Quantitative Assay and Subcellular Distribution of Enzymes Acting on Dolichyl Phosphate in Rat Liver

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ABSTRACT

To establish on a quantitative basis the subcellular distribution of the enzymes that glycosylate dolichyl phosphate in rat liver, preliminary kinetic studies on the transfer of mannose, glucose, and N-acetylglucosamine-1-phosphate from the respective 14C-labeled nucleotide sugars to exogenous dolichyl phosphate were conducted in liver microsomes. Mannosyltransferase, glucosyltransferase, and, to a lesser extent, N-acetylglucosamine-phosphotransferase were found to be very unstable at 37°C in the presence of Triton X-100, which was nevertheless required to disperse the membranes and the lipid acceptor in the aqueous reaction medium. The enzymes became fairly stable in the range of 10-17°C and the reactions then proceeded at a constant velocity for at least 15 min. Conditions under which the reaction products are formed in amount proportional to that of microsomes added are described. For N-acetylglucosaminephosphotransferase it was necessary to supplement the incubation medium with microsomal lipids. Subsequently, liver homogenates were fractionated by differential centrifugation, and the microsome fraction, which contained the bulk of the enzymes glycosylating dolichyl phosphate, was analyzed by isopycnic centrifugation in a sucrose gradient without any previous treatment, or after addition of digitonin. The centrifugation behavior of these enzymes was compared to that of a number of reference enzymes for the endoplasmic reticulum, the Golgi complex, the plasma membranes, and mitochondria. It was very similar to that of enzymes of the endoplasmic reticulum, especially glucose-6-phosphatase. Subcellular preparations enriched in Golgi complex elements, plasma membranes, outer membranes of mitochondria, or mitoplasts showed for the transferases acting on dolichyl phosphate relative activities similar to that of glucose-6-phosphatase. It is concluded that glycosylation of dolichyl phosphate into mannose, glucose, and N-acetylglucosamine-1-phosphate derivatives is restricted to the endoplasmic reticulum in liver cells, and that the enzymes involved are similarly active in the smooth and in the rough elements.

Biosynthesis of the core portion of N-glycosidically linked saccharide chains of glycoproteins involves a number of membrane-bound enzymes and glycoside derivatives of dolichyl phosphate (reviewed in reference 42). Glycoproteins of the Asn-glycoside type include many secretory and membrane proteins. Therefore, the subcellular distribution of the enzymes acting on dolichyl phosphate is an essential piece of information in the understanding of secretion process and membrane biogenesis.

Biochemical data reported for various subcellular preparations suggest that the enzymes involved in core glycosylation of proteins are widely distributed among the cell membranes (10, 13, 17, 18, 19, 23–25, 35, 41). Similar specific activities have been found in rough and in smooth microsomes from rat liver for the transfer of mannose (41) and glucose (13) from nucleotide sugars to lipid in the presence of added dolichyl phosphate, for the transfer of glucose to endogenous proteins (13), and for the transfer of an oligosaccharide from a lipid precursor to endogenous proteins (35). However, mannose is transferred from labeled guanosine diphosphate (GDP)-mannose to endogenous dolichyl phosphate and protein more efficiently by rough than by smooth microsomes prepared from hen oviduct (18) or from rat liver (41). In contrast, N-acetylglucosamine is transferred from uridine diphosphate (UDP)-
N-acetylglucosamine to endogenous proteins more efficiently by smooth than by rough microsomes (10) (see, however, reference 13), but it is unclear whether these transferred N-acetylglucosamine residues are internal or peripheral. Incorporation of N-acetylglucosamine into lipid derivatives in the presence of exogenous dolichyl phosphate is similar (13), or fourfold lower (10), in smooth microsomes as compared to rough microsomes. It has also been claimed that enzymes of the pathway for core glycosylation of proteins occur in mitochondria (17, 19, 23-25) and in the Golgi complex (13, 19, 41) of liver cells. In addition, studies on rat spleen lymphocytes (34), hen oviduct cells (51), and mouse fibroblasts (44) suggest that proteins can be glycosylated through the pathway of lipid intermediates at the surface of intact cells.

In fact, quantitative data on the subcellular distribution of enzymes involved in the synthesis of the core portion of the saccharide chain of glycoproteins are still lacking. The meaning of sugar incorporation into endogenous acceptors is ambiguous because the values found may reflect the amount of acceptor present as well as the enzyme activity, depending on which one is limiting. Even when a lipid acceptor was added in excess, most results published so far were not truly quantitative measurements of enzyme activities, as evidenced by the nonlinear time-course of the reaction (13, 17, 23, 25, 41) or by the complex dependence of reaction rate upon the concentration of the detergent used for dispersion of the lipid reagent and membranes (9, 10, 17, 30, 35, 41). To remove these uncertainties, we have undertaken kinetic studies on the transfer of mannose, glucose, and N-acetylglucosamine-1-phosphate from nucleotide sugars to dolichyl phosphate in rat liver microsomes. The subcellular distribution of enzymes involved was determined by quantitative fractionation methods. In addition, various membrane preparations were examined for their ability to carry out these reactions. The results, which have been presented earlier in abstract form (46), show that mannosyltransferase, glucosyltransferase, and N-acetylglucosaminephosphotransferase belong to the endoplasmic reticulum membranes and are in no way restricted to the rough portions of this cell component. Neither mitochondria, nor the Golgi complex membranes that synthesize the peripheral saccharide portion of complex glycoproteins contain detectable activities of these enzymes.

