Glucuronosyl-N-acetylglucosaminyl Pyrophosphoryldolichol

FORMATION IN SV$_{40}$-TRANSFORMED HUMAN LUNG FIBROBLASTS AND BIOSYNTHESIS IN RAT LUNG MICROSUMAL PREPARATIONS*

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Incubation of SV$_{40}$-transformed human lung fibroblasts with [H]glucosamine for 1 h, followed by chloroform:methanol extraction and thin layer chromatographic analysis, revealed the presence of a major radioactive lipid that was isolated and characterized as GlcUA-(1 → 4)-GlcNAc-P-P-dolichol. An identical lipid was formed in smaller quantities under similar incubation conditions in several fibroblastic lines, HeLa cells, and in mouse L cells.

Rat lung microsomal preparations catalyze the synthesis of the disaccharide lipid in the following sequence of reactions:

$$\text{UDP-['H]GlcNAc + dolichol-P} \rightarrow \text{['H]GlcNAc-P-P-dolichol (1)}$$

$$\text{['H]GlcNAc-P-P-dolichol + UDP-[C]GlcUA} \rightarrow \text{[C]GlcUA-['H]GlcNAc-P-P-dolichol (2)}$$

The double-labeled lipid was identical to the lipid isolated from SV$_{40}$-transformed fibroblasts with regard to its behavior on thin layer and silicic acid chromatography. Further, the double-labeled disaccharide released from the lipid by mild acid hydrolysis was identical to GlcUA-(1 → 4)-GlcNAc in its chromatographic and electrophoretic behavior and in its composition. The occurrence of a polyprenol derivative of GlcUA-(1 → 4)-GlcNAc suggests a possible role for this lipid in the biosynthesis of the repeating disaccharide units of proteoglycans, such as heparin.

The possible role of mono- and oligosaccharide derivatives of polyprenols in the biosynthesis and assembly of complex carbohydrate-containing macromolecules has received considerable attention in recent years. The obligatory participation of polyprenol derivatives was first established in microbial systems regarding the biosynthesis of the heteropolysaccharides of bacterial cell envelopes (reviewed in Ref. 2). In these systems, the C$_{10}$ polyprenol, undecaprenol, was shown to function as a "carrier lipid" in the formation of these polymers. More recently, studies in our laboratory, as well as in the laboratories of many others (reviewed in Ref. 3), indicate that mono- and oligosaccharide derivatives of the C$_{10}$ (α-saturated) polyprenol, dolichol, participate in the biosynthesis of the carbohydrate side chains of eukaryotic glycoproteins.

On the basis of the currently available evidence regarding the biosynthesis of eukaryotic glycoproteins, the polyprenol derivatives appear to participate primarily in the preassembly of the "core" portion of the carbohydrate side chain and its subsequent attachment through an N-glycosidic linkage to the polypeptide chain; in contrast, the bacterial polyprenol derivatives appear to function primarily in the biosynthesis and assembly of oligosaccharides that are subsequently polymerized as repeating structural units in the macromolecular products.

A class of eukaryotic glycoproteins in which the major carbohydrate component is composed of repeating structural units are the proteoglycans such as hyaluronate, the chondroitin sulfates, and heparin. In these macromolecules, the elongated, unbranched carbohydrate side chains are composed of repeating disaccharide structures, each containing one uronic acid and one hexosamine moiety, which is attached to a core oligosaccharide composed of galactose and xylose; attachment to the polypeptide chain occurs by way of an O-glycosidic linkage to serine or threonine residues. The mode of biosynthesis of the carbohydrate side chains of the proteoglycans has been studied extensively and has been comprehensively reviewed (4). The majority of these studies indicate that the carbohydrate side chains are initiated and elongated by a series of highly specific glycosyltransferases that sequentially transfer single carbohydrate residues from their corresponding nucleotide derivatives to the growing side chains. Thus, in spite of several specific efforts to design experiments that would detect lipid-linked disaccharide intermediates, it was concluded that the polymerized disaccharide side chains of chondroitin sulfate (5) and hyaluronate (6) are formed by the sequential transfer of the individual uronic acid and hexosamine residues from their corresponding nucleotides without the formation of a disaccharide intermediate.

In this paper, we present evidence for the biosynthesis and partial structural characterization of glucuronosyl-1 → 4- N-acetylglucosaminyl pyrophosphoryldolichol. The polyprenol disaccharide was isolated and characterized from chloroform/methanol extracts of SV$_{40}$-transformed human lung fibroblasts.
and, using rat lung microsomes as a source of enzyme, it was shown that the lipid-linked disaccharide is biosynthesized according to the following sequence of reactions:

\[
\text{UDP-GlcNAc + dolichol-P} \rightarrow \text{GlcNAc-P-P-dolichol} + (\text{LIMP})
\]

1 Preliminary experiments suggest that the disaccharide moiety of the polyprenol derivative is enzymatically transferred to macromolecular products in the rat lung microsomal system. It is possible, therefore, that this derivative may serve as a donor of glucuronyl-hexosamine disaccharide residues in the biosynthesis of the carbohydrate side chains of proteoglycans. In fact, the analogous osamine-containing family of polyphosphoryldolichol, as well as the prominent occurrence of the disaccharide polyprenol in lung tissue, suggests a possible role for this derivative in the biosynthesis of heparin and related proteoglycans.

