Enrichment of the intracellular dolichol pool in isolated liver cells

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Abstract Isolated hepatocytes were incubated with egg lecithin liposomes containing dolichol(C55), dolichol(C95), and dolichol phosphate(C55) in order to enrich intracellular membranes with these polyprenols. After incubation, the lipids were recovered from various membrane fractions and from the supernatant. The highest concentration was found in the microsomes. A part of the dolichol in microsomes, as well as in other fractions, was phosphorylated. This phosphorylation is mediated by the CTP-specific kinase that is present only on the outer surface of the microsomes and uses α-saturated polyprenols as substrates. The isolated microsomes enriched with dolichol in vivo exhibited increased lipid and protein glycosylation upon incubation with nucleotide sugars and it was demonstrated that the increased lipid glycosylation was due to transfer of the sugar to the exogenous incorporated dolichol.—**Ekström, T., T. Chojnacki, and G. Dallner.** Enrichment of the intracellular dolichol pool in isolated liver cells. *J. Lipid Res.* 1982. **23:** 972–983.

Supplementary key words transfer of liposomes • hepatocyte fractionation • dolichol phosphorylation

The glycoproteins synthesized in liver contain mostly or exclusively oligosaccharide chains bound to the protein with an N-glycosidic linkage between asparagine and GlcNAc (N-acetyl-glucosamine) (1). During biosynthesis the core of the oligosaccharide chain is accumulated on a dolichol pyrophosphate molecule. The initial sugar residues are transferred directly from the nucleotide-activated form while the last four mannoses and the three glucoses are added from dolichol phosphate. This type of oligosaccharide is then transferred to the protein where processing and completion take place.

Available data indicate that the amount of total dolichol in the different tissues of the same animal or the same tissue of different species varies greatly and only a small part of the dolichol is present in activated, phosphorylated form (2). There is also a wide variety of types of dolichols in nature and the number of isoprene residues in the molecule may vary between 11 and 22.

Recent investigations indicate that dolichol phosphate functions not only as a coenzyme in protein N-glycosylation but also exerts a control in this process. In cultured smooth muscle cells, inhibition of dolichol phosphate biosynthesis reduces protein N-glycosylation (3); such an inhibition has been shown to block the development of sea urchin embryos (4). On the other hand, hypercholesterolemia increases dolichol phosphate concentration in the liver of both rat and rabbit with a consequent increase of protein N-glycosylation (5, 6). These types of experiments suggest that the amount, nature, form, and distribution of dolichol may directly regulate the amount and type of glycoprotein produced and a study of this regulatory role requires a selective and controlled change of specific dolichols in intracellular membranes. In this investigation we used isolated hepatocytes capable of taking up liposomal dolichol which is then transferred to microsomal membranes.

MATERIALS AND METHODS

Isolation of hepatocytes

Non-starved male rats weighing 250–300 g were used. Isolation of hepatocytes was done by first perfusing the liver with an EGTA-containing buffer, followed by collagenase perfusion as described earlier (7). The isolated hepatocytes were washed once in Krebs-Hensleit buffer, pH 7, centrifuged at 50 *g* for 2 min, resuspended in the same buffer, and counted in a Bürker chamber. The viability was measured by suspending the cells in Krebs-Hensleit buffer with trypane blue as vital stain.

Preparation of liposomes

Twenty mg of egg lecithin (Lipid Products, South Nutfield, England) in chloroform–methanol 2:1 was

Abbreviations: GlcNAc, N-acetyl-glucosamine; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; CTP, cytidine triphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate.

mixed with 1 μmol of [^{3}H]dolichol (sp act 8–10 × 10^6 dpm/μmol) or with 1 μmol of unlabeled dolichol. The solvent was evaporated under nitrogen and then in vacuo. To the lipid residue was added 4.5 ml of 0.9% NaCl and the suspension was subjected to a 50-min pulsed sonication in an ice-water bath under nitrogen. The output effect was about 100 W. After sonication the slightly opalescent suspension was stored at 4°C for a maximum of 2 days before use. The suspensions were centrifuged at 105,000 *g* for 1 hr before use.