Preparation and Subfractionation of Mitochondria

The livers from three rats were processed separately. They were chopped into pieces, added with 3 ml of ice-cold solution A (70 mM sucrose, 0.21 M mannitol, 0.1 mM EDTA, 1 mM Tris-HCl, pH 7.2 [43]) per gram of tissue, and homogenized by one passage of the teflon pestle of a tissue grinder model C (A. H. Thomas Co., Philadelphia, Pa.). The homogenate was centrifuged for 10 min at 1,700 rpm in the no. 225 rotor of the DFR-6000 centrifuge (Damon/IEC Division, Needham Heights, Mass.) and refrigerated at 2°C. The sediment was washed by two cycles of resuspension in solution A and centrifugation. Mitochondria were obtained by centrifuging the pooled supernates for 5 min (acceleration time included) at 12,500 rpm in the no. 30 rotor (Beckman Instruments Inc., Spincio Division, Palo Alto, Calif.). After decantation, the pellets were washed twice by resuspension in solution A and centrifugation; they were finally transferred into a Dounce homogenizer ( Kontes Glass Co., Vineland, N.J.), added with a solution (3 ml/g of liver) of 20 mM sodium phosphate, pH 7.2, and 0.02% (wt/vol) bovine serum albumin (43), and suspended by means of the loose-fitting pestle. After 30 min at 0°C, the swollen mitochondria were given six strokes of the tight-fitting pestle to disassociate the outer membranes from the mitoplasts, and centrifuged for 20 min, 20,000 rpm in the Beckman no. 30 rotor. After decantation, the sediment was washed by resuspension in 1 ml of 0.25 M sucrose containing 0.75 mg digitonin/ml (~0.3 mg digitonin/mg protein) and finally resuspended in 0.25 M sucrose. This preparation, called disintegrated mitochondria, was subfractionated by centrifugation for 60 min at 39,000 rpm in the E-40 rotor (4), loaded with 10 ml sample corresponding to ~10 g of liver, 32 ml of a sucrose gradient extending linearly with respect to the volume from density 1.10 to 1.27, and 6 ml sucrose solution of density 1.32. Subfractions were recovered and processed for density measurement and analysis as described earlier (7).

Golgi Complex Preparation

Golgi elements were prepared by the following procedure described by Wilbo et al. (58).

Preparation of Plasma Membranes

Plasma membranes were prepared according to Song et al. (49), as described in a previous paper (58). Samples were withdrawn for analysis at various stages of the purification procedure. In short, the preparations consisted in membranes spun down at 1,800 rpm for 20 min in the IEC rotor no. 259 (low speed sediment), brought to equilibrium in a sucrose gradient at a density ~<1.18 U, and washed and resuspended in 0.25 M sucrose (type I preparation). These membranes were further purified taking advantage of the density perturbation of plasma membranes caused by digitonin (2.1 ml of 0.25 M sucrose containing 0.75 mg digitonin/ml (~0.3 mg digitonin/mg protein) was added dropwise at 0°C under continuous stirring. A 15 ml aliquot of the suspension was laid over a step gradient loaded in the SW 25.2 Beckman rotor, and made of the following layers of sucrose solutions: 8 ml at 52.2%, 15 ml at 41.2%, 15 ml at 39.3% and 5 ml at 37.1%, and centrifuged for 90 min at 39,000 rpm in the E-40 rotor. 259 (low speed sediment). Samples were withdrawn for analysis at various stages of the purification procedure. In short, the preparations consisted in membranes spun down at 1,800 rpm for 20 min in the IEC rotor no. 259 (low speed sediment), brought to equilibrium in a sucrose gradient at a density ~<1.18 U, and washed and resuspended in 0.25 M sucrose (type I preparation). These membranes were further purified taking advantage of the density perturbation of plasma membranes caused by digitonin (2.1 ml of 0.25 M sucrose containing 0.75 mg digitonin/ml (~0.3 mg digitonin/mg protein) was added dropwise at 0°C under continuous stirring. A 15 ml aliquot of the suspension was laid over a step gradient loaded in the SW 25.2 Beckman rotor, and made of the following layers of sucrose solutions: 8 ml at 52.2%, 15 ml at 41.2%, 15 ml at 39.3% and 5 ml at 37.1%, and centrifuged for 90 min at 39,000 rpm in the E-40 rotor. After centrifugation, the sediment was washed by resuspension in 1 ml of 0.25 M sucrose. This preparation, called disintegrated mitochondria, was subfractionated by centrifugation for 60 min at 39,000 rpm in the E-40 rotor (4), loaded with 10 ml sample corresponding to ~10 g of liver, 32 ml of a sucrose gradient extending linearly with respect to the volume from density 1.10 to 1.27, and 6 ml sucrose solution of density 1.32. Subfractions were recovered and processed for density measurement and analysis as described earlier (7).

