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An-Fei Hsu

John W. Baynes
University of South Carolina - Columbia, john.baynes@sc.edu

Edward C. Heath

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The Role of a Dolichol-Oligosaccharide as an Intermediate in Glycoprotein Biosynthesis

(myeloma proteins/mannosyl-dolichol/lipid intermediate)

AN-FEI HSU, JOHN W. BAYNES*, AND EDWARD C. HEATH†

Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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ABSTRACT **Incubation of mouse myeloma microsomes with GDP-[^{14}C]mannose results in the biosynthesis of [^{14}C]mannose phosphoryl dolichol [Baynes, J. W., Hsu, A.-F. & Heath, E. C. (1973) *J. Biol. Chem.* 248, 5693–5704] and a [^{14}C]mannose- and *N*-acetylglucosamine (GlcNAc)-containing oligosaccharide derivative of dolichol. Thus, [^{14}C]mannose phosphoryl dolichol and [^{14}C]mannose-labeled oligosaccharide pyrophosphoryl dolichol were isolated from incubation mixtures by solubilization in 2% (w/v) Triton X-100 and the lipids were separated from small molecules by gel filtration fractionation. After removal of radioactive protein from the preparation, the two lipid derivatives were separated quantitatively by fractionation on a concanavalin A–Sepharose column; [^{14}C]mannose phosphoryl dolichol was not retained by the affinity resin but [^{14}C]mannose-oligosaccharide pyrophosphoryl dolichol adsorbed to the gel and was eluted with α-methylmannoside. [^{14}C]Mannose-oligosaccharide pyrophosphoryl dolichol appeared to be homogeneous when fractionated on DEAE-cellulose and in several thin-layer chromatographic systems. Treatment of [^{14}C]mannose oligosaccharide pyrophosphoryl dolichol with 10% (w/v) NH_4OH at 100° for 1 hr resulted in the formation of a water-soluble radioactive oligosaccharide phosphate which was isolated and characterized as $[Man]_5 \rightarrow$ [GlcNAc → GlcNAc → P. Incubation of [^{14}C]mannose-oligosaccharide pyrophosphoryl dolichol with myeloma microsomal preparations results in the transfer, presumably, of the entire oligosaccharide to endogenous protein. Kinetic studies indicate that the dolichol derivatives serve as intermediates in the glycosylation of protein as follows:**

$$\text{GDP-Man} \xrightarrow{P\text{-Dol}} \text{Man-}P\text{-Dol} \xrightarrow{[\text{GlcNAc}]_2\text{-}P\text{-}P\text{-Dol}} [\text{Man}]_5\text{-}[\text{GlcNAc}]_2\text{-}P\text{-}P\text{-Dol} \xrightarrow{\text{Protein}} [\text{Man}]_5\text{-}[\text{GlcNAc}]_2\text{-Protein}.$$

The mouse myeloma tumor, MOPC-46B, synthesizes a kappa-type immunoglobulin light chain. This light chain is a glycoprotein of approximately 25,000 molecular weight with a single carbohydrate side chain attached to the peptide chain by an *N*-glycosidic linkage between *N*-acetylglucosamine (GlcNAc) and asparagine residue 34 of the protein (1). We recently reported (2) that microsomal preparations from this myeloma tumor catalyze the biosynthesis of mannosyl-phosphoryl-dolichol (Man-*P*-Dol), and that this mannolipid serves as a donor of mannosyl residues in the glycosylation of endogenous protein. Similar observations were reported with enzyme preparations from hen oviduct (4) and rat liver (5).