Incubation of hepatocytes with dolichol containing liposomes

Hepatocytes from a 250–300 g rat were usually divided into three incubation flasks, one of which was a blank incubation without liposomes. To the others were added 3 ml of the liposomal suspension and Krebs-Henseleit buffer to a final volume of 25 ml. The number of cells in each flask was usually 2–3 × 10^8. The cells were incubated for 90 min at 37°C and subjected to a continuous stream of carbogen (93.5% O_2 and 6.5% CO_2). The incubation was terminated by centrifugation at 50 *g* for 4 min after which the cells were washed four times in 20 ml of 0.9% NaCl. The final pellet was suspended in 0.25 M sucrose.

Fractionation of hepatocytes

After incubation and washing, the cells were suspended in 0.25 M sucrose to a protein concentration of 30 mg/ml, then disrupted by a short sonication (30 sec) at the lowest output with a Branson sonicator (8). Protein was determined by the biuret procedure (9). The suspension was centrifuged for 10 min at 300 *g* which pelleted primarily unbroken cells and nuclei. The mitochondria were isolated by centrifugation at 3,000 *g* for 20 min and the pellet was washed twice. The 3,000 *g* supernatant was then centrifuged at 10,000 *g* for 15 min, which gave a fraction containing both light mitochondria and heavy microsomes. The 10,000 *g* supernatant was used to obtain microsomes (105,000 *g*, 60 min). The microsomes were then suspended in 0.15 M Tris–HCl, pH 8.0, and recentrifuged in order to remove adsorbed basic proteins from the membranes. The 300 *g* pellet, the mitochondrial fraction, the 10,000 *g* pellet, and washed microsomal pellet were then suspended in 0.25 M sucrose to appropriate protein concentrations and stored at −20°C.

In some experiments, microsomes were isolated from rat liver homogenate as described earlier (10). Inner and outer mitochondrial membranes were separated according to Sottocasa et al. (11) and total Golgi fractions were prepared according to Bergeron et al. (12). Proteolytic treatment of microsomes was performed as described earlier (13) with trypsin (Boehringer-Mannheim), 25 μg/mg protein, and unspecific protease (type VII from *B. amyloliquefaciens*, Sigma, St. Louis, MO), 25 μg/mg protein in the absence or presence of 0.05% deoxycholate. Suitable markers (nucleoside diphosphatase release, mannose-6-phosphatase latency, and measurement of intramicrosomal water space) were used to show that proteolytic treatment, in the absence of detergent, did not change microsomal permeability and affected only the outer surface of the intact vesicles.

Microsomal incubation with nucleotide-activated sugars

The incubation mixture contained: 30 mM Tris–HCl, pH 7.8, 2.5 mM EDTA, 10 mM $MnCl_2$, 1.5 mM ATP, 12.5 mM β-mercaptoethanol 0.2 μCi of a labeled nucleotide sugar (GDP-[^{14}C]mannose (108 μCi/μmol), UDP-[^{14}C]glucose (240 μCi/μmol), or UDP-N-acetyl[^{14}C]glucosamine (330 μCi/μmol) (The Radiochemical Centre, Amersham, England), and total microsomes (2 mg protein) in a final volume of 800 μl. In experiments including all three sugars, the mixture contained 0.2 μCi of the radioactive species and 2 nmol each of the other two.

Extraction of lipid-linked sugars

The incubations were terminated by addition of 1.6 ml of methanol and 2.4 ml of chloroform. After extraction for 20 min at 40°C and centrifugation, the upper phase was discarded and the lower phase was collected by decantation. The remaining protein was extracted again with 4 ml of chloroform–methanol 3:2 after which 0.8 ml of water was added. After centrifugation, the lower phase was pooled with the first and washed two times with "theoretical upper phase" (chloroform–methanol–water, 3:48:47). The solvent was evaporated and dissolved in 1 ml of chloroform–methanol–water 1:1:0.3 (CM extract). The remaining protein was washed once with 1 ml of water and extracted two times with 2 ml of chloroform–methanol–water 1:1:0.3 (15 min, 40°C). These latter two extracts were pooled and evaporated in a scintillation vial (CMW extract). The final protein pellet was washed once with 1 ml of methanol and dissolved in 1 ml of 2% sodium dodecyl sulfate after which 10 ml of Aqua Luma Plus was added for scintillation counting.

