chiefly cholesterol, cholesteryl esters, and ubiquinones) was 18,130 cpm/million cells for wild type cells (clone E78), 31,000 cpm/million cells for 3Ell - TM, and 42,250 cpm/million cells for 3Ell + TM (n = 4). Total incorporation into dolichols was 2,270 cpm/million cells for wild type cells, 4,165 cpm/million cells for 3Ell - TM, and 10,820 cpm/million cells for 3Ell + TM (n = 2). Part B, incorporation into total neutral lipids was 14,830 cpm/million cells for wild type cells (Pro-5) (n = 3) and 18,750 cpm/million cells for wild type cells + TM (n = 2). Total incorporation into dolichols was 3,380 cpm/million cells for wild type cells (n = 3) and 4,237 cpm/million cells for wild type cells + TM (n = 2).

A. Expressing increased transferase activity

<table>
<thead>
<tr>
<th>Dolichol derivative</th>
<th>Cell type</th>
<th>3Ell</th>
<th>3Ell + TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolichol + dolichyl esters</td>
<td>Wild type</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Dolichyl phosphate</td>
<td>11</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Man-P-dolichol</td>
<td>9</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td>Glc-P-dolichol</td>
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<td>42</td>
</tr>
<tr>
<td>Oligosaccharyl-P-P-Dol</td>
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<td>66</td>
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</table>

B. Expressing decreased transferase activity

<table>
<thead>
<tr>
<th>Dolichol derivative</th>
<th>Cell type</th>
<th>Wild type</th>
<th>Wild type + TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolichol + dolichyl esters</td>
<td>15</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Dolichyl phosphate</td>
<td>10</td>
<td>15</td>
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</tr>
<tr>
<td>Man-P-dolichol</td>
<td>14</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Glc-P-dolichol</td>
<td>21</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Oligosaccharyl-hexosil-poly</td>
<td>40</td>
<td>6</td>
<td></td>
</tr>
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</table>

3Ell cells in the presence and absence of TM (data not shown).

Since the most striking effect of adding TM to 3Ell cells was the increase in Man- and Glc-P-Dol, several additional experiments were performed to show this was a specific effect involving changes in the levels of dolichol derivatives. First, we confirmed that the increased amount of material in the anionic lipid fraction was long-chain dolichol, not short-chain derivatives (see Miniprint Supplement) or unsaturated poly-prenyl derivatives (data not shown). Second, we determined that there was no increase in the activity of Man-P-Dol synthase in membranes of 3Ell in the presence of drug. Glc-P-Dol synthase was 10-fold lower in activity in membranes of 3Ell cells grown in the presence of tunicamycin than in membranes of 3Ell cells grown without drug. By mixing membranes from cells grown in the presence and absence of the drug, it appeared that TM inhibited the Glc-P-Dol synthase activity, since the mixture had less activity than would be expected for that amount of membranes prepared from cells grown in the absence of TM (data not shown). The inhibition of Glc-P-Dol synthase by TM has been reported earlier by Elbein et al. (1979). Despite less Glc-P-Dol synthase activity in detergent extracts, the relative level of Glc-P-Dol increased dramatically in 3Ell cells grown in the presence of TM (Table 4). Therefore, the increases in the amounts of Man- and Glc-P-Dol seem to be a specific effect involving changes in the levels of the dolichol derivatives.

Effect of Inhibiting GlcNAC-P-Transferase in Wild Type Cells by TM—We predicted that a short-term treatment of wild type cells with TM would lead to a decrease in the amount of oligosaccharyl-P-P-Dol and an increase in the level of dolichyl phosphate. As shown in Table 4, the relative level of oligosaccharyl-P-P-Dol did in fact decrease 7-fold. However, as we had seen above in 3Ell cells, there was not a compensatory increase in the level of dolichyl phosphate. Instead, there was a modest increase in the level of dolichyl phosphate and larger increases in the levels of Man-P-Dol and Glc-P-Dol (Table 4). Again, we confirmed that the increase detected in the anionic lipid fraction was an increase in long-chain dolichols, not short-chain intermediates. Also, there was no increase in the amount of activity of Man-P-Dol synthase or Glc-P-Dol synthase in the membranes of cells treated with TM, which might have accounted for the increased levels of Man-P-Dol and Glc-P-Dol. Therefore, it appeared that in the presence of TM, as oligosaccharyl-P-P-Dol is used for glycosylation, it is not readily resynthesized, thus the steady state level of this intermediate decreased and the resultant dolichyl phosphate was primarily shunted into monosaccharyl-P-lipids.