The purpose of this communication is to present evidence for the enzymatic synthesis of a mannose- and glucosamine-containing oligosaccharide dolichol derivative (oligosaccharide-*P*-*P*-Dol) and that these glycosyl derivatives of dolichol participate in the glycosylation of protein in the following sequence of reactions:

$$\text{GDP-Man} \rightarrow \text{Man-}P\text{-Dol} \rightarrow [\text{Man}]_5\text{-}[\text{GlcNAc}]_2\text{-}P\text{-}P\text{-Dol} \rightarrow [\text{Man}]_5\text{-}[\text{GlcNAc}]_2\text{-Protein}$$

MATERIALS AND METHODS

The mouse myeloma tumor, MOPC-46B, was kindly made available to us by Dr. Michael Potter of the National Institutes of Health; the tumor was maintained and enzyme fractions were prepared as described (2). Except where noted otherwise, incubation mixtures were prepared, incubated, and analyzed under conditions essentially the same as those described (2). A preparation of purified α-mannosidase (EC 3.2.1.24) (6) was a gift from Dr. Yu-Teh Li, Department of Biochemistry, Tulane University School of Medicine, New Orleans, La. 70112.

All chemicals and reagents were the highest grades available commercially. GDP-[^{14}C]Man (160 μCi/μmole) and sodium [^{3}H]borohydride (160 μCi/μmole) were obtained from New England Nuclear Corp. Concanavalin A–Sepharose and Sephadex gels were purchased from Pharmacia Fine Chemicals, Inc. Pure *Escherichia coli* alkaline phosphatase was purchased from Sigma Chemical Co. Radioactivity measurements were conducted on particulate, water-soluble and organic solvent-soluble fractions of incubation mixtures as described (2).

RESULTS

Isolation and Characterization of Oligosaccharide Phosphate. During the course of studies designed to elucidate the role of [^{14}C]Man-*P*-Dol in the glycosylation of protein (see Fig. 8; ref. 2), we observed that while considerable [^{14}C]mannose was incorporated into protein, a larger portion of the radioactivity rapidly appeared in the water-soluble fraction of the incubation mixture. Preliminary analysis of this fraction indicated that virtually all of the radioactivity was contained in an 1800–2000 molecular weight substance. Further characterization of this radioactive substance involved purification by gel filtration and DEAE-cellulose chromatography; analysis of the purified material by paper chromatography

Abbreviations: The term dolichol (Dol) refers to a family of C-100, α-saturated polyisoprenols (ref. 3); GlcNAc, *N*-acetylglucosamine.

* Present address: Department of Clinical Biochemistry, University of Minnesota Graduate School, Minneapolis, Minn. 55455.
† To whom requests for reprints should be sent at the University of Pittsburgh.