Chromatography of the chloroform–methanol extract on DEAE-Sephadex

The CM extract from above was poured into columns (0.8 × 15 cm) of DEAE-Sephadex (acetate form), equilibrated with chloroform–methanol–water 1:1:0.3, and then washed with the same solvent to elute uncharged molecules. The dolichol derivatives were then eluted with 200 mM ammonium acetate in chloroform–meth-

anol–water 1:1:0.3. The eluate was evaporated in a scintillation vial and counted after addition of a toluene scintillator.

Separation of dolichol and dolichol phosphate

The different fractions were extracted with chloroform–methanol 3:2. The extract was adjusted to chloroform–methanol–water 1:1:0.3, and put on a DEAE-Sephadex column in acetate form (0.8 × 15 cm). Dolichol was eluted with chloroform–methanol–water 1:1:0.3, and dolichol phosphate was eluted with 200 mM ammonium acetate in chloroform–methanol–water 1:1:0.3. The eluates were evaporated and counted as above.

Incubation of different rat liver membrane preparations with [γ-^{32}P]CTP and polyprenols

Membrane preparations corresponding to 1–7 mg of protein were incubated with 27 mM Tris–HCl, pH 7.0, 0.5 mM EDTA, 20 mM UTP, 5 mM β-mercaptoethanol, 30 mM $CaCl_2$, 5–7 μCi [γ-^{32}P]CTP, and 40 μM unlabeled CTP in a final volume of 500 μl. When polyprenols were added, 50 μl was taken from a solution containing 1 mg/ml in 0.5% Triton X-100. For blank incubations, 50 μl of Triton X-100 was added. [γ-^{32}P]CTP was obtained from ICN (Irvine, CA).

Isolation of [^{32}P]dolichol phosphate after incubation of different rat liver membranes with dolichol and [γ-^{32}P]CTP

The incubation was terminated by addition of 1 ml of methanol and 1.5 ml of chloroform and extracted at 40°C. After centrifugation the water phase was discarded and the lower phase was decanted. Extraction was repeated once and the two extracts were pooled and then washed twice with "theoretical upper phase". The solvents were then evaporated and the extract was subjected to a mild alkaline methanolysis in 1 ml of 0.1 M KOH in methanol–toluene 3:1, at 0°C for 1 hr. After evaporation the extract was dissolved in a small amount of chloroform and put on a SiO_2-column (0.8 × 3 cm) equilibrated with chloroform. The column was washed with 10 ml of chloroform and eluted with 15 ml of chloroform–methanol 1:1. The eluate was evaporated, dissolved in 2 ml of chloroform–methanol 2:1, and washed with 0.9 ml of 0.9% NaCl. After evaporation it was dissolved in a small amount of chloroform–methanol 2:1, and placed on a thin-layer chromatography (TLC) plate and run in chloroform–methanol–water 60:25:4. After radioautography for 60 hr, visible spots, corresponding to dolichol phosphate, were scraped and counted for radioactivity. Large excesses of polyprenols (35 nmol) were used as substrates for comparison with the low phosphorylating activity (11 pmol/mg protein per 5 min).

Preparation of mannosyl derivatives of labeled or unlabeled dolichol phosphates

Phosphorylation of dolichols with radioactive or nonradioactive orthophosphate was done according to Cornforth and Popják (14). C35, C55, C75, or C95 phosphates (10 μM) were incubated with labeled (0.2 μCi) or unlabeled (40 μM) GDP-mannose in the presence of 0.1% Triton X-100 and microsomes by using the same conditions as described above. Microsomes from C35 and C55 dolichol preincubated hepatocytes were incubated with GDP-[^{14}C]mannose in the absence of detergent. The reaction mixture was extracted with chloroform–methanol as described above and placed on a chloroform–methanol–water 1:1:0.3-equilibrated DEAE-Sephadex column (1 × 20 cm). The mannosyl lipid was isolated with a 0-133 mM gradient of ammonium formate in chloroform–methanol–water 1:1:0.3. Four-ml fractions were taken by using gravitational force and the mannosylated dolichol phosphates were found in fractions 7–8. TLC analyses demonstrated that no dolichol-PP-oligosaccharide was present in these two fractions.