**EXPERIMENTAL PROCEDURES**

**Materials** - d-[6-3H]Glucosamine hydrochloride, 10.1 Ci/mmol; sodium [3H]borohydride, 205 Ci/mmol; [32P]Phosphoric acid, carrier-free; UDP-N-[6-3H]glucosamine, 6.6 Ci/mmol; and UDP-[13C]glucuronic acid, 231 Ci/mmol were purchased from New England Nuclear Corp. Other reagents were obtained as follows: dmitone, Tris/maleate, and DEAE-cellulose from Sigma Chemical Co.; Sephadex LH-20 from Pharmacia Fine Chemicals, Inc.; Unil (activated silicic acid) from Clarkson Chemical Co.; precoated Avicel TLC plates from Analtech, Inc.; Silica Gel G from E. Merck; and Bio-Gel P-2 from Bio-Rad Laboratories. The following reagents were generous gifts: mannosylphosphoryldolichol, dolichyl phosphate (Dr. Roger W. Janloz, Harvard University, Cambridge, Mass.); N-acetylcysteinhydroxydes (Dr. Don M. Carlson, Purdue University, West Lafayette, Ind.); N,N'-diacetylatedbisole (Dr. Saul Rosenman, Johns Hopkins University, Baltimore, Md.); ω- or β-mannosidase prepared from bromein by the method of Li and Lee (7) (Dr. Gary L. Firestone, of this laboratory); β-acylacylglycerinaminidase, ω- or β-galactosidase prepared from Aspergillus niger by the method of Bayer and Agrawal (8) (Dr. Bryan Rigby, of this laboratory); and the tetrasaccharide, Gal-Man-(GlcUA)-Gal, prepared from bacterial来源. All other chemicals and reagents were of the highest quality available commercially. In experiments involving incorporation of [3H]glucosamine by cultured cells, a glucose-free modified Eagle's Minimal Essential Medium was used. This medium was mixed from mannosephosphoryldolichol, Glicoh phosphat (Dr. Roger W. Janloz, Harvard University, Cambridge, Mass.); N-acetylcysteinhydroxydes (Dr. Don M. 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utilized a procedure developed by Dr. Roger K. Bretzhauser in this laboratory. The incubation mixture contained 30 µmol of MnCl₂, 30 µmol of MgCl₂, 15 µmol of KF, 1 µmol of UDP-N-acetyl[14C]glucosamine (10⁶ cpm), 10 µg of dolichol phosphate, and 30 to 50 mg of rat lung microsomal protein in a final volume of 3 ml, and was incubated for 30 min at 37°; larger incubation mixtures were scaled up proportionately. Reactions were terminated by the addition of 15 ml of CHCl₃:CH₂OH (2:1) and processed as described above for lipid extraction. The washed lipid extract was evaporated to dryness, redissolved in 5 ml of toluene:methanol (1:1), and deacylated by mild alkaline methanolysis (0.1 N KOH, 15 min, room temperature) according to the method of White and Freeran (13). The saponified mixture was neutralized by the addition of 1 M acetic acid and then washed according to the method of Folch et al. (11). The organic phase was evaporated to dryness, redissolved in 50 ml of CHCl₃:CH₂OH (4:1), and the solvent was re-removed by evaporation. To each tube were added 4 µmol of MnCl₂, 2 µmol of MgCl₂, 1 µmol of KF in a final volume of 100 µl, and the mixture was sonicated for 30 s in order to disperse the radioactive lipid; finally, 2 to 5 µmol of UDP-[14C]glucuronic acid (4 x 10⁶ cpm), and 2 to 4 mg of rat lung microsomal protein were added in a final total volume of 200 µl and the mixture was incubated for 15 min at 37°. For larger scale preparations of the lipid, a series of incubation mixtures, identical to those described, was used. Reactions were terminated by the addition of 1 ml of CHCl₃:CH₂OH (3:2) and processed as described above for lipid extraction. The washed lipid extract was evaporated to a small volume and then applied to an Avicel thin layer plate which was developed in Solvent System F, butanol-1:pyridine:H₂O (7:3) and on Schleicher and Schuell No. 589 Green Ribbon paper. The migration of sugars and sugar derivatives was determined relative to the solvent front (Rₘ). Radioactivity Measurements - Quantitative determination of radioactivity was obtained using a Packard Tri-Carb liquid scintillation spectrometer, model 3390. The preparation of particulate, water-soluble, and organic solvent-soluble samples, and the composition of scintillation mixtures were as previously described (17). In double label experiments, radioactive values for each isotope were corrected for efficiency, crossover, and background.

RESULTS

These studies were directed toward the comparison of various aspects of glycoprotein biosynthesis in normal human lung fibroblasts and in viral transformants of these cells, particularly with regard to the structure and distribution of polyisopren-linked carbohydrate intermediates. Initially, experiments were conducted to evaluate the relative rates of incorporation of [14C]glucosamine into acid-insoluble macromolecules and into organic solvent-soluble lipid derivatives. The results of these preliminary experiments indicated that cultured fibroblasts produce a heretofore unreported polyisopren derivative that contains hexosamine and uronic acid and that SV₄-transformed lung fibroblasts synthesize significantly greater quantities of this lipid than normal cells or other cultured cells. Because of the unique composition of this polyisopren derivative and because of its possible role in the biosynthesis of complex carbohydrate-containing macromolecules, our major effort was directed toward the elucidation of the structure and mode of biosynthesis of the lipid.