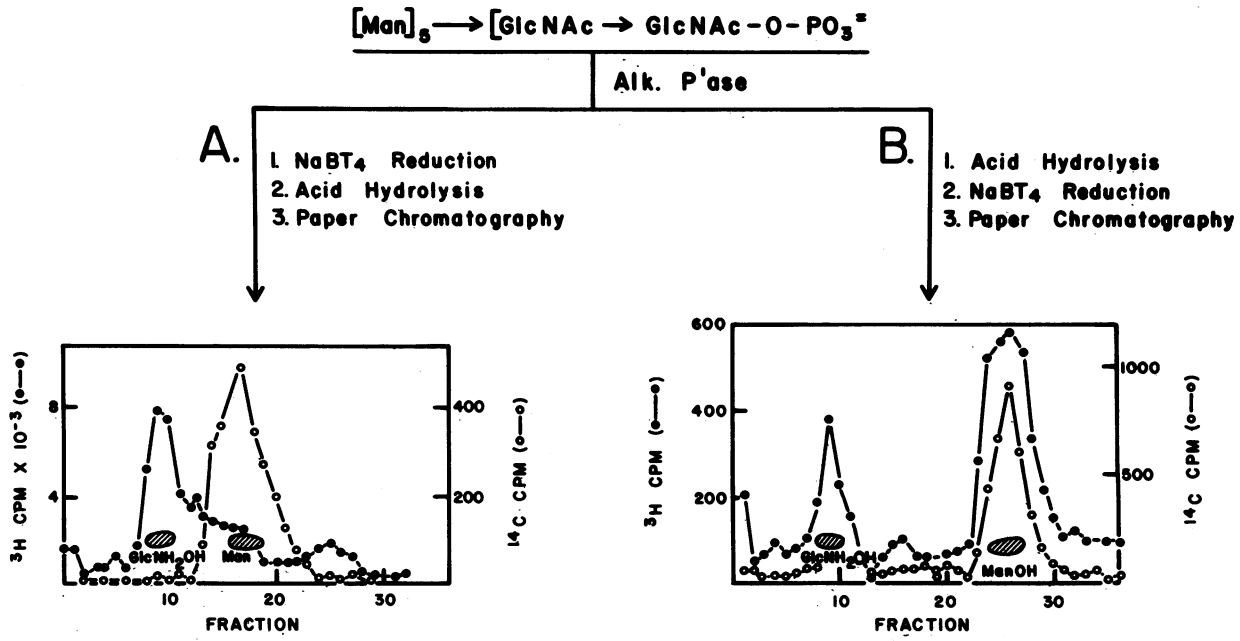

FIG. 1. Structural analysis of oligosaccharide phosphate. The radioactive oligosaccharide phosphate was isolated from the aqueous soluble fraction of an incubation mixture containing [^{14}C]Man-*P*-Dol as substrate. The radioactive material was purified by paper electrophoresis, gel filtration chromatography, and finally by DEAE-cellulose chromatography. The final product appeared homogeneous by paper chromatographic and electrophoretic criteria. Two aliquots *A* and *B* were treated as follows: *A* was successively reduced with sodium [^{3}H]borohydride, acid hydrolyzed, and analyzed by paper chromatography; and *B* was successively acid hydrolyzed, reduced with sodium [^{3}H]borohydride, and analyzed by paper chromatography. Acid hydrolysis was conducted in 3 N HCl at 100° for 3 hr; reduction was carried out in 1000-fold excess of sodium [^{3}H]borohydride for 60 min at pH 9; paper chromatography was conducted with Whatman 3 MM paper in ethyl acetate–acetic acid–formic acid–water 18:3:1:4. Chromatograms were cut in 1-cm strips and each was suspended in scintillation fluid for determination of radioactivity. *Alk. P'ase*, alkaline phosphatase. ^{3}H cpm in *A* have been multiplied by 10^{-3}, as indicated.

and paper electrophoresis indicated that: (*a*) it is composed of a single radioactive compound; (*b*) when subjected to electrophoresis the compound migrates as an anion and is converted to a neutral compound by treatment with alkaline phosphatase; (*c*) the dephosphorylated, neutral compound exhibits a molecular weight of 1350, as determined by gel filtration analysis (7); and (*d*) acid hydrolysis (3 N HCl, 3 hr, 100°) quantitatively liberates all of the radioactivity as free mannose.

On the basis of further structural analysis, as shown in Fig. 1, we have concluded that the water-soluble, radioactive compound is a [^{14}C]mannose-containing oligosaccharide phosphate. No detectable radioactivity is incorporated into the oligosaccharide phosphate when it is treated directly with sodium [^{3}H]borohydride, indicating that the phosphate residue occupies the potential reducing terminus of the oligosaccharide. After removal of the phosphate group with alkaline phosphatase, subsequent treatment of the neutral oligosaccharide with sodium [^{3}H]borohydride followed by acid hydrolysis and paper chromatographic analysis indicated that only glucosaminitol contains ^{3}H, whereas all of the ^{14}C is present in free mannose. Conversely, after alkaline phosphatase treatment, acid hydrolysis of the oligosaccharide prior to reduction with sodium [^{3}H]borohydride and paper chromatographic separation revealed the presence of ^{3}H-labeled glucosaminitol, and mannitol labeled with both ^{14}C and ^{3}H. We have concluded that this compound is an oligosaccharide phosphate containing mannose and *N*-acetylglucosamine with a potential terminal reducing *N*-acetylglucosamine residue substituted with a phosphomonoester residue. From the relative ^{3}H content of glucosaminitol (3058 cpm) and mannitol (7560 cpm), the molar proportions of glucosamine and mannose in the oligosaccharide were estimated to be five mannose residues per two glucosamine residues per reducing terminus. On the basis of the relative specific activity of [^{14}C]mannose in the oligosaccharide, it was estimated that approximately three of the five mannose residues had been incorporated into the oligosaccharide from [^{14}C]Man-*P*-Dol; presumably, unlabeled endogenous mannose as well as unlabeled *N*-acetylglucosamine were present in the oligosaccharide. Treatment of the oligosaccharide with α-mannosidase resulted in liberation of 85% of the radioactivity as free mannose.