Effect of Reduced Man-P-Dol Synthase Activity on Levels of Dolichyl Derivatives—We determined the relative levels of the various dolichyl derivatives in wild type cells and in B4-2-1 cells (Table 5). B4-2-1 cells have reduced Man-P-Dol synthase activity compared with wild type cells (Stoll et al., 1982). Again, one might have predicted that B4-2-1 cells would have higher dolichyl phosphate levels than wild type cells, since one of the enzymes which utilize this substrate is missing. However, as shown in Table 5, the relative amounts of dolichyl phosphate in wild type and mutant cells were identical. However, B4-2-1 cells have higher levels of oligosaccharyl-P-P-Dol. When treated with TM to reduce these levels of oligosaccharyl-P-P-Dol, the relative amount of Glc-P-Dol increased rather than the level of dolichyl phosphate.

In summary, all of the data indicated that dolichyl phosphate levels in cells are buffered against a change in concen-

TABLE 5
Relative amounts of dolichol derivatives in cells expressing different amounts of active mannosylphosphoryldolichol synthase

Dolichols were labeled by incubating cells with [3H]mevalonate for 72 h either with or without 1 μg/ml TM for the last 12 h of the labeling period as described under “Experimental Procedures.” The addition of TM to B4-2-1 cells reduced the rate of [2-3H]mannose incorporation into protein 10-fold whereas minimally reducing (10%) the rate of incorporation of [3H]methionine into protein. Dolichol intermediates were extracted and analyzed as described under “Experimental Procedures” and the Miniprint Supplement. The total incorporation into dolichols was 18,750 cpm/million cells for wild type cells (clone NIH) (n = 3), 17,680 cpm/million cells for B4-2-1 cells (n = 5), and 24,850 cpm/million cells for B4-2-1 cells + TM (n = 2). Total incorporations into dolichols was 2,515 cpm/million cells for wild type cells, 4,237 cpm/million cells for wild type cells (data not shown). Dolichol + dolichyl esters 16 15 15
Dolichyl phosphate 14 16 20
Man-P-dolichol 14 2 4
Glc-P-dolichol 29 30 54
Oligosaccharyl-P-P-Dol 26 38 7
tion by its availability to three biosynthetic enzymes which, therefore, appear to share a common pool of dolichyl phosphate. A transient increase in the amount of dolichyl phosphate above a certain level resulted in an increase in the level of Man-P-Dol, Glc-P-Dol, or oligosaccharyl-P-P-Dol.

**DISCUSSION**

In this study, we describe a procedure to label dolichol and its many forms with [3H]mevalonate to steady state levels in continuously growing CHO cells. Mevalonate is a substrate for the synthesis of dolichol, a long-chain polyisoprenoid lipid (Brown and Goldstein, 1980; Betsyia and Porter, 1976). The phosphorylated derivative, dolichyl phosphate, is an important precursor in the synthesis of the lipid-linked oligosaccharide intermediates. These oligosaccharides are transferred en bloc after their assembly on dolichyl phosphate to the asparagine residues of nascent proteins (Struck and Lennarz, 1980). This study was undertaken in an effort to characterize the role of dolichyl phosphate and its many glycosylated derivatives in the regulation of glycosylation. Although cholesterol, cholesteryl esters, and ubiquinone are the major quantitative metabolites of [3H]mevalonate (see Miniprint Supplement), we found that it was possible to analyze dolichol, its acyl esters, and its phosphorylated and glycosylated forms. Steady state labeling of dolichol metabolites has been described previously by incubating insect cells, Drosophila Kc, cells, with [3H]mevalonate (Sagami and Lennarz, 1987), but this work is the first report of an analysis of individual dolichol metabolites in mammalian cells.