Formation of Disaccharide Lipid in Cultured Cells

Incorporation of [14C]Glucosamine in Normal and Transformed Fibroblasts - In preliminary studies it was observed that incubation of human embryonic lung fibroblasts, WI-38, and the SV₄-transformed counterpart, WI-38 (VA-13), with [14C]glucosamine resulted in a greater accumulation of labeled material in organic-extractable and trichloroacetic acid-precipitable material in the transformed cells. These results are illustrated in Fig. 1 and indicate that the transformed cells accumulate 3- to 4-fold greater quantities of radioactivity than normal cells in all of the fractions analyzed. On the basis of previous experiences of this laboratory, acid-insoluble material should represent protein; chloroform:methanol-soluble material should represent simple carbohydrate-containing lipids; and the chloroform:methanol:water fraction should contain complex carbohydrate-containing lipids. The accumulation of carbohydrate-containing material in transformed cells does not appear to be a phenomenon related to all carbohydrate precursors of macromolecules. Thus, when similar experiments were conducted with radiolabeled fucose, no significant quantitative or qualitative difference between normal and transformed cells was observed in the distribution of radioactive products in these various fractions. Since glucosamine and fucose are the two carbohydrate precursors which exhibit the least equilibration with other intermediary metab-

Unless otherwise indicated, the terms "normal" and "transformed" cells refer to WI-38 and WI-38 (VA-13), respectively.
Cultured Cells - A variety of cultured cell lines were evaluated regarding their capacity to incorporate \[^{3}H\]glucosamine into lipid and protein of normal and transformed cells. Five cultures of each cell line (approximately \(1 \times 10^{9}\) cells/plate) were incubated with 1 ml of \[^{3}H\]glucosamine-containing medium (10 \(\mu\)Ci/ml). Radioactivity present in lipid and protein fractions was determined as described under "Experimental Procedures." Protein concentration was estimated by analysis of three duplicate cultures of each cell line as follows: the cell layer was washed 3 times with NaCl/P, dissolved in 0.5 ml of 0.06 N NaOH, and pooled. The protein values obtained on the pooled samples were used to calculate the average protein concentration per culture dish. Symbols used are: \(\times\times\), chloroform:methanol (3:2) extract; \(\Delta\Delta\), chloroform:methanol:water (1:1:0.3) extract; \(\bullet\bullet\), trichloroacetic acid-precipitable material.

In order to evaluate the significance of the increased accumulation of \[^{3}H\]glucosamine-labeled products in the lipid and protein fractions of \(SV_{tr}\)-transformed cells, the qualitative distribution of radioactive products in the various fractions was undertaken. Analysis of the acid-precipitable material by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and analysis of the chloroform:methanol:water fraction by thin layer chromatography revealed qualitatively identical radioactivity profiles from the normal and transformed cells. Thus, while these fractions from transformed cells contained 3 to 4 times the amount of radioactivity as those from normal cells, it appears that these observations may be due to increased biosynthetic rates rather than the biosynthesis of unique compounds or different proportions of the same compounds. However, further analysis of the \[^{3}H\]glucosamine-labeled products in the chloroform:methanol fraction by thin layer chromatography revealed a marked difference in normal and transformed cells in the proportions of the various labeled compounds in this fraction. These results, illustrated in Fig. 2, show that 70 to 90% of the radioactivity accumulated in 1 h by transformed cells is present in a lipid which migrates with a \(R_{man-P-Dol}\) of 0.35 whereas less than 5% of the radioactivity in this fraction from normal cells was contained in a similar compound.

Formation of \[^{3}H\]Glcua-containing Lipid in Other Cultured Cells - A variety of cultured cell lines were evaluated regarding their capacity to incorporate \[^{3}H\]glucosamine into the lipid observed in \(SV_{tr}\)-transformed human lung fibroblasts. Log phase monolayer cultures of HeLa cells, mouse L cells, and skin fibroblasts, in addition to normal and \(SV_{tr}\)-transformed lung fibroblasts, were incubated with \[^{3}H\]glucosamine as described and the labeled lipid was isolated. As shown in Table I, the transformed fibroblasts are clearly more efficient in producing the labeled lipid; normal lung and skin fibroblasts formed approximately \(1/6\) and \(1/4\) the quantities, respectively, and the lipid was formed in only trace quantities in HeLa and mouse L cells. In all cell lines, the incorporation of radioactive glucosamine into the lipid was markedly diminished in aged or dense cultures. Whether the basis for the variation in quantities of the lipid formed in the different cell lines is due to variation in biosynthetic capacities, such as efficiency of utilization in a metabolic pathway, or due to other factors of the culture conditions remains to be established. It is possible that these variations could be due to differences in the glucosamine transport mechanism or in the cellular glucosamine pool sizes causing a dilution of the specific activity of \[^{3}H\]glucosamine.