Chromatography of mannosylated dolichol phosphates on Lipidex-1000

The lipid extract obtained in the previous section was chromatographed on columns (0.7 × 22 cm) of Lipidex-1000 equilibrated in water–methanol–n-butanol–chloroform 60:40:7:3. Lipidex-1000 is an alkoxy group derivative of Sephadex LH-20 and is highly hydrophobic. By using this matrix, separation of hydrophobic components can be made with decreasing polarity gradient. The extract was applied in the following way. The solvent was evaporated and the residue was dissolved in 0.5 ml of methanol–n-butanol–chloroform 40:7:3. One half (0.5) ml of a thick suspension of equilibrated Lipidex-1000 was added to the tube after which 0.6 ml of water was added during extensive vortexing. The slurry was then transferred to the top of the column. The elution was carried out in a linear gradient of decreasing water in the solvent system, *i.e.*, methanol–*n*-butanol–chloroform 40:7:3 in the reservoir. The extract was eluted by gravitational force and 4-ml fractions were taken at a rate of 4 ml/hr at the beginning and 2.5 ml/hr at the end of the gradient. The fractions were dried and counted for radioactivity in a toluene scintillator. Lipidex-1000 was obtained from Packard-Becker, Groningen, The Netherlands.

Preparation of dolichols

C35, C55, and C75 dolichols were prepared by selective hydrogenation of the respective plant polyprenols isolated from *Betula verrucosa*, *Rhus typhina*, and *Pinus silvestris* (15). The isolation of pure C95 prenologue

from human liver dolichol (16) and its identification by TLC, reversed phase TLC, and nuclear magnetic resonance spectrometry was performed as described earlier (15). Tritium-labeled dolichols were prepared according to Keenan and Kruczek (17). Quantitation of dolichol was performed by acetylation with [^{14}C]acetic anhydride as described by Keller and Adair (18) and the individual dolichols were separated by reversed phase thin-layer chromatography on paraffin-impregnated cellulose plate (15). In some experiments the dolichols were quantitated with high pressure liquid chromatography (HPLC). After incubation the suspension was subjected to alkaline hydrolysis and dolichol was extracted with diethyl-ether. The extract was placed on an Al_2O_3 column and eluted with increasing concentrations of diethyl-ether in n-hexane. The dolichol fraction, determined by TLC, was analyzed on HPLC. Eluates were monitored at 210 nm. A convex gradient (No. 4 Waters solvent programmer) was used, starting from 100% propanol–methanol–H_2O 40:60:5 to 50% n-hexane–2-propanol 70:30 in a C18 μBondapac column (Waters).

RESULTS

Endogenous dolichols in rat and human liver

Acetylation of isolated and purified dolichol preparations with [^{14}C]acetic anhydride demonstrated that the interaction, as expected, is strictly stoichiometric and, by using a known amount and type of standard dolichols, the amount can be calculated (15, 18).

Table 1 shows the distribution of dolichol in rat liver; for comparison, that of the human liver is also given. Rat liver contains 42 μg of total dolichol per gram wet weight which is in sharp contrast to the human liver where the amount exceeds 0.5 mg per gram wet weight (16). Reversed phase chromatography of the acetylated rat liver dolichol gave four components, both C85 and C100 make up around 10% each of the total and the remaining 80% is divided between the C90 and C95 fractions. The composition of human dolichol is different; C90, C105, and C110 are present in relatively smaller amounts and the majority of the dolichol is recovered as C95 and C100.

TABLE 1. Distribution of different types of dolichols in rat[a] and human liver[b]

Type of Dolichol[c]	Amount[d]	Rat	Human
	μg/g liver	*% of total*	
Total, rat	42 ± 2.6		
Total, human	652 ± 49		
C85		13	
C90		40	9
C95		36	30
C100		11	44
C105			13
C110			4

[a] Liver from nonstarved rat.
[b] Autopsy sample of liver showing histologically normal or almost normal structure.
[c] Dolichols were acetylated with [^{14}C]acetic anhydride and separated by reversed phase chromatography. The designation C85–C110 refers to the number of carbon atoms in the separated dolichols, i.e., isoprene residues between 17–22.
[d] The values are the means ± S.E.M. (n = 7).