The amount of dolichyl phosphate is thought to be an important factor in determining the extent of N-linked glycosylation that occurs in cells and tissues. Evidence for this hypothesis has come from many different lines of investigation. For example, it has been found that addition of exogenous dolichyl phosphate to cells and tissue slices increases the level of glycosylation (Carmi et al., 1981, Ekstrom et al., 1982; Kousvelari et al., 1983). Also it has been shown that when thyroid microsomes are given dolichyl phosphate directly, or agents that allow dolichyl phosphate to recycle (i.e. glycosylation acceptor peptides), the amount of glycosylation increases (Spiro and Spiro, 1986). The extent of glycosylation also appears to increase in response to hormonal stimulation (Lucas and Levin, 1977; Carson et al., 1988). By using [3H]-labeled sugars as tracers, these workers indirectly inferred that the increase in glycosylation was the result of an increase in dolichyl phosphate concentration because they were unable to demonstrate increases in the specific activities of the glycosyl P (P) synthases which use dolichyl phosphate as a substrate. The amount of dolichyl phosphate may be regulated by both changes in the amount of de novo biosynthesis and by regulating the activity of dolichol modifying enzymes, such as dolichol kinase and dolichyl phosphate phosphatase. For example, concentrations of phosphorylated dolichols appear to be modulated during progression through the cell cycle. This is accomplished by changes in the specific activities of dolichol kinase, dolichyl pyrophosphatase, and cis-prenyltransferase (Adair and Caffmeier, 1987). However, as will be discussed below, our results indicate that in CHO cells the steady state levels of dolichyl phosphate are not significantly altered under a variety of conditions.

Of the anionic polyenols, approximately half were found in the anionic lipid fraction under normal conditions (Table 3). Glc-P-Dol, Man-P-Dol, and dolichyl phosphate appeared to be approximately equally abundant. Molecules such as GlcNAc-P-P-dolichol, GlcNAc-GlcNAc-P-P-dolichol, and dolichyl pyrophosphate would be expected to be in this fraction, but mevalonate-labeled species with the mobility expected for these intermediates (Warren and Jeanloz, 1975) were not detected under normal conditions. GlcNAc-P-P-transferease is thought to be a committed step in oligosaccharyl-P-P-Dol biosynthesis (Waldman et al., 1987); therefore it is expected that the amount of GlcNAc-P-P-dolichol in cells would be very low. Dolichyl pyrophosphate is generated from transfer of oligosaccharide to protein (Struck and Lennarz, 1980) and is an intermediate in the de novo synthesis of dolichyl phosphate (Chojnacki and Dallner, 1988). In either event, a dolichyl pyrophosphatase must convert dolichyl pyrophosphate to dolichyl phosphate. Since dolichyl phosphate is detectable in cells and appears to account for approximately 10% of the total dolichols, it can be inferred that there is sufficient pyrophosphatase to convert the majority of dolichyl pyrophosphatase to dolichyl phosphate in cells. Such an enzyme has been described in lymphocytes (Wedgwood and Strominger, 1980) and in calf brain (Scher and Waechter, 1984). Occasionally, in the presence of TM, we detected a species with the expected mobility for dolichyl pyrophosphate, but this intermediate was not detected consistently.

The other half of the anionic polyenols were found in the oligosaccharide-lipid fraction. The most abundant molecule found in this fraction was GlcManGlcNAc1-P-P-Dol. This is consistent with the results from incubation of CHO cells with [3H]sugar labels (Rosenwald et al., 1989). We found that GlcManGlcNAc1-P-P-Dol was the single most abundant molecule of all the dolichol species found in CHO cells under normal conditions (Table 3). Also, oligosaccharyl-P-P-Dol accumulated in 3E11 cells cultured in the absence of TM (Table 4). These results suggest that one of the rate-limiting steps for the early stages of glycosylation of asparagine residues may be the transfer of oligosaccharides from the lipid carrier to proteins.

Our results show that a transient increase in the amount of dolichyl phosphate would result directly in an increase in the amounts of glycosylated dolichol intermediates. The amount of dolichyl phosphate did not vary significantly in CHO cells under a variety of conditions. These conditions included decreased activity of GlcNAc1-phosphotransferase by the inclusion of TM in cultures of wild type and mutant cells, increased activity of GlcNAc1-phosphotransferase in a TM-resistant cell line, 3E11, and decreased activity of Man-P-Dol synthase in the cell line, B42-1. We have also found that dolichyl phosphate levels did not vary with cell density (data not shown). Finally, we found that although the level of oligosaccharyl-P-P-Dol varied 16-fold under a variety of conditions (Tables 3–5), the rate and extent of glycosylation only varied 2-fold as measured by mannosine incorporation into protein normalized to methionine incorporation into protein (data not shown). This suggests that the activity of the oligosaccharide transferase is not the only rate-limiting step in glycosylation.