Purification and Properties of \[^{3}H\]Glcua-labeled Lipid - A large scale preparation of \[^{3}H\]glucosamine-labeled lipid (\(R_{man-P-Dol} = 0.35\), Fig. 1) from transformed cells was extracted with chloroform:methanol:water in a proportion of 3:2:0.6 rather than the ratio 3:2:1 which is normally used for lipid extraction. It was observed that the latter solvent permitted extensive partitioning (20 to 30%) of the radioactive lipid into the aqueous phase, and consequently, significant losses of material. Therefore, to maximize the efficiency of extraction of the labeled lipid, the proportion of water in the solvent was lowered as indicated. \[^{3}H\]Glcua-labeled disaccharide lipid was extracted from 3 to 5 \(\times\) \(10^{9}\) transformed cells and the crude lipid extract was evaporated to dryness and the residue was dissolved in 4 ml of anhydrous toluene:methanol (1:1). Four milliliters of 0.2 N KOH in absolute methanol was added and the solution was allowed to stand for 10 min at room temperature. The mixture was then neutralized by the addition of 1 N acetic acid. The saponified sample was evaporated to dryness, dissolved in chloroform:methanol (4:1), and applied to a column of silicic acid (5 to 10 ml), equilibrated in the same solvent. The column was washed successively with 20 to 30 ml each of chloroform:methanol solutions in proportions of 4:1,
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"Experimental Procedures"; aliquots were hydrolyzed in 0.2 N HCl for 1 h and the radioactive disaccharide was isolated by paper electrophoresis for determination of radioactivity.

### Table I

**Formation of [\(^{14}\)C]glucosamine-labeled lipid in cultured cells**

Monolayer cultures of each cell line were incubated with [\(^{14}\)C]glucosamine for 1 h and lipids were extracted as described under "Experimental Procedures"; aliquots were hydrolyzed in 0.2 N HCl for 20 min at 100° and the radioactive disaccharide was isolated by paper electrophoresis for determination of radioactivity.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disaccharide lipid cpm/mg protein</th>
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<tbody>
<tr>
<td>Normal human lung</td>
<td>97</td>
</tr>
<tr>
<td>SV-40 human lung</td>
<td>631</td>
</tr>
<tr>
<td>Normal skin</td>
<td>145</td>
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<tr>
<td>HeLa</td>
<td>33</td>
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<tr>
<td>Mouse L</td>
<td>23</td>
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2, 21, and finally 1:1. The chloroform:methanol (1:1) eluate was concentrated to a small volume, applied to a Silica Gel G thin layer plate which was developed in Solvent System C, and the radioactive material exhibiting an \(R_{	ext{naneo}}\) value of 0.55 was eluted from the gel with chloroform:methanol (1:1). This sample was applied to a column of DEAE-cellulose (5 to 10 ml), equilibrated in chloroform:methanol (1:1), and washed successively with 20 to 30 ml of chloroform:methanol (1:1), methanol, and finally 0.2 N ammonium bicarbonate in methanol. The final eluant containing the radioactive lipid was fractionated on a column of Sephadex LH-20 as illustrated in Fig. 3. Fractions were analyzed throughout the purification by analytical thin layer chromatography (Silica Gel G in Solvent System A) and by paper electrophoresis of the anionic disaccharide obtained by mild acid hydrolysis of the disaccharide lipid (see below). The radioactive lipid was judged to be homogeneous on the basis of the symmetry of its elution profile from Sephadex LH-20 and by thin layer chromatography in several solvent systems as illustrated in Table II. In all instances, the disaccharide lipid migrated as a single radioactive component corresponding to the migration of a single iodine- and anisaldehyde-staining material. The overall yield of the disaccharide lipid, purified by this procedure, was approximately 60%, based on the total radioactivity present in the crude lipid extract.

On the basis of its behavior during purification, we concluded that the [\(^{14}\)C]glucosamine-labeled lipid possesses the following characteristics: (a) amphiphatic, based on its behavior on silicic acid (column and thin layer) and on its ability to partition between aqueous and organic solvents; (b) absence of esters, based on its resistance to alkaline methanolysis; (c) acidic, based on its behavior on DEAE-cellulose; and (d) exhibits molecular properties consistent with a carbohydrate derivative of dolichol in that it elutes from a Sephadex LH-20 column in a position similar to that of mannosphosphoryldolichol and exhibits a positive staining reaction with anisaldehyde on thin layer chromatography (16).