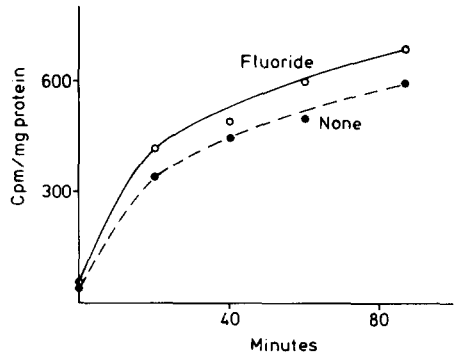

Fig. 1. Transfer of [^{14}C]phosphatidylcholine into isolated hepatocytes. Liposomes containing [^{14}C]phosphatidylcholine to a specific activity of 300,000 cpm/mg of egg lecithin were prepared as described and 2 ml was used for incubation with hepatocytes in the presence or absence of 10 mM NaF at 37°C. At various time points, aliquots were removed and radioactivity of the washed, total hepatocytes was measured. The values represent the means of three experiments.

Transfer of dolichol into isolated hepatocytes

Isolated intact hepatocytes have the ability to take up liposomal vesicles. This is demonstrated by incubation of hepatocytes with liposomes consisting of egg lecithin and radioactive synthetic lecithin. The radioactivity appearing in the washed cells increases in the 90-min incubation period in a nonlinear fashion (**Fig. 1**). If incubation was performed in the presence of 25 mM NaF, which inhibits dolichol phosphate dephosphorylation in in vitro systems (19), the incorporation pattern was similar. During this 90-min incubation period the hepatocytes remained in an intact state and no leakage could be detected after measurement of NADH-permeability and the extracellular lactate dehydrogenase activity.

There are two ways that liposomes can be incorporated into hepatocytes: through an exchange reaction or by means of a net increase of lipids in the cell. This point is important in the study of the function of externally incorporated dolichol and therefore this question was investigated. Nonincubated hepatocytes or hepatocytes incubated in buffer or with lecithin liposomes contain about 16 nmol total dolichol per 10^8 cells and one-third of it is dolichol(C95) (**Table 2**). After incubation with dolichol(C95)-containing liposomes, the cellular dolichol content increased about four times,

TABLE 2. Net transfer of dolichol into hepatocytes during incubation with liposomes[a]

Incubation	Dolichol in Hepatocytes	
	Total	Dolichol(C95)
	nmol / 10^8 cells	
None	16.0	5.1
Buffer	15.8	5.1
Lecithin-liposomes	16.0	5.0
Dolichol(C95)-lecithin-liposomes	61.8	51.4

[a] The hepatocytes were incubated for 90 min at 37°C. The dolichol fraction was isolated and quantitation was performed by using HPLC on C_{18} reversed phase column.

an increase which was attributed completely to dolichol(C95).

The chromatogram of the dolichol fraction after incubation is shown in **Fig. 2**. Hepatocytes contain dolichols mainly between C85 and C105. After incubation with dolichol(C95), the peak corresponding to this lipid was greatly increased while the amount of other dolichols was unchanged. These experiments demonstrated that the liposomal system resulted in a net transfer of dolichol into the cell and under the conditions employed no exchange took place.

In the following experiments hepatocytes were subfractionated and the isolated mitochondrial and microsomal fractions were analyzed. Consequently, it is important to deal with reasonably pure fractions. **Table 3** shows the measurements of contaminations in isolated mitochondrial and microsomal fractions by using various marker enzymes. Only 3% of the protein in the mitochondrial fraction was microsomal contamination, judged by measurement of the marker enzyme NADPH-cytochrome *c* reductase activity. Contamination of the mitochondrial fraction with Golgi and plasma membranes was limited but more extensive with lysosomes and peroxisomes. In the microsomal fraction, about 12% of the protein belonged to lysosomes and outer mitochondrial membranes (marker for the latter is monoamino oxidase), and contamination with the other intracellular membranes was more restrictive. Thus, contamination in isolated fractions from hepatocytes was at a relatively low level not exceeding that found in fractionation of rat liver homogenate.