We did find, however, that there were substantial effects on the levels of the products of the reactions for which dolichyl phosphate is a substrate. Thus, it appears that the three activities, GlcNAc1-phosphotransferase, Man-P-Dol synthase, and Glc-P-Dol synthase, are generally in balance with one another. When one activity is more abundant, as is the GlcNAc1-phosphotransferase in 3E11 cells grown in the absence of TM, the levels of the product oligosaccharyl-P-Dol increase at the expense of the other two products, Glc-P-Dol and Man-P-Dol. When one activity is reduced, as is the GlcNAc1-phosphotransferase in wild type and 3E11 cells incubated in the presence of TM, oligosaccharyl P-P-Dol levels decreased while the levels of the other two products,
Man-P-Dol and Glc-P-Dol, are increased. Interestingly, the level of oligosaccharyl P-P-Dol rather than Glc P-Dol was increased in cells B4-2-1 lacking Man-P-Dol synthase (B4-2-1) relative to parental cells. Again, GlcNAc-P-P-Dol and GlcNAc-GlcNAc-P-P-Dol were not detected. When glycosylation was further altered in B4-2-1 cells by the addition of TM, so that those cells now lacked both Man-P-Dol synthase and GlcNAc-1-phosphotransferase activity, again we found that dolichyl phosphate levels were not significantly altered and the most abundant species was now Glc-P-Dol.

We have not yet been able to determine the effect of a lack of Glc-P-Dol synthase activity on the levels of the various dolichol forms. We would predict that the levels of Man-P-Dol and oligoaglycosyl P-P-Dol would increase while dolichyl phosphate levels remained unchanged. However, presently, neither a specific inhibitor nor a mutant in Glc-P-Dol synthase is available.

Our results support the hypothesis that all three enzymes use a common pool of dolichyl phosphate. The results of Spiro and Spiro (1987), using calf thyroid microsomes, also suggested this. Furthermore, dolichyl phosphate levels are maintained at a fixed concentration because this concentration is too low to be utilized effectively by the enzymes. When one enzyme utilizing dolichyl phosphate is inactive, more substrate is available and is used by the remaining activities. Glycosylation is not dependent on a single rate limiting step, but rather the amount of glycosylation appears to depend on the interplay of a number of enzymes and substrates.

Acknowledgments—We thank Jun Zou and Asad Umar for their help with development of some of the TLC procedures shown here. We also acknowledge Maria Bakaj and Dr. Paul Bachorik (The Johns Hopkins Hospital) for their help with the cholesterol oxidase assay and for sharing their modifications of the Heider and Boyett procedure and Mr. Irving Robbins who obtained the Prusius leaves for us, which were the source of the cis-a-unsaturated polyene. We thank Helen Lei for her skillful technical assistance, for preparing the samples as the experiments to determine the rate of glycosylation. Finally, we thank Dr. John Scocca for his constant help and insight.

REFERENCES

Kraü S. S. (1979) J. Biol. Chem. 254, 9167-9177
Three Enzymes Compete for Common Pool of Dolichyl Phosphate

by Anne G. Rossmann, James Stoll, and Sharon S. King

Development of the Labeling Procedure

Monodeuterated Dolichyl Phosphate (MDP) (2000 P) in the labeled enzyme pool (see Materials and Methods). Cells were labeled with 0.5 μCi of MDP and harvested in the presence of actinomycin D (0.1 μg/ml), cycloheximide (10 μg/ml), and 5 μM actinomycin D. The labeled enzymes were extracted from the cells and analyzed by gel filtration on a Sephadex G-25 column.

Results and Discussion

The results of the experiment (Table 1) demonstrated that the labeling of dolichyl phosphate in the absence of actinomycin D was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool.

Table 1: Labeling Efficiency of Cells Grown in MDP and MDP

<table>
<thead>
<tr>
<th>MDP (μg/ml)</th>
<th>MDP (μg/ml)</th>
<th>MDP (μg/ml)</th>
<th>MDP (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>1.5</td>
<td>2.5</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>0.05</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Counts per minute/million cells**

- **0**: 0
- **5**: 5
- **0.05**: 0.05
- **0.1**: 0.1
- **1.5**: 1.5
- **2.5**: 2.5
- **3.5**: 3.5
- **4.5**: 4.5
- **0.05**: 0.05
- **0.1**: 0.1
- **0.2**: 0.2
- **0.3**: 0.3
- **0.4**: 0.4

**N/P** - number of colonies recovered on day 3 from the number of cells plated on day 1.

**Colony Size** - relative size of colonies on day 3 from day 2 of growth compared to control (no MDP or unlabeled). L = large, M = medium, S = small, 1S = very small.