Isolation of Oligosaccharide Pyrophosphoryl Dolichol. After characterization of the structural features of the [^{14}C]mannose-containing oligosaccharide phosphate, we postulated that this compound may have been formed in the incubation mixtures as a result of degradation of an oligosaccharide-phosphoryl (or pyrophosphoryl) dolichol derivative and that the latter compound may also be an intermediate in the glycosylation of protein. Therefore, incubation mixtures derived from experiments conducted with GDP-[^{14}C]Man were re-examined for the possible presence of [^{14}C]mannose-labeled oligosaccharide lipid. The entire incubation mixture was solubilized in 2% (w/v) Triton X-100 and applied to a column of Sephadex G-75 which was developed with a solution of 0.05 M ammonium bicarbonate. The voided material contained a small amount of radioactive protein and a larger proportion of radioactivity that was soluble in organic solvent. Analysis of the chloroform–methanol-soluble fraction by thin-layer chromatography indicated the presence of two radio-

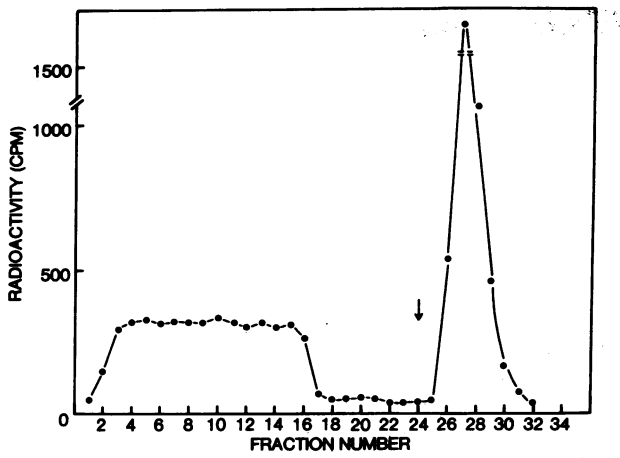

FIG. 2. Fractionation of [^{14}C]mannose-containing lipids on concanavalin A–Sepharose. A microsomal preparation was incubated with GDP-[^{14}C]Man as described, adjusted to 2% Triton X-100, and centrifuged at 105,000 × *g* for 1 hr. The supernatant fluid was applied to a Sephadex G-75 column and eluted with a solution of 0.05 M ammonium bicarbonate. The radioactive material voided from the column was pooled and applied to a column (1 × 10 cm) of concanavalin A–Sepharose, which was then eluted with a solution containing 0.05 M ammonium bicarbonate, 1% Triton X-100, and 0.5 M sodium chloride; when the effluent was devoid of radioactivity, the eluting solution was adjusted to 10% (w/v) α-methylmannoside (*arrow*) and elution was continued until all of the radioactivity was released from the column.