Egg lecithin liposomes containing C55 or C95 ^{3}H-labeled dolichol were taken up by hepatocytes and distributed in the different fractions after a 90-min incubation period (**Table 4**). The 300 *g* pellet (which is a contaminated nuclear fraction), the mitochondria fraction, the 10,000 *g* pellet (which is a mixture of mainly mitochondria and microsomes), the total microsomes, and the particle-free supernatant all contained labeled dolichol.

Incubations of hepatocytes with dolichol(C55)- and dolichol(C95)-containing liposomes were performed with the same concentration of dolichol, 0.7 μmol in 25 ml of incubation medium. In spite of this, the uptake was different since the total cellular dolichol(C95) was 60% greater than the C55 dolichol. In all fractions, the amount of C95 exceeded that of the C55.

Not only was the free alcohol present in the liver fractions, but a part of the lipid was recovered in phosphorylated form. Under the conditions employed, the cellular phosphorylation of dolichol(C55) was more effective than phosphorylation of C95, since 25% of the former and only 10% of the latter lipid was recovered in monophosphate form. Consequently, in contrast to the distribution of the free dolichol, the amount of dolichol(C55) phosphate exceeded the amount of dolichol(C95) phosphate.

Glycosylation of the incorporated dolichol in microsomes

Preincubation of hepatocytes with dolichol-containing liposomes resulted in the appearance of both dolichol(C55) phosphate and dolichol(C95) phosphate in microsomes. In both cases, incubation of microsomes with GDP-mannose gave an increased incorporation in the chloroform–methanol extract (**Table 5**). Consequently, a part of the incorporated mannose is associ-

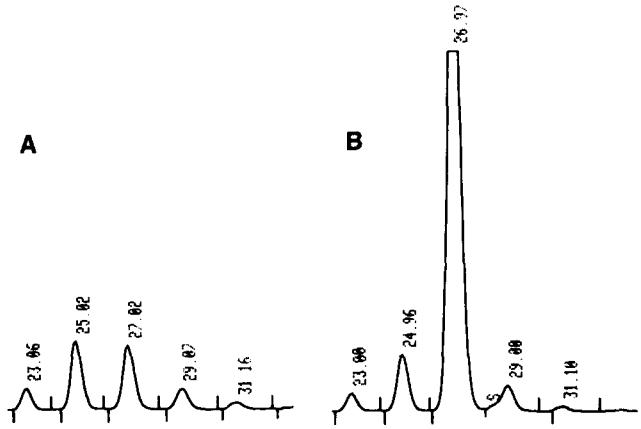

Fig. 2. Transfer of dolichol(C95) into hepatocytes during incubation with liposomes. Isolated hepatocytes were incubated with dolichol(C95) containing liposomes at 37°C for 90 min. After alkaline hydrolysis the unsaponifiable lipids were extracted with diethyl ether and dolichol fractions were isolated on Al_2O_3 columns in a gradient with increasing concentration of diethyl ether in hexane. The dolichol fraction was subjected to reversed phase chromatography on HPLC. A, Dolichol from isolated hepatocytes incubated in buffer with egg lecithin liposomes; B, dolichol from hepatocytes incubated with liposomes containing dolichol(C95). The numbers above the individual peaks give the retention time in minutes.

TABLE 3. Distribution of the various marker enzymes in the mitochondrial and microsomal fractions isolated from hepatocytes[a]

Marker Enzyme	Mitochondria		Microsomes	
	Sp Act	Calculated Contamination	Sp Act	Calculated Contamination
		% of total protein		*% of total protein*
Cytochrome *c* oxidase[b]	1.35		0.03	0.5
Monoamino oxidase[c]	15.2		0.84	6
NADPH-cytochrome *c* reductase[d]	0.002	3	0.042	
Acid phosphatase[e]	0.07	6	0.08	6
Urate oxidase[f]	0.04	11	0.01	2
UDP-galactosyl transferase[g]	0.05	3	0.05	3
AMPase[e]	0.01	1	0.025	3

[a] Enzyme activities were measured in lysosomes (acid phosphatase), peroxisomes (urate oxidase), Golgi (UDP-galactosyl transferase), and plasma membranes (AMPase) isolated from liver tissue of rat. These values were used to calculate the contamination in the mitochondrial and microsomal fractions of the isolated hepatocytes.
[b] μmol Cytochrome *c* oxidized/min per mg protein.
[c] nmol Benzaldehyde/min per mg protein.
[d] μmol NADPH oxidized/min per mg protein.
[e] μmol Orthophosphate/min per mg protein.
[f] μmol Urate oxidized/min per mg protein.
[g] nmol Transferred/30 min per mg protein.

ated with the exogenous dolichol phosphate, that is, a partial mannosylation takes place both in the case of C55 and C95.