The data showed that the labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool. The labeling of dolichyl phosphate was at least 95% of the total pool.

Identification of Dolichyl Phosphate Enzymes in Cells

The cells were harvested in the presence of 20 mM MDP and analyzed by gel filtration on a Sephadex G-25 column. The labeled enzymes were extracted from the cells and analyzed by gel filtration on a Sephadex G-25 column.

Figure 2: Separation of [3H]Dolichyl-Labeled Neutral Lipids by Gel Filtration Chromatography and Thin-Layer Chromatography

Counts per minute/million cells

- **0**: 0
- **5**: 5
- **0.05**: 0.05
- **0.1**: 0.1
- **1.5**: 1.5
- **2.5**: 2.5
- **3.5**: 3.5
- **4.5**: 4.5
- **0.05**: 0.05
- **0.1**: 0.1
- **0.2**: 0.2
- **0.3**: 0.3
- **0.4**: 0.4

**Migration (cm)**

- **0**: 0
- **5**: 5
- **0.05**: 0.05
- **0.1**: 0.1
- **1.5**: 1.5
- **2.5**: 2.5
- **3.5**: 3.5
- **4.5**: 4.5
- **0.05**: 0.05
- **0.1**: 0.1
- **0.2**: 0.2
- **0.3**: 0.3
- **0.4**: 0.4

**Counts per minute/ml (mL)**

- **0**: 0
- **5**: 5
- **0.05**: 0.05
- **0.1**: 0.1
- **1.5**: 1.5
- **2.5**: 2.5
- **3.5**: 3.5
- **4.5**: 4.5
- **0.05**: 0.05
- **0.1**: 0.1
- **0.2**: 0.2
- **0.3**: 0.3
- **0.4**: 0.4
Three Enzymes Compete for Common Pool of Dolichyl Phosphate

Figure 3: Separation of Tri-O-mannosylated Lactose Lipids by Paper Chromatography. C57 Black mice were killed by cervical dislocation. The liver and brain were removed, and the glycogen was extracted. The glycogen was dissolved in water and applied to a Whatman No. 1 paper (Fig. 4). The paper was then developed with a solvent system (Fig. 4), and the glycogen was visualized with an infrared lamp. The presence of glycogen was confirmed with an ultraviolet lamp.

Table 1: Analysis of Neutral Lipid Phenolic Lipids

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Galactose</td>
<td>1.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The presence of glycogen was confirmed with an ultraviolet lamp.

Figure 4: Analysis of Separated Dolichol Fractions by Thin-Layer TLC. C57 Black mice were killed by cervical dislocation. The liver and brain were removed, and the glycogen was extracted. The glycogen was dissolved in water and applied to a Whatman No. 1 paper (Fig. 4). The paper was then developed with a solvent system (Fig. 4), and the glycogen was visualized with an infrared lamp. The presence of glycogen was confirmed with an ultraviolet lamp.

Table 2: Analysis of Neutral Lipid Phenolic Lipids

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The presence of glycogen was confirmed with an ultraviolet lamp.
Three Enzymes Compete for Common Pool of Dolichyl Phosphate

The same method used to determine the mass amounts of cholesterol in cells (See Table 2) was used to demonstrate the labeled product which coeluted with cholesteryl during gel filtration (Fig. 2, top panel) was authentic cholesterol. The product of the cholesteryl esterification, cholesteryl oleate, had higher mobility on silica gel in solvent systems A compared to the substrate cholesteryl. When [1-14C]cholesteryl oleate was treated with cold cholesterol oxidase, the majority of the radioactive product migrated with cholesterol (Fig. 4). Similarly, labeled material comigrating with the cholesterol oleate standard by gel filtration migrated with cholesteryl oleate on silica gel in system A (Fig. 2, bottom panel) incubated with cholesterol oxidase, and treated with cholesteryl oleate after saponification and treatment with cholesterol oxidase (data not shown).

Enzymatic Assay of Cholesterol Oxidase Activity

From the data shown in Fig. 3, it seemed that even after 24 hr of incubation with [1-14C]cholesteryl oleate, the neutral lipid fraction was still containing a large amount of unreacted material. Therefore, it was necessary to test whether the unreacted material could be catalyzed into cholesterol. The material was incubated with cholesterol oxidase under the same conditions as described above, and then extracted with ethyl ether. A significant increase in radioactivity was observed in the ethyl ether fraction. The results indicate that the unreacted material could be catalyzed into cholesterol.