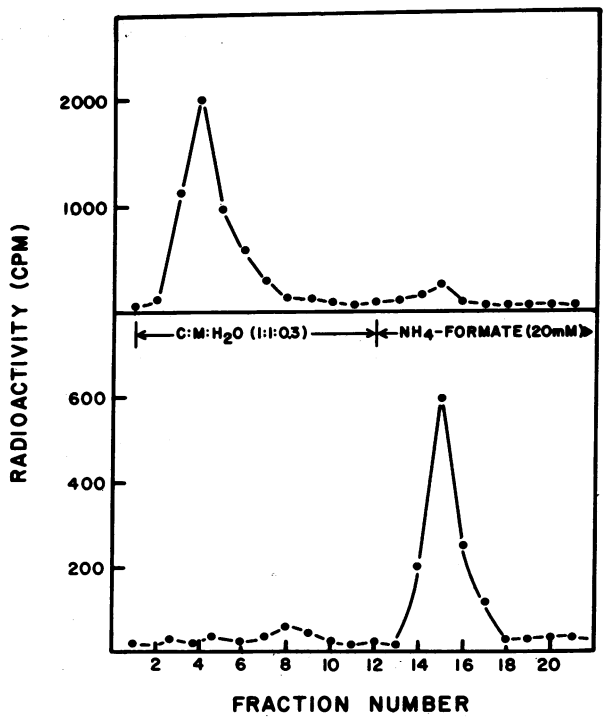

FIG. 3. Fractionation of Man-*P*-Dol (*top*) and oligosaccharide-*P*-*P*-Dol (*bottom*) on DEAE–cellulose. [^{14}C]Mannose-labeled preparations of each of the lipids were isolated from the concanavalin A–Sepharose eluate (Fig. 2), dissolved in chloroform–methanol–water 1:1:0.3, and applied to a column (1 × 10 cm) of DEAE–cellulose, equilibrated in the same solvent. Each of the columns was eluted as shown in Fig.; 2-ml fractions were collected and aliquots were removed from each for determination of radioactivity.

active constituents, one of which corresponded to [^{14}C]Man-*P*-Dol. The two radioactive constituents were separated quantitatively by fractionation of the mixture on a concanavalin A–Sepharose column in 1% Triton X-100 as shown in Fig. 2. The voided material from the Sephadex G-75 column was applied to a concanavalin A–Sepharose column and the column was washed with buffer containing 1% Triton X-100, resulting in elution of a portion of the radioactivity at the breakthrough volume in a broad, diffuse peak; addition of α-methylmannoside to the same eluting buffer eluted the remainder of the radioactivity as a single peak. Further analysis of these two radioactive fractions by thin-layer chromatography (see solvents below) indicated that the material not adsorbed to the affinity column is pure [^{14}C]Man-*P*-Dol, and that the material that bound to the gel and that was eluted with α-methylmannoside is a glucosamine- and [^{14}C]mannose-containing oligosaccharide-lipid derivative.

The two mannose-containing lipids are readily distinguished when fractionated on DEAE–cellulose as shown in Fig. 3. Man-*P*-Dol is eluted from DEAE–cellulose in the chloroform–methanol–water solvent while the oligosaccharide-lipid appears to be more acidic in that elution requires 20 mM ammonium formate. In addition, several thin-layer chromatographic systems distinguish the two mannolipids from each other. The relative migration (R_F) of Man-*P*-Dol and the oligosaccharide-lipid, respectively, are: chloroform–methanol–acetic acid–water (25:15:4:2), 0.90 and 0.16; chloroform–methanol–ammonium hydroxide (75:25:4), 0.43 and 0.16; and *n*-propanol–water (8:2), 0.54 and 0.25.

Treatment of purified [^{14}C]mannose-labeled oligosaccharide-lipid with 10% NH_4OH for 1 hr at 100° results in conversion of the radioactivity to a water-soluble form; this material was isolated and shown to be identical in all respects to the oligosaccharide phosphate described in Fig. 1. These results, in addition to the observations that the oligosaccharide-lipid is more acidic than Man-*P*-Dol, suggest that the oligosaccharide is linked to the lipid moiety by a pyrophosphate bond (see Fig. 6). While the lipid moiety of the oligosaccharide derivative has not yet been fully characterized, its properties are consistent with the assumption that the lipid is dolichol, as described by Behrens *et al.* (8). We concluded, therefore that the appearance of the [^{14}C]mannose-labeled oligosaccharide phosphate in the aqueous-soluble fraction of incubation mixtures is a result of degradation (enzymatic?) of [^{14}C]mannose-labeled oligosaccharide-*P*-*P*-Dol, which had been synthesized enzymatically during the incubation period.