Biochemical Determinations

Aliquots of subcellular fractions and membrane preparations were quickly frozen at ~80°C and thawed immediately before the assays.

Mannosyltransferase, Glucosyltransferase, and N-acetylglucosamine-phosphotransferase Assays

The dolichyl phosphate solution was diluted 20 times in chloroform/methanol (2/1, vol/vol), and 200-ml portions were stored at ~20°C in teflon-stoppered culture tubes (Kimax; Kimble Div., Owens-Illinois, Inc. Toledo, Ohio). Immediately before use, 0.5 pmol EDTA and 1 pmol MgCl2 (mannosyltransferase and glucosyltransferase) or MnCl2 (N-acetylglucosaminiphosphotransferase) were added in each tube (8). The content of the tubes was thoroughly mixed and evaporated at ambient temperature under a stream of nitrogen. The dried deposit was resuspended in 50 ml of 0.2 M Tris-HCl buffer, pH 7.4, 5.8 mM MgCl2, 11.5 mM MnCl2, and 2.5 mM dithiothreitol, 1 mM ATP to minimize enzymic hydrolysis of the nucleotide sugars (10, 54), and the additional reagents given below.

Mannosyltransferase was assayed at 10°C and pH 7.5 in the presence of 80 mM Tris-HCl buffer, 5.8 mM GDP-[3H]Man and 11.5 mM MgCl2. For glucosyl-
transf erase, the assay conditions were 17°C and pH 6.5, with 80 mM 2-(N-
morpholino)ethane sulfonic acid (MES)-KOH buffer, 3.3 mM UDP-14C
Glc and 11.5 mM MgCl₂. N-acetylglucosaminephosphotransferase was assayed at 17°C
and pH 7.5 in the presence of 80 mM MES-glycylglycine-KOH, 28 μM UDP-
14C GlcNac, 10 mM MgCl₂, 4 mM MnCl₂, and heat-denatured (5 min, 100°C)
microsomes from 3 mg liver. Radioactivity was ~500,000 cpm/assay. After 10
min, the reaction was stopped by adding 2.5 ml chloroform/methanol (3/2, vol/ vol)
and 0.4 ml of 4 mM MgCl₂. After mixing thoroughly, the phases were
separated by centrifugation; the aqueous upper layer was removed, and the lower
layer plus the insoluble material was washed twice with 1.25 ml chloroform/
methanol/4 mM MgCl₂ (1/16/16, vol/vol/vol). An aliquot of the lower phase
was assayed for radioactivity.

**Chromatographic Analysis**

Another aliquot of the lower phase was chromatographed on silica gel plates
(Kieselgel 60, Merck A.G., Darmstadt, W. Germany) with chloroform/methanol/
water (65/25/4, vol/vol/vol) as developing solvent (48). Radiolabeled glycolipids
produced from the endogenous dolichyl phosphate of liver rough microsomes
after incubation with UDP-14C GlcNac or GDP-14C Man in the presence of
CTP (28, 29) were used as standards.

To determine the amount of the nucleotide precursor left after incubation, the
aqueous phase was analyzed by descending chromatography on Whatman no. 1
paper in ethanol/1 M acetic acid adjusted at pH 3.8 with ammonia (2/1, vol/ vol).

**Other Biochemical Determinations**

Protein (40). NADH- and NADPH-cytochrome c reductases, cytochrome c
oxidase, glucose-6-phosphatase, alkaline phosphodiesterase 1, and galactosyl-
transferase (6), monoamine oxidase (59), N-acetylglucosaminyltransferase (with
ovalbumin acting as acceptor [58]) and RNA (21) were assayed according to the
methods described in the quoted articles. Assay of 5'-nucleotidase was performed
by the concentration of added dolichyl phosphate in a complex
facilitating the phosphotransferase reaction. In most fractionation experiments reported below,
the monoglycoside derivative (see below), even at the highest
concentrations was due to denaturation of the enzyme protein.

RESULTS

**Enzyme Kinetic Studies**

Kinetic studies were conducted to find out optimum assay
conditions for N-acetylglucosaminyltransferase, mannosyltransferase, and glucosyltransferase, and to determine the
range within which reaction velocity is proportional to enzyme
concentration. Microsomes were used as a source of enzyme.
The results obtained may be summarized as follows.