**Characterization of Disaccharide Structure**—By analogy with the chemical properties of known carbohydrate derivatives of polypropens (2), the [\(^{14}\)C]glucosamine-labeled lipid was subjected to mild acid hydrolysis in order to determine whether the carbohydrate moiety of the lipid is linked by an acid-labile bond. After the labeled lipid was hydrolyzed in 0.02 N HCl for 20 min at 100°, the radioactivity was quantitatively recovered in the aqueous-soluble fraction indicating that the glucosamine-containing portion of the lipid was linked to the lipophilic moiety by an acid-labile linkage. These results suggest that this linkage point may involve a phosphodiester or pyrophosphoryl bridge between the carbohydrate and lipid moieties, a property that is consistent with the structure of known polypropen derivatives of carbohydrates. In order to pursue this observation as an approach to further structural characterization, the [\(^{14}\)C]glucosamine-labeled lipid was subjected to acid hydrolysis under various conditions and the aqueous-soluble products were analyzed by paper chromatography and paper electrophoresis; these results are shown in Figs. 4 and 5. The untreated lipid migrated slightly slower than the solvent front upon paper chromatography and was immobile in an electrophoretic field. Hydrolysis of the lipid in 3 N HCl for 3 h at 100° resulted in the quantitative liberation of the radioactivity in a compound which corresponded to glucosamine upon chromatography and electrophoresis. However, the product of mild acid hydrolysis (0.02 N HCl, 20 min, 100°) was distinct from glucosamine and derivatives of glucosamine, migrating very slow (\(R_f = 0.16\)) on the chromatogram and as an anion upon electrophoresis. Of particular significance is the observation that the mild acid-labile product exhibits proper-
Purification of the lipid was achieved by repeated extractions with chloroform:methanol (2:1) and by paper chromatography in solvent system F. The material was subjected to hydrolysis with 3 N HCl at 100°C for 3 h and with 0.02 N HCl for 20 min at 100°C. The hydrolysate was then subjected to paper chromatography in solvent system F. The results indicated that the carbohydrate moiety of the lipids consists of the glucosamine residue (presumably N-acetylglucosamine) covalently linked to an acidic component.

To evaluate the possibility that the mild acid-labile product contains other neutral sugars or phosphomonoester residues, the compound was treated exhaustively with the following enzymes: β-N-acetylglucosaminidase, α- or β-mannosidase, α- or β-galactosidase, and alkaline phosphatase (Escherichia coli, Worthington). These results indicated that the chromatographic and electrophoretic properties of the mild acid-labile product were unaltered as a result of treatment with any of these hydrolytic enzymes or combinations of these enzymes.

On the basis of these results, we concluded that the carbohydrate moiety of the lipids consists of the glucosamine residue (presumably N-acetylglucosamine) in addition to another constituent(s) which conferred anionic properties on the acid-released product. However, the possibility that the lipid contains additional neutral sugars was not ruled out by the experiments described above.

In order to determine the carbohydrate composition and relative structure of the mild acid-released [3H]glucosamine-labeled product, the material was subjected to analysis with tritium-labeled sodium borohydride. An unlabeled sample of the aqueous-soluble material released by mild acid hydrolysis was prepared from transformed cells by the procedure outlined in the legend to Fig. 6. The glucosamine-containing material was reduced with sodium [3H]borohydride prior to, and after strong acid hydrolysis and analyzed by paper chromatography as described under "Experimental Procedures." The results of this experiment, illustrated in Fig. 6, indicated that the carbohydrate moiety released from the lipid by mild acid hydrolysis is the disaccharide glucuronic acid-glucosamine. Thus, reduction of the mild acid-released product by [3H]borohydride prior to strong acid hydrolysis yielded a single radioactive component that corresponded to [3H]glucosaminol (Fig. 6, I), indicating that the reducing terminus of the compound is glucosamine. Conversely, reduction of the products of strong acid hydrolysis with sodium [3H]borohydride yielded three radioactive compounds as indicated by paper chromatography (Fig. 6, II). A major radioactive component corresponding to [3H]glucosaminol was observed, in addition to two minor components that corresponded to the chromatographic mobility of gulonic acid and gulonolactone, the predicted products of reduction of glucuronic acid. It was determined that the radioactive material at the origin and near the solvent front of the chromatogram were radioactive contaminants unrelated to the unknown compound since they also were observed in blank experiments. The molar ratio of glucosamine and glucuronic acid in the oligosaccharide was estimated by comparison of the total radioactivity in glucosaminol (3110 cpm) to the combined total radioactivity in gulonic acid and gulonolactone (2710 cpm). These results indicated that each of the two carbohydrate constituents is present in equimolar quantities and that the carbohydrate moiety of the lipid is the disaccharide.
behaves as an anion at neutral pH indicating that the amino acetylated is based on the fact that the intact disaccharide hexosamine moiety of the unhydrolyzed disaccharide is N-cosamine would have been hydrolyzed; our conclusion that the products of reduction, the N-acetyl group of N-acetylglucosamine residues of the hexosamine residue is not free.