Role of Man-P-Dol and Oligosaccharide-P-P-Dol in the Glycosylation of Protein. Substrate quantities of [^{14}C]mannose-labeled Man-*P*-Dol and oligosaccharide-*P*-*P*-Dol were prepared in order to determine the metabolic relationship of these two lipid derivatives and their roles in the enzymatic sequence resulting ultimately in the glycosylation of protein. As shown in Fig. 4, incubation of myeloma microsomal preparations with pure [^{14}C]Man-*P*-Dol results in the disappearance of radioactive mannose from the substrate with the concomitant, stoichiometric appearance of [^{14}C]mannose in oligosaccharide-*P*-*P*-Dol; only small amounts of radioactivity appear in either the aqueous phase or in the protein fraction. Thus, there appears to be a rapid transfer of mannose from the monomannosyl lipid to the oligosaccharide lipid.

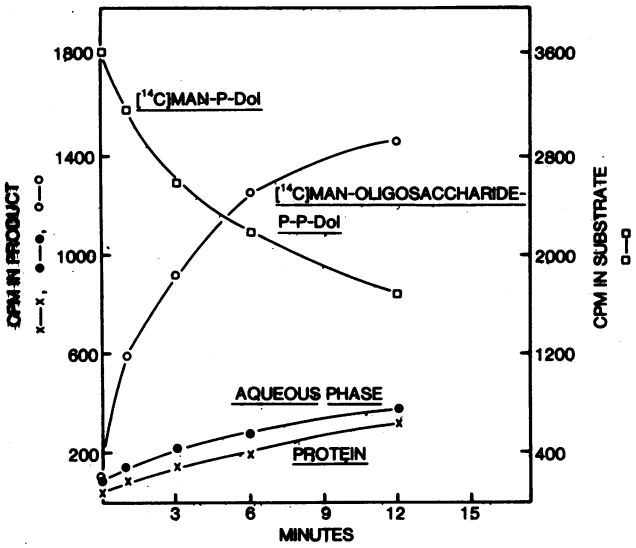

FIG. 4. Transfer of radioactivity from [14C]Man-*P*-Dol to oligosaccharide-*P*-*P*-Dol by myeloma microsome preparation. Aliquots of the incubation mixture were adjusted to 1.5% sodium chloride and then extracted with chloroform–methanol (3:2); the organic phase contained [14C]Man-*P*-Dol. The insoluble interphase was extracted with chloroform–methanol–water (1:1:0.3); the soluble fraction contained [14C]oligosaccharide-*P*-*P*-Dol and the insoluble fraction contained protein. The protein fraction was washed several times in 10% trichloroacetic acid.

Conversely, as shown in Fig. 5, when isolated [14C]mannose-labeled oligosaccharide-*P*-*P*-Dol is used as substrate with the microsomal membrane preparation, radioactivity disappearas from the oligosaccharide lipid with the concomitant appearance of radioactivity in protein and in the aqueous phase of the fractionated incubation mixture. Kinetically, the oligosaccharide-*P*-*P*-Dol is synthesized, at least partially, by the transfer of mannose from [14C]Man-*P*-Dol and in a subsequent reaction, the [14C]mannose-labeled oligosaccharide-*P*-