(a) Triton X-100 augmented noticeably the enzyme activities.

However, at 37°C, its concentration in the incubation medium of
mannosyltransferase was extremely critical in agreement with results from others (41, 57).
A sharp peak of activity was observed at 1 mg/ml (Fig. 1 a).
The inhibition noted at higher
concentrations was due to denaturation of the enzyme protein.
This effect is strongly temperature-dependent, as shown by the
time-course of mannose transfer at 37°C, 25°C, and 13°C (Fig.
1 b). In the presence of 2 mg Triton X-100/ml, the enzyme was
completely inactivated after 4 min at 37°C; it was somewhat
more stable at 25°C and, consequently, more mannose could be
transferred despite the lower initial velocity of the reaction at this
temperature; at 13°C the reaction proceeded almost linearly for 16 min. In view of these results, mannosyltransferase
was subsequently assayed at 10°C. Under these conditions,
the activity increases with the concentration of Triton X-100
up to a plateau reached at 2–3 mg detergent/ml (Fig. 1 c),
and corresponding to 40-fold the activity measured in the absence of detergent, an enhancement which is much higher than that
found at 37°C (Fig. 1 a, see references 41, 57). Glucosyltransf erase and N-acetylglucosaminephosphotransferase also lose activity at 25°C or above in the presence of Triton X-100
(results not shown). These reactions proceed linearly for >15
min at 17°C, the temperature at which the enzymes were
primarily assayed. Their dependence on the Triton X-100
concentration was similar to that shown for mannosyltransferase
as shown in Fig. 1 b, leading to 20- and 40-fold activations for N-
acetylglucosaminylphosphotransferase and glucosyltransferase, respectively.

(b) As shown in Fig. 2, the transfer reactions were influenced by the concentration of added dolichyl phosphate in a complex
manner. Glucosyltransferase and, to a lesser extent, N-acetyl-
glucosaminylphosphotransferase (see also reference 32) activities
were slightly inhibited at high dolichyl phosphate concentra-
tion. At the concentration used in the standard assay (80
μg/ml), the amount of label transferred was 40-fold (N-acetyl-
glucosamine-1-phosphate), 60-fold (glucose), and 500-fold
(mannose) greater than in the absence of added lipid. Such
enhancement factors are far above the values currently reported
(8, 17, 26, 33, 52, 57).

(c) The dependence of reaction rates upon the nucleotide sugar
concentration is shown in Fig. 3. Kₐ₅ values of 1.0 and
0.8 μM were calculated for GDP-Man and UDP-Glc, respective-
ly. The non-Michaelian relationship observed for UDP-
GlcNac suggested that two enzymes of different Ka might be
involved. Potentially, UDP-GlcNac could react with dolichyl
phosphate according to the following sequence:

(i) Dolichyl phosphate + UDP-GlcNac → dolichyl pyrophosphoryl
N-acetylglucosamine + UMP.

(ii) Dolichyl pyrophosphoryl N-acetylglucosamine + UDP-
GlcNac → dolichyl pyrophosphoryl N,N' diacetylchitobiose
+ UDP.

However, the reaction did not proceed beyond formation of the
monoglycoside derivative (see below), even at the highest
concentration of UDP-GlcNac used in this study. The kinetics
shown in Fig. 3 b is thus characteristic of the phosphotransferase
reaction. In most fractionation experiments reported below,
the activity was assayed at low (0.5 μM) and high (28 μM)
FIGURE 2 Dependence of transferase activities on the concentration of dolichyl phosphate. Mannosyltransferase ( ), glucosyltransferase (O), and N-acetylglucosaminephosphotransferase (Δ) activities of microsomes derived from 2.5 mg liver were assayed at various concentrations of dolichyl phosphate. Other conditions were as given under Materials and Methods.

concentration of UDP-GlcNAc, but this did not evidence any difference in the distribution patterns (not shown). In addition, tunicamycin abolished the activity at either concentration of the nucleotide sugar, although more tunicamycin was required in the presence of 28 μM UDP-GlcNAc to achieve the same inhibition (not shown). Consequently, if distinct N-acetylglucosaminephosphotransferases occur in the liver cells, they have identical subcellular distributions, and they are similarly sensitive to the antibiotic.

(d) Influence of pH was studied using Na cacodylate-HCl (pH 5.0–7.5), glycylglycine-KOH (pH 7.5–9.5), glycylglycine-MES-KOH (pH 7.0–9.5), Tris-HCl (pH 7.0–9.0), and MES-KOH (pH 5.5–7.0) buffers. Glucosyltransferase and N-acetylglucosaminephosphotransferase activities differed noticeably according to the buffer used (not shown); optimal conditions are those given in Materials and Methods.