The linkage between the two components of the disaccharide must involve a glycosidic bond between C-1 of the glucuronic acid moiety and either C-3, C-4, or C-6 of the N-acetylgalactosamine moiety. Substitution at C-6 of the N-acetylgalactosamine residue was eliminated by the results of periodate oxidation studies on the isolated N-acetyl[6-3H]glucosamine-labeled disaccharide. As shown in Fig. 7, periodate oxidation of the disaccharide resulted in quantitative conversion of the radioactivity to a low molecular weight product, presumably [3H]formaldehyde, which co-eluted with the product obtained by oxidation of standard [6-3H]glucosamine, and was distinct from either the untreated disaccharide or glucosamine as determined by gel filtration chromatography. Direct evidence to confirm that the radioactive product of periodate oxidation of the disaccharide is [3H]formaldehyde was obtained by precipitation and recrystallization to constant specific activity of the dimeric derivative of formaldehyde (19). As shown in Fig. 8, the specific radioactivities of the dimedone derivatives of the periodate products of the disaccharide and of standard glucosamine were constant from the second through the sixth recrystallizations indicating that the C-6 of the N-acetylgalactosamine moiety of the 6-[3H]glucosamine-labeled disaccharide is susceptible to periodate oxidation and, therefore, unsubstituted. These results indicate that linkage between the monosaccharide residues must be either 1, 4 or 1, 3. The latter was ruled out as a possibility on the basis of resistance of the bond to alkali (20, 21). Thus, treatment with 0.05 N NaOH for 1 h at 37°C of the disaccharide, N-acetylchitobiose (O-β-D-glucopyranosylofronic acid (1→3) 2-acetamido-2-deoxy-D-galactopyranose) resulted in quantitative cleavage into the component monosaccharides whereas the disaccharide, N,N-diacetylcitolobiose (O-β-D-acetamido-2-deoxy-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-galactopyranose) was stable to these conditions (data not shown). Treatment of the glucuronosyl-N-acetyl[6-3H]glucosamine disaccharide under identical alkaline conditions resulted in quantitative recovery of the intact disaccharide as shown in Fig. 9. Since the radioactivity in the disaccharide residues exclusively in the hexosamine moiety, alkaline degradation would have resulted in the formation of a neutral radioactive product which would have been immobile upon electrophoresis. Therefore, by eliminating the possibilities of substitution of the hexosamine moiety at C-3 and C-6, we have concluded that the disaccharide component of the lipid possesses the structure, glucuronosyl-(1→4)-N-acetylgalactosamine.

The anomeric configuration of the glucuronosyl bond remains to be established. The disaccharide is resistant to cleavage by rat liver β-D-glucuronidase ( Worthington), but these results are equivocal since this enzyme exhibits maximum specificity for 1,4-unsaturated D-glucuronic acid disaccharides or higher molecular weight D-glucuronic acid oligosaccharides (22). It may be possible in the future to determine this anomeric configuration with other preparations of glucuronidases or by nuclear magnetic resonance techniques if sufficient amounts of the disaccharide become available.

Preparation of [3H]-labeled GlcUA-GlcNAc-Lipid—In order to evaluate further the possibility that the disaccharide is linked to the lipophilic moiety through a phosphodiester or

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FIG. 6. Analysis of the sodium [3H]borohydride-reduced products of the disaccharide. Unlabeled lipid was extracted from transformed cells, purified by saponification and thin layer chromatography, and analyzed by paper chromatography in Solvent System F. Conditions for acid hydrolysis, reduction, and location of samples on chromatograms are described under "Experimental Procedures." A sample of [3H]glucosamine-labeled lipid was co-purified to ascertain the location of the unlabeled material during the purification steps. Correction was made for a background of 150 cpm in plotting the data. Standards used are: a, L-galactonic acid; b, L-gulonic acid; c, L-idonic acid; d, D-glucosaminol; e, D-sorbitol; f, L-idonolactone; and g, L-gulonolactone.

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Fig. 6. Analysis of the sodium [3H]borohydride-reduced products of the disaccharide. Unlabeled lipid was extracted from transformed cells, purified by saponification and thin layer chromatography, and hydrolyzed in 0.02 N HCl for 20 min at 100°C. The aqueous soluble material was purified by paper electrophoresis and gel filtration on Bio-Gel P-2. Two aliquots I and II of the final product were treated as follows: I was successively reduced with sodium [3H]borohydride, hydrolyzed in 3 N HCl for 3 h at 100°C, and analyzed by paper chromatography in Solvent System F; II was successively hydrolyzed in 3 N HCl for 3 h at 100°C, reduced with sodium [3H]borohydride, and analyzed by paper chromatography in Solvent System F. Conditions for acid hydrolysis, reduction, and location of samples on chromatograms are described under "Experimental Procedures." A sample of [3H]glucosamine-labeled lipid was co-purified to ascertain the location of the unlabeled material during the purification steps. Correction was made for a background of 150 cpm in plotting the data. Standards used are: a, L-galactonic acid; b, L-gulonic acid; c, L-idonic acid; d, D-glucosaminol; e, D-sorbitol; f, L-idonolactone; and g, L-gulonolactone.
The lipid was hydrolyzed in 0.02 N HCl for 20 min at 100° and the legend to Table II, possessed a 52P/3H of approximately 1.2.

mental Procedures" except that [32P]orthophosphoric acid and incubated under the usual conditions described under "Experi-

mental Procedures." Standard [6-3H]glucosamine hydrochlo-
riddle (800,000 cpm) served as a control and was treated in the same manner as the disaccharide. Illustrated by x are 10-μl aliquots of disaccharide-treated sample and • are 5μl aliquots of glusac-
mine-treated sample.