The data in **Table 6** are from experiments in which the microsomes were incubated with the three sugars both individually and together. Table 6 shows both total radioactivity/mg protein and also the values that represent the incorporation over the endogenous level. In all cases, incorporation into proteins was slightly stimulated, indicating that the incorporated dolichols participated in the glycosylation system of the microsomes. Both dolichols transferred to microsomal membranes in the liposomal system were good acceptors for mannose when the test system used consisted of isolated microsomes and the nucleotide-actived sugar as substrate. These exogenous dolichols were less effective for glucose and they were poor acceptors for N-acetyl-glucosamine. It also appears that the two dolichols applied exhibited some substrate specificity and C55 was more effective as mannose acceptor than C95, while the opposite was true in the case of GlcNAc. In the CMW extract, which contains the dolichol-PP-oligosaccharide,

TABLE 4. Transfer of liposomal [^{3}H]dolichol(C95) and (C55) into isolated hepatocytes[a]

Fraction	Type of Dolichol	Dolichol[b]		Dolichol-P[b]	
		cpm	*pmol/mg protein*	*cpm*	*pmol/mg protein*
300 *g* Pellet	C95	35,916	51.9	3,828	5.1
	C55	24,072	26.9	11,388	12.2
Mitochondria	C95	15,010	46.0	1,970	5.9
	C55	6,870	20.5	3,140	9.1
10,000 *g* Pellet	C95	34,248	139.4	2,616	10.5
	C55	28,002	101.9	7,032	24.8
Microsomes	C95	32,216	197.4	4,968	27.7
	C55	18,472	115.5	9,016	50.1
Supernatant	C95	34,706	51.8	1,702	2.6
	C55	20,683	33.6	1,221	2.0

[a] The hepatocytes were incubated with dolichol(C95) or dolichol(C55) containing liposomes for 90 min at 37°C.
[b] Dolichol and dolichol-P were separated by chromatography on DEAE-Sephadex. The values represent the means of five experiments.

TABLE 5. Mannosylation of dolichol phosphate in microsomes prepared from hepatocytes preincubated with dolichol(C95) and (C55)[a]

Preincubation	[^{3}H]Dolichol-P[b]	[^{14}C]Mannose Incorporated (CM extract)[c]	Mannosylation of Dolichol-P	
			Endogenous	Exogenous
	pmol / mg protein	*cpm / mg protein*	*pmol / mg protein*	
None		2,248	10.2	
[^{3}H]Dolichol(C95)	27.7	3,369	10.2	5.1
[^{3}H]Dolichol(C55)	50.1	6,005	10.2	17.2

[a] Isolated hepatocytes were incubated with liposomes containing either dolichol(C95) or dolichol(C55) followed by preparation of microsomes from these cells.
[b] Dolichol phosphate was isolated by chromatography on DEAE-Sephadex.
[c] The isolated microsomes were incubated with GDP-[^{14}C]mannose and the radioactivity in the chloroform–methanol 3:2 (CM) extract was measured. The incubation medium contained 2×10^5 cpm GDP-[^{14}C]mannose.
All values represent the means of five experiments.

C95 seems to have had a slightly higher incorporation of both mannose and glucose. When the radioactive GDP-mannose-containing medium was complemented with UDP-GlcNAc and UDP-Glc, glycosylation of the protein could not be further increased.