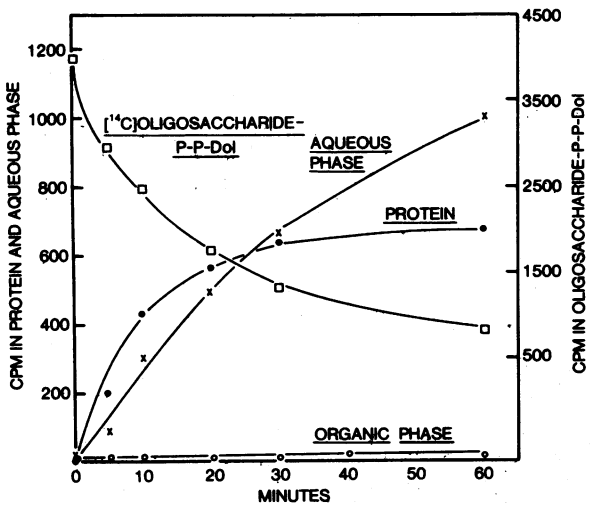

FIG. 5. Transfer of [14C]mannose-labeled oligosaccharide from oligosaccharide-*P*-*P*-Dol to endogenous protein by myeloma microsome preparation. At intervals indicated, aliquots of the incubation mixture were fractionated as described in the legend to Fig. 4 in order to determine the radioactive content of each constituent.

P-Dol appears to be the primary donor of radioactivity in the glycosylation of protein. Again, the radioactivity that appears in the water-soluble fraction of the incubation mixture under these conditions was isolated and characterized as [14C]mannose-labeled oligosaccharide phosphate as described previously. Because of the rapidity with which the oligosaccharide lipid is degraded, it seems likely that enzymatic hydrolysis of the compound may be occurring during the incubation.

The nature of the glycoprotein product formed in these experiments has not yet been fully characterized. The radioactive protein isolated from incubation mixtures containing myeloma microsomal preparations and [14C]mannose-labeled oligosaccharide-*P*-*P*-Dol as substrate exhibited the following properties: (*a*) it is insoluble, particulate bound, and is solubilized with 1% sodium dodecyl sulfate or 2% Triton X-100; (*b*) gel filtration of solubilized protein indicates that most of the labeled protein has a molecular weight in the range of 20,000–50,000; (*c*) after proteolytic digestion (2), essentially all of the radioactivity is recovered as low molecular weight species that exhibit electrophoretic properties of glycopeptides; and (*d*) approximately 10–20% of the radioactive protein is immunoprecipitable with specific anti-kappa 46B antibody.

DISCUSSION

The observation that radioactivity from a [14C]mannose-labeled oligosaccharide-*P*-*P*-Dol is transferred to protein when incubated with mouse myeloma microsomes strongly suggests that this oligosaccharide lipid may play a key role in the biosynthesis of the core region of the carbohydrate oligosaccharide residues of glycoproteins. Thus, it is assumed that the entire "pre-assembled" oligosaccharide is transferred intact to protein, since the only activated glycosidic bond in oligosaccharide-*P*-*P*-Dol is the *N*-acetylglucosaminyl bond, linking the oligosaccharide to dolichol through a pyrophosphate bridge. Further support of this contention may be gained from the observations that several glycoproteins possess carbohydrate sidechain core region structures which, in general features, are identical to the oligosaccharide described in these studies. Hickman, *et al.* (9), studying γ-M-immunoglobulin, and Arima and Spiro (10), studying thyroglobulin, concluded that a common carbohydrate sidechain core structure of both of these glycoproteins consists of an oligosaccharide containing five mannose residues and two *N*-acetylglucosamine residues with one of the *N*-acetylglucosamine residues serving as the site of attachment of the oligosaccharide to an asparagine residue of the protein through an *N*-glycosidic linkage. If the oligosaccharide dolichol derivative proves to be an obligatory intermediate in the biosynthesis of the core region of oligosaccharide sidechains of glycoproteins, it would establish an interesting analogy with oligosaccharide-polyisoprenol intermediates that function in the biosynthesis of bacterial polymers (11).