(e) Owing probably to the presence of EDTA in the incubation medium, and in agreement with previous reports (8, 16, 26, 37, 55), the enzyme activities were undetectable in the absence of added divalent cations. In each case MgCl₂ was a more efficient activator than MnCl₂. The two salts have been included in the incubation medium for N-acetylglucosaminephosphotransferase because its activity has been demonstrated in the presence of Mg²⁺ (36, 56) or Mn²⁺ (10, 26, 31, 53).

(f) The reaction of mannosyltransferase and glucosyltransferase proceeded at a rate proportional to the amount of microsomes added up to ~8 and 4 mg liver (~0.4 and 0.2 mg microsomal protein), respectively (Fig. 4a and c). The relationship was also linear up to 4 mg liver when heat-denatured microsomes were included in the incubation medium of N-acetylglucosaminephosphotransferase reaction (Fig. 4b); otherwise, it consistently diverged from linearity in the range of 0–2 mg liver, where the activity was unexpectedly low. Addition of total lipid extracted from liver microsomes restored the expected level of activity, whereas the addition of bovine sera albumin had no effect (not shown). The added lipid could contain endogenous dolichyl pyrophosphoryl N-acetylglucosamine that acts as substrate for the next glucosyltransferase. We rule out this possibility, because tunicamycin completely inhibited the reaction, and owing to the behavior of the labeled products in thin layer chromatography (see below). Most likely, some microsomal phospholipids are required for full activity of the enzyme. A curve, concave upwards, was obtained previously with solubilized enzyme preparations from hen oviduct (36) and Acanthamoeba castellani (56), and with rat lung membranes (45). In the latter case addition of acidic phospholipids linearized the plot of activity vs. enzyme concentration.

For identification of the reaction products, the chloroform/methanol extracts were analyzed by thin layer chromatography. Radioautography revealed a single component, which ran with dolichyl phosphoryl mannose after incubation with GDP-
FIGURE 4 Dependence of transferase activities on amount of microsomes. Mannosyltransferase (A), N-acetylglucosaminephosphotransferase (B), and glucosyltransferase (C) were assayed at various microsome concentrations, as described under Materials and Methods. Reaction mixtures for N-acetylglucosaminephosphotransferase contained 0 (□), 1 (△), or 3 mg (○) (liver weight equivalent) of heat-denatured microsomes (5 min at 100°C).

[14C]Man, or UDP-[14C]Glc. Mannose and glucose derivatives of dolichyl phosphate have the same mobility in this chromatographic system (33, 57). The bulk of labeled products formed upon incubation with UDP-[14C]GlcNAC had the chromatographic properties of dolichyl pyrophosphoryl N-acetylglucosamine. The amount of dolichyl pyrophosphoryl N,N′-diacetylchitobiose represented <5% of the label extracted, allowing to take the total label extracted as a satisfactory measure for the N-acetylglucosaminephosphotransferase reaction. However, the fraction of label present as a diglycoside derivative became greater upon incubation for >10 min, or at 25-37°C. Aliquots of the medium were also analyzed by paper chromatography to establish the extent of hydrolytic cleavage of the nucleotide sugars during incubation. Except for the soluble fraction, it did not exceed 10% and had no effect on the measurement of transferase activities.

Activity in Various Subcellular Preparations

To seek for the possible occurrence of the transferases acting on dolichyl phosphate in membranes other than those of the endoplasmic reticulum, various membrane preparations were isolated and assayed for these enzyme activities. The morphological characteristics of similar membrane preparations have been published elsewhere (58).

Golgi complex preparations appeared purified, judging from...
Table I
Quantitative Distribution of Enzymes Acting on Dolichyl Phosphate after Differential Centrifugation

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Total activity in liver</th>
<th>Activity in subcellular fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol min⁻¹/g tissue</td>
<td>N</td>
</tr>
<tr>
<td>Mannosyltransferase</td>
<td>8.62 ± 0.99</td>
<td>10.1±1.6</td>
</tr>
<tr>
<td>Glucosyltransferase</td>
<td>1.11 ± 0.17</td>
<td>11.6±1.6</td>
</tr>
<tr>
<td>N-Acetylglucosaminephosphotransferase</td>
<td>0.79 ± 0.13</td>
<td>12.3±0.8</td>
</tr>
<tr>
<td>Protein</td>
<td>245.5 ± 25</td>
<td>16.4±0.8</td>
</tr>
</tbody>
</table>

Statistics refer to the mean ± SD in six experiments (five for N-acetylglucosaminephosphotransferase).