Fig. 8 (center). Specific activity profile of radioactive dimedone derivative. Fractions 61 to 71 of the Bio-Gel P-2 column (Fig. 7) were pooled in a total volume of 10 ml, reacted with dimedone, and pyrophosphoryl bridge, an effort was made to incorporate [32P] into the lipid synthesized by transformed cells. Cultures were inoculated under the usual conditions described under "Experi-

mental Procedures" except that [32P]orthophosphoric acid and [1H]glucosamine were included in the media. The double-
labeled disaccharide lipid, isolated and purified as outlined in the legend to Table II, possessed a 32P/1H of approximately 1.2. The lipid was hydrolyzed in 0.02 N HCl for 20 min at 100° and then layer chromatographic analysis in Solvent System D indicated 32P and H-labeled material at the origin, the ex-
pected position of the H-disaccharide, 32P and 32P1. In addi-
tion, approximately 50% of the 32P-labeled material migrated with a Rf1 of 0.63 which corresponds to the relative mobility of dolichol pyrophosphate according to Warren and Jeanloz (23). These workers demonstrated that pyrophosphoryl deriv-
atives of dolichol yield multiple products including dolichol pyrophosphate, dolichol monophosphate, and free dolichol. In our experiment 32P-labeled dolichol monophosphate was not observed in significant quantities, indicating that the 32P was apparently incorporated primarily into the β-phosphoryl group of dolichol under the conditions used for labeling the cells. In any case, these results indicate that the disaccharide lipid is most likely a pyrophosphoryl derivative of dolichol.

Enzymatic Synthesis of GlcUA-GlcNAc-P-P-Dolichol

Because of limitations in the quantities of cells and cell fractions inherent in the use of cultured cells as a source of enzymes, attempts were made to study the sequence of enzy-
matic steps involved in the biosynthesis of GlcUA-GlcNAc-P-
P-dolichol using microsomal preparations from rat lung as an enzyme source. By analogy to known reactions, we proposed that the disaccharide dolichol derivative is synthesized in a two-step process according to the scheme outlined in Fig. 10. Reaction 1, studied by others in a variety of tissues as re-
viewed by Waechter and Lennarz (24), involves the transfer of

UDP-α-GlcUA as substrates to permit isolation and charac-
terization of a double-labeled disaccharide lipid.

Preparation of [1H]GlcNAc-P-P-Dolichol—UDP-[6-3H]Glc-
Nac and synthetic dolichol monophosphate were incubated with rat lung microsomal membrane preparations under con-
ditions as described under "Experimental Procedures." [6-3H]GlcNAc-P-P-dolichol was isolated and purified from the incubation mixtures as described and its identity was con-

The double-labeled product, [14C]GlcUA-[3H]GlcNAc-P-P-doli-

chol—Incubation of rat lung microsomes with [14C]GlcUA-P-
P-dolichol and UDP-[3H]GlcNAc-P-P-dolichol using microsomal preparations from rat lung as an enzyme source. By analogy to known reactions, we proposed that the disaccharide dolichol derivative is synthesized in a two-step process according to the scheme outlined in Fig. 10. Reaction 1, studied by others in a variety of tissues as re-
viewed by Waechter and Lennarz (24), involves the transfer of

UDP-α-GlcUA as substrates to permit isolation and charac-
terization of a double-labeled disaccharide lipid.
**Glucuronosyl-N-acetylglucosaminyl Pyrophosphoryldolichol**

\[
\text{UDP-}[{^3}\text{H}]-\text{GlcNAc} + \text{Dolichol-P} \rightarrow [{^3}\text{H}]-\text{GlcNAc-P-P-Dolichol (+ UMP)}
\]

**Fig. 10.** Proposed pathway of biosynthesis of glucuronosyl-N-acetylglucosaminyl pyrophosphoryldolichol.

\[
\text{[3H]-GlcNAc-P-P-Dolichol + UDP-[}'\text{VI}'\text{-GlcUA} \rightarrow [{']\text{GlcUA}-[}'\text{HI}'\text{-GlcNAc-P-P-Dolichol (+ UDP)}
\]

**Fig. 11.** Purification of \([^{14}\text{C}]{\text{GlcUA}}-{[^{3}\text{H}]}\text{GlcNAc}\) by thin layer chromatography. The double-labeled disaccharide lipid was extracted and partially purified from eight identical incubation mixtures as described under "Experimental Procedures." The chloroform:methanol (1:1) eluate from the silicic acid column chromatography step was concentrated, applied to a thin layer Silica Gel G plate, chromatographed in Solvent System A, and the fractions containing \(^{14}\text{C}\) and \(^3\text{H}\) were pooled as indicated by the cross-hatched area (Panel I). In Panels II and III, this procedure was repeated using Solvent Systems B and D. Purified preparations of \(^{14}\text{C}\)GlcUA-\([^{3}\text{H}]\text{GlcNAc-P-P-dolichol}\), isolated from transformed cells, and \(^{14}\text{C}\)\text{GlcNAc-P-P-dolichol}\), isolated from microsomal incubation mixtures, were used as standards a and b, respectively.

**Fig. 12.** Chromatography of \([^{14}\text{C}]{\text{GlcUA}}-{[^{3}\text{H}]}\text{GlcNAc}\) on Bio-Gel P-2. Double-labeled disaccharide lipid was purified from 12 identical incubation mixtures that were extracted and processed as described in the legend to Fig. 11. The double-labeled disaccharide was isolated from the mild acid hydrolysate of the lipid by paper electrophoresis as described in the text, and applied to a column (1 x 40 cm) of Bio-Gel P-2, equilibrated in 0.5 N NaCl. Fractions (0.8 ml) were collected and counted in toluene/Triton X-100 scintillation fluid. Standards are: V\(_o\), blue dextran; a, Gal-Man-(GlcUA)Gal; b, glucuronosyl-N-acetyl[\(^3\text{H}\)]glucosamine; c, stachyose; d, glucuronic acid; e, lactose; f, galactose.