Is is possible, however, that the oligosaccharide dolichol derivative may play a minor role in glycoprotein biosynthesis and the bulk of the core portion of the carbohydrate sidechains of glycoproteins may be synthesized by a series of individual glycosyl transferases functioning in sequence, as has been clearly demonstrated to be the case with regard to the addition of distal sugar residues in glycoprotein oligosaccharide sidechains (12).

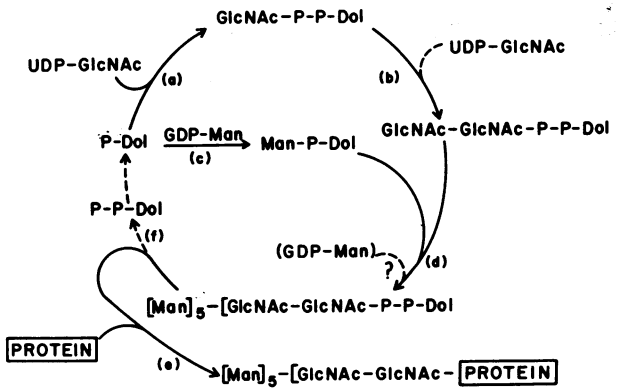

FIG. 6. Proposed role of dolichol derivatives in mammalian glycoprotein biosynthesis. Reactions *a* through *f* are discussed in the *text*.

We have not yet elucidated each of the individual steps in the metabolism of the mannose-containing dolichol derivatives. However, on the basis of the results of our studies and those currently available from the work of others, a cyclic sequence involving the mannose-containing dolichol derivatives, ultimately resulting in the glycosylation of protein, may be proposed. Several years ago, Molnar (13) reported that a microsomal particulate preparation catalyzes the transfer of *N*-acetylglucosaminyl phosphate from UDP-GlcNAc to an endogenous lipid. In the proposed sequence shown in Fig. 6, a cyclic sequence may be initiated by such a phosphoglycosyl transferase reaction (reaction *a*) resulting in the transfer of *N*-acetylglucosaminyl phosphate to dolichol phosphate to form GlcNAc-*P*-*P*-Dol. A second *N*-acetylglucosamine residue may be transferred from UDP-GlcNAc to form GlcNAc-GlcNAc-*P*-*P*-Dol (reaction *b*), as reported by Leloir *et al.* (14). We previously reported (2) the biosynthesis of Man-*P*-Dol (reaction *c*), using microsome preparations from mouse myeloma tumors, and the formation of this mannolipid has been reported in various tissues by others (4, 5, 15). The formation of mannose-, glucose-, and *N*-acetylglucosamine-containing oligosaccharide derivatives of dolichol has been reported by Leloir and his coworkers (5, 8, 14). The results of our current experiments establish that Man-*P*-Dol and GlcNAc-GlcNAc-*P*-*P*-Dol participate in the biosynthesis of $[Man]_5$-$[GlcNAc]_2$-*P*-*P*-Dol (reaction *d*). However, it is possible that only the α-linked mannose residues of the complete oligosaccharide are derived from Man-*P*-Dol; thus, GDP-Man or some other mannosyl donor could serve as precursor to a portion of the mannose residues in the oligosaccharide. In the proposed sequence the transfer of the oligosaccharide to protein (reaction *e*) would generate *P*-*P*-Dol which, presumably, would then be converted to the monophosphoryl derivative (reaction *f*) in order to re-initiate the cycle.

Note Added in Proof. [^{14}C]Mannose-labeled oligosaccharide, isolated by alkaline borohydride hydrolysis (16) from protein synthesized *in vitro* (Fig. 5), was shown to possess a reducing terminal GlcNAc residue and to be chromatographically identical to [^{14}C]mannose-labeled oligosaccharide isolated from oligosaccharide-*P*-*P*-Dol. These results support the contention that intact oligosaccharide is transferred from oligosaccharide-*P*-*P*-Dol to form an *N*-glycosidic linkage to protein.

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