Figure 5 Distribution of the transferases acting on dolichyl phosphate, compared to that of reference enzymes, after fractionation of liver by differential centrifugation. These graphs were constructed as described in reference 4 from the average results of three to six experiments. Complementary data are given in Table I. Fractions are plotted from left to right in the following order: nuclear fraction (N), large granules (ML), microsomes (P), and final supernate (S). Each fraction is represented separately in the ordinate scale by the relative specific activity of the enzyme, i.e., the percentage amount of total recovered activity divided by the percentage amount of total recovered protein. In the abscissa scale, the protein content of fractions is represented cumulatively. N-Acetylglucosaminephosphotransferase (A), mannosyltransferase (B), and glucosyltransferase (C) were assayed on samples of particulate fractions which contained 40-50 μg phospholipid. The other enzymes shown for comparison are galactosyltransferase (D), glucose-6-phosphatase (E) and 5'-nucleotidase (F), which gave average recoveries of 79%, 101%, and 101%, respectively.

Figure 6 Density distribution of microsomal transferases acting on dolichyl phosphate and comparison with reference enzymes. Average results of three or four experiments in which microsomes have been centrifuged to equilibrium in a linear gradient of sucrose, as described under Materials and Methods. Frequency histograms have been normalized and averaged (see reference 4). The represented portion of histograms, divided into 15 normalized fractions of identical density increment, extends from 1.10 to 1.27 and includes >95% of the enzyme activities. Vertical lines through histogram bars give standard deviations. Solid lines are galactosyltransferase (A), 5'-nucleotidase (B), NADPH cytochrome c reductase (C), glucosyltransferase (D), mannosyltransferase (E), and N-acetylglucosaminephosphotransferase (F). The distribution of glucose-6-phosphatase (shading) is superimposed on each plot to facilitate comparison. Percentage values give the average recoveries from the microsome fraction. Recovery of glucose-6-phosphatase was 101%.

The 80-fold increase in specific activity for galactosyltransferase and N-acetylglucosamine transferase acting on ovalbumin, when compared to total liver (Table II). For the transfer of mannose, glucose, and N-acetylglucosamine-1-phosphate to dolichyl phosphate, the relative specific activities were much lower (0.8-1.7), and comparable to those found for NADPH cytochrome c reductase and glucose-6-phosphatase (0.7-1.6).

Table II also shows the properties of plasma membrane preparations at different stages of their isolation: the low speed sediment, the type I preparation, and the final preparation after treatment by digitonin and isolation of the material that has acquired a higher density (type II fraction). The relative specific activities of 5'-nucleotidase and alkaline phosphodiesterase I increase in this order; those of the other enzymes, including the transferases acting on dolichyl phosphate, decrease, the final levels being 1-5% of the relative specific activity of alkaline phosphodiesterase I. The transferases acting on dolichyl phosphate are in a twofold excess over glucose-6-phosphatase and NADPH cytochrome c reductase at all stages.

Other experiments were devised for settlement of the question as to whether the enzymes of interest occur in the outer or inner mitochondrial membranes (Table III and Fig. 8). Mitochondria prepared by differential centrifugation were successively subjected to osmotic swelling, gentle mechanical disruption, and density equilibrium in a linear gradient of sucrose. In these experiments the transferases acting on dolichyl phosphate behaved like glucose-6-phosphatase, with respect to their yield in the mitochondrial preparations, which ranged from 6.5 to 8.5% as compared to 64-69% for monoamine oxidase and cytochrome c oxidase (Table III), and to their density distri-
Equilibrium Density

FIGURE 7 Effect of digitonin on the density distribution of the microsomal transferases acting on dolichyl phosphate. Microsomes have been treated with digitonin according to reference 3 before density gradient analysis which was carried out exactly as described in Fig. 6. Enzymes are galactosyltransferase (A), alkaline phosphodiesterase I (B), glucosyltransferase (C), N-acetylgalcosaminylphosphotransferase (D), and mannosyltransferase (E). The distribution obtained after digitonin treatment (solid lines) is compared to that of the same enzyme in untreated microsomes (shading, redrawn from Fig. 6, except for alkaline phosphodiesterase I). Enzyme activities of the microsome fraction were not significantly modified by digitonin treatment. Percentage values give the recovery of enzyme activities in the gradient fractions, which was 104% for glucose-6-phosphatase.

TABLE II
Enzyme Activities in Plasma Membrane and Golgi Complex Preparations

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Type I</th>
<th>Type II</th>
<th>Golgi complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>9.45</td>
<td>0.99</td>
<td>0.45</td>
</tr>
<tr>
<td>Relative specific activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannosyltransferase</td>
<td>1.23</td>
<td>1.11</td>
<td>1.03</td>
</tr>
<tr>
<td>Glucosyltransferase</td>
<td>1.29</td>
<td>1.15</td>
<td>0.83</td>
</tr>
<tr>
<td>N-Acetylgalcosaminylphosphotransferase</td>
<td>1.32</td>
<td>1.21</td>
<td>1.08</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>N-Acetylgalcosaminyltransferase</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase I</td>
<td>5.04</td>
<td>16.6</td>
<td>22.2</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>4.11</td>
<td>15.4</td>
<td>21.0</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>0.71</td>
<td>0.85</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>0.86</td>
<td>0.65</td>
<td>0.61</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>0.97</td>
<td>1.17</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Subcellular fractions were obtained as described under Materials and Methods. Relative specific activities are as defined in the legend of Fig. 5. The percentage amount of enzyme activity recovered in a subcellular preparation is given by the product: percent yield of protein times relative specific activity. ND, not determined.