**Discussion**

To the best of our knowledge, the results presented in this paper represent the first documented evidence for the occurrence of a glucuronate-containing oligosaccharide derivative of the polyenol, dolichol. Previous reports (27, 28) suggested...
the possibility of the existence of such compounds or have observed the incorporation of radioactive glucuronate into organic solvent-soluble fractions of cells, but the detailed structure of these compounds was not studied.

We have utilized two distinct approaches to demonstrate the occurrence of the glucuronate-containing polypropenol: (a) isolation and characterization of the lipid from SV40-transformed fibroblasts; and (b) by establishing in vitro with rat lung microsomes, the sequence of enzymatic reactions (Fig. 10) that participate in the biosynthesis of the oligosaccharide derivative. The compound from either source was shown to possess the structure, glucuronosyl-N-acetylglucosaminyl pyrophosphoryldolichol. The structure of the carbohydrate moiety was determined by isolation and characterization of the disaccharide, glucuronosyl-(1 → 4)-N-acetylglucosamine, after its release from the polypropenol by mild acid hydrolysis. The anomic configuration of the linkage between the carbohydrate components remains to be established, either by obtaining sufficient mass of the disaccharide for unambiguous structural analysis (NMR or chemical methods) or by obtaining preparations of specific glucuronidases that would distinguish between α- and β-anomeric configuration in disaccharides of this type.

Our conclusions that the lipid component of the compound is dolichol and that the polypropenol and the disaccharide are linked by a pyrophosphoryl bridge are based on the chemical and chromatographic characteristics of the compound as well as by elucidation of the enzymatic steps in its biosynthesis. Thus, the disaccharide is released by hydrolysis with mild acid or by strong alkali. The chromatographic properties of the compound on silica gel thin layer plates and on paper are consistent with those of a dolichol pyrophosphoryl disaccharide, but, more importantly, elution of the compound from columns of DEAE-cellulose and silicic acid are characteristic of dolichol pyrophosphoryl oligosaccharides and distinguish between monophosphoryl- and pyrophosphoryl-linked derivatives.

The metabolic role of the disaccharide dolichol derivative is not clear at the present time. We have conducted preliminary experiments in an effort to demonstrate transfer of the disaccharide moiety of the polypropenol derivative to endogenous macromolecular acceptors available in microsomal membranes. The results of these experiments indicate that the disaccharide moiety is transferred to an endogenous acceptor(s) that exhibited the general characteristics of a macromolecular product. However, extensive study of this system will be necessary in order to firmly establish the nature of this reaction and the identity of the components that serve as acceptors.

Because of the similarity in structure of the disaccharide moiety of the dolichol derivative characterized in our studies with the disaccharide structure that constitutes the primary repeating structural component of the proteoglycans, heparin and heparan sulfate (29), it is tempting to speculate that glucuronosyl-(1 → 4)-N-acetylglucosaminyl pyrophosphoryldolichol may represent an intermediate in the biosynthesis of the carbohydrate side chains of these macromolecules. The pyrophosphoryl side chains of these proteoglycans are composed primarily of repeating disaccharide sequences of glucuronic acid and N-acetylglucosamine in β-1,4 linkage; the long chain polysaccharides are linked to a serine or threonine residue in the polypeptide backbone through a, so-called, core oligosaccharide composed of GlcUA-Gal-Gal-Xyl. As reviewed by Roden and Schwartz (4), presently available information indicates that the core oligosaccharide is synthesized by the sequential transfer of each of the sugar residues directly from their corresponding nucleotide derivatives to the polypeptide chain followed by formation of the long chain disaccharide repeating structures, also presumably by the individual alternating transfer of glucuronate and N-acetylgalactosamine from their nucleotide derivatives. The introduction of sulfate groups (30, 31) and the epimerization of glucuronate to iduronate (32) apparently are accomplished after extensive polymerization of the side chain has occurred. Most of the studies concerning the enzymatic synthesis of these polyns have been conducted with crude microsomal preparations from a variety of tissues, and thus, it is quite possible that involvement of a lipid-linked glucuronate-N-acetylgalactosamine disaccharide in this process may have been undetected.

The suggestion that glucuronosyl-N-acetylglucosaminyl pyrophosphoryldolichol is, in fact, an intermediate in the biosynthesis of proteoglycans is strengthened by the increasing evidence (24) implicating mono- and oligosaccharide derivatives of dolichol in the biosynthesis of glycoproteins. In addition, our observations that enzymatic preparations from lung and fibroblasts derived from lung tissue are efficient in forming the disaccharide dolichol derivative lend further support to the suggestion that this compound plays a role in heparin or heparan sulfate, or both, biosynthesis since the lung is a major site of biosynthesis of these proteoglycans.

Further work is currently underway in our laboratory to evaluate the metabolic significance of glucuronosyl-N-acetylgalactosaminyl pyrophosphoryldolichol in the biosynthesis of proteoglycans and possibly other macromolecules as well.

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REFERENCES


3. Lennarz, W. J. (1975) Science 188, 896-901


Glucuronosyl-N-acetylglucosaminyl Pyrophosphoryldolichol

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