Equilibrium Density

FIGURE 8 Density distribution of the transferases acting on dolichyl phosphate, compared to that of several reference enzymes, in a mitochondrial fraction subjected to hypotonic conditions and equilibrated in a sucrose gradient. Conditions for isolation of mitochondria, disintegration in an hypotonic medium, and isopycnic centrifugation are given under Materials and Methods. The represented portion of frequency histograms extends from 1.08 to 1.28 and corresponds to >95% of the enzyme activities in the mitochondrial preparation. Solid lines are monoamine oxidase (A), cytochrome c oxidase in a distinct experiment (B), mannosyltransferase (C), glucosyltransferase (D), and N-acetylgalcosaminylphosphotransferase (E). The distribution of glucose-6-phosphatase (shading) is superimposed on each plot to facilitate comparison. Quantitative biochemical data on the mitochondrial fraction used, and on the properties of the gradient subfractions characteristically enriched in outer (Υ) and inner (Ω) mitochondrial membranes are reported in Table III. Percentage values give the recovery of enzyme activities in the gradient fractions. The recovery of glucose-6-phosphatase was 98%.

TABLE III
Enzyme Activities in Mitochondrial and Submitochondrial Preparations

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Disintegrated mitochondria</th>
<th>Subfraction of density</th>
<th>Subfraction of density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>17.3</td>
<td>0.56</td>
<td>6.9</td>
</tr>
<tr>
<td>Relative specific activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannosyltransferase</td>
<td>0.38</td>
<td>1.03</td>
<td>0.09</td>
</tr>
<tr>
<td>Glucosyltransferase</td>
<td>0.41</td>
<td>1.31</td>
<td>0.08</td>
</tr>
<tr>
<td>N-Acetylgalcosaminylphosphotransferase</td>
<td>0.48</td>
<td>1.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>4.0</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Monoamine oxidase</td>
<td>3.7</td>
<td>57.0</td>
<td>0.26</td>
</tr>
<tr>
<td>NADH cytochrome c reductase</td>
<td>0.90</td>
<td>8.1</td>
<td>0.15</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>0.49</td>
<td>1.22</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Average results of two experiments. Relative specific activities are as defined in the legend of Fig. 5; see also Table II for estimation of the yield of enzyme activities.
These distributions are broad and centered around the middle of the gradient; they clearly differ from those of monoamine oxidase and cytochrome c oxidase, which sharply peak at modal densities of 1.12 and 1.23 U, respectively. Quantitative data on the corresponding subfractions are reported in Table III. It is apparent from these data that light (density 1.12) and heavy (density 1.23) subfractions are fairly purified outer and inner membranes of mitochondria, respectively. Like glucose-6-phosphatase, the transferases acting on dolichyl phosphate reach relative specific activities that are only 2–2.5% of those of monoamine oxidase in the outer membrane-rich fraction, and of cytochrome c oxidase in the inner membrane-rich fraction. In contrast, NADH cytochrome c reductase, an established constituent of both the endoplasmic reticulum and the outer membrane of mitochondria (50) reveals a distinctly higher specific activity in the light subfraction.

DISCUSSION

The enzymes forming the monoglycoside derivatives of dolichyl phosphate are highly susceptible to denaturation in the presence of a detergent. This property, exemplified for mannosyltransferase by the bell-shaped curve of the activity at 37°C when the concentration of Triton X-100 is varied (Fig. 1a), and by the nonlinear time-course at 25°C or above (Fig. 1b), is also apparent from other studies (17, 35, 41). We have found that mannosyltransferase, glucosyltransferase, and N-acetylglucosaminemembrane transferase activities can nevertheless be accurately measured provided the temperature be kept low enough, i.e., 10–17°C. In the case of N-acetylglucosaminemembrane transferase, the presence of enough phospholipid in the reaction medium is an additional requirement. Our assay methods differ from those used in other laboratories only in these few aspects which turned out to be essential. The fair recovery values after subcellular fractionation (see Table I and Figs. 6–8) strengthen the kinetic evidence (Fig. 4), and argue for the validity of the methods used and of the distributions obtained.

According to these distributions, the three enzymes may be assigned to subcellular elements derived from the endoplasmic reticulum. They closely follow markers of this cell component in differential centrifugation (Fig. 5), and when microsomes or mitochondrial preparations are subfractionated by density equilibrium in a linear gradient of sucrose (Figs. 6 and 8). In addition, their host membranes in the microsome fraction are unaltered in their equilibrium density after treatment with digitonin (Fig. 7). This differentiates these microsomal membranes from the Golgi complex and plasma membrane elements.

In agreement with our analytical data, several subcellular preparations enriched in either the Golgi complex, the large plasma membrane sheets derived from the surface of hepatocytes (see 58), or the outer or inner membranes of mitochondria contain only a very small part of the total activity of the enzymes acting on dolichyl phosphate. We have recently discussed the significance of the low activities of endoplasmic reticulum–associated enzymes that are found in various preparations enriched in other subcellular components (58). The conclusion is that it reflects cross-contamination by membranes biochemically undistinguishable from those derived from the endoplasmic reticulum. This conclusion holds for the transferases acting on dolichyl phosphate because their yields are similar to those of glucose-6-phosphatase, or NADPH cytochrome c reductase (Tables II and III). However, the three transferases are in slight excess over the reference enzymes of the endoplasmic reticulum in our plasma membrane preparations (Table II), which might be a clue in favor of their occurrence at the cell surface. Nevertheless, this possibility will not be considered, because upon increasing purification of plasma membranes the specific activity of the enzymes acting on dolichyl phosphate decreases in parallel to that of the authentic enzymes of endoplasmic reticulum.

It is thus most likely that the enzymes which form the monoglycoside derivatives of dolichyl phosphate are restricted to the endoplasmic reticulum in liver cells. This conclusion conflicts with studies reporting activity of mannosyltransferase (13, 17, 23, 41), glucosyltransferase (13, 17, 19, 24), or N-acetylglucosaminemembrane transferase (10, 13, 17, 25) in various other subcellular membrane entities, including the mitochondria and Golgi apparatus. The main reason for this discrepancy is the quantitative comparison with other reference enzymes, from which our conclusion is drawn, was not possible in these earlier works. For the mitochondrial membranes, our results illustrate how misleading the presence of enzyme activities in a single membrane fraction may be (Table III). The enzyme distributions in the density gradient (Fig. 8), from which the outer and inner membrane fractions were obtained, make it evident that some enzyme activities in these fractions reflect contamination by a particular subcellular component, broadly distributed through the gradient, and peaking halfway between the two kinds of mitochondrial membranes. Contamination of mitochondrial or submitochondrial fractions by endoplasmic reticulum elements probably remained unnoticed, or underestimated in other works (17, 19, 23, 24, 25). For the Golgi apparatus, the reason for the discrepancy is perhaps also in the structural heterogeneity of this membrane complex and its close relationship with the endoplasmic reticulum. Our statement is that the enzymes acting on dolichyl phosphate, like other enzymes of the endoplasmic reticulum, occur in membranes distinct from those which carry the terminal glycosyltransferases. As discussed in detail elsewhere (5, 58), the real limits of these membrane domains are still uncertain, and may not coincide with the endoplasmic reticulum–Golgi complex junction as it is currently established on a morphological basis.

The confinement of the transferases acting on dolichyl phosphate to the endoplasmic reticulum implies that proteins glycosylated via the dolichol pathway must go through, or depend on this cell component at some stage of their biosynthesis. This is easily envisioned for glycoproteins that are synthesized by membrane-bound ribosomes. Several studies carried out on intact cells (11, 27, 38), or on cell-free systems that synthesize proteins in the presence of microsomal membranes (12, 14, 39, 47), have shown that sugar moieties are attached to various nascent chains, demonstrating that core glycosylation of proteins may occur as a cotranslational event at the level of the rough endoplasmic reticulum. According to our results, the transferases acting on dolichyl phosphate are present in both the rough and smooth portions of the endoplasmic reticulum. They markedly differ from RNA in their density distribution (see reference 7) and compare better with glucose-6-phosphatase than with NADPH cytochrome c reductase. The biochemical heterogeneity within the endoplasmic reticulum reflects the fact that some enzymes, e.g., glucose-6-phosphatase, assume nearly random distribution through the whole endoplasmic reticulum, whereas others, e.g., NADPH cytochrome c reduc-
tase, are distinctly more prominent in the smooth than in the rough portions (discussed in reference 1). Thus, the transferases acting on dolichyl phosphate belong to the first category, or even occur in slight excess in the smooth endoplasmic reticulum (15). This is in keeping with the finding that smooth microsomes from rat liver effectively glycosylate the nascent α subunit of human chorionic gonadotropin during synthesis in an heterologous cell-free system (15). Besides, some proteins appear to be glycosylated after release of the completed peptide chain from polysomes (12). Together with the information obtained from these other works, our results keep open the possibility that some proteins be glycosylated while they are in transit in the rough or in the smooth portions of this subcellular compartment. Quantitative studies on the other enzymes of the dolichol pathway for protein glycosylation, particularly on the enzyme that transfer the oligosaccharide from the lipid donor to the protein, may provide some clue to this matter.

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