Glycosylation of incorporated dolichol monophosphate

Liposomal phosphorylated dolichol, labeled with ^{32}P, could also be transferred to isolated hepatocytes. The labeled dolichol phosphate appeared in the microsomal membranes and the amount was not significantly increased when the incubation with liposomes was performed in the presence of NaF (**Fig. 3A**). The main difference in the dolichol phosphate eluate is that in the presence of NaF an additional peak appeared at the end of the concentration gradient. We did not identify this peak, but it is probable that it corresponds to dolichol pyrophosphate oligosaccharide. When the microsomes containing [^{32}P]dolichol phosphate were incubated with GDP-mannose, a part of the labeled lipid disappeared and a new peak appeared (Fig. 3B). The peak was identified on silica gel-TLC in chloroform-methanol-water 60:25:4, where the R_f of the lipid was identical to that of synthetic dolichol-P-mannose. Thus, phosphorylated

TABLE 6. Glycosylation of dolichol(C55) phosphate and dolichol(C95) phosphate in microsomes prepared from hepatocytes preincubated with dolichols[a]

Substrate[b]	Type of Exogeneous Dolichol	CM Extract[c]		CMW Extract[d]		Protein
		cpm / mg protein	*pmol sugar / nmol exogenous dol-P available*	*cpm / mg protein*	*pmol sugar / nmol exogenous dol-P available*	*pmol sugar / mg protein*
[^{14}C]UDP-GlcNAc	None	857		99		0.37
	C95	1.173	16.9	148	2.5	0.68
	C55	945	2.6	199	2.8	0.73
[^{14}C]GDP-Man	None	2.248		240		3.98
	C95	3.369	184.8	574	55.2	7.92
	C55	6.005	342.5	525	25.9	7.29
[^{14}C]UDP-Glc	None	2.654		885		2.91
	C95	3.261	38.9	1.449	35.7	4.64
	C55	3.943	44.7	1.601	25.1	3.99
UDP-GlcNAc	None	2.014		332		4.08
[^{14}C]GDP-Man	C95	2.990	163.2	442	18.4	6.09
UDP-Glc	C55	5.488	316.5	493	15.0	6.93

[a] Hepatocytes, enriched in dolichol(C95) or dolichol(C55) by preincubation, were fractionated.
[b] Microsomes were incubated with nucleotide sugars in the absence of detergents.
[c] CM extract denotes extraction with chloroform–methanol 3:2.
[d] CMW extract denotes extraction with chloroform–methanol–water 1:1:0.3 of the chloroform–methanol extracted sample.
All values represent the means of four to nine experiments.

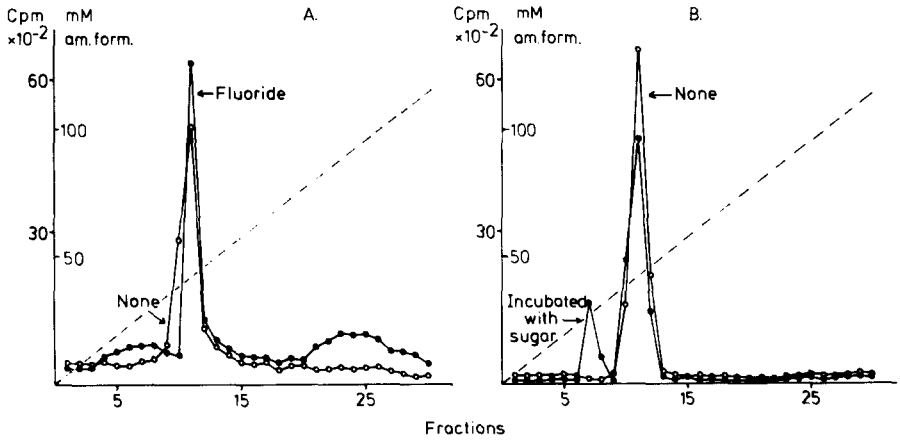

Fig. 3. Transfer and mannosylation of [^{32}P]dolichol(C55) phosphate in microsomes. Isolated hepatocytes were incubated with dolichol-P containing liposomes at 37°C for 90 min in the presence or absence of 10 mM NaF. The labeled dolichol phosphate from microsomes was extracted with chloroform–methanol 3:2 and chromatographed on DEAE-Sephadex using an ammonium formate gradient (0–133 mM) in chloroform–methanol–water 1:1:0.3 (A). Microsomes from [^{32}P]dolichol phosphate-preincubated hepatocytes were incubated with and without nonlabeled GDP-mannose, and the chloroform–methanol 3:2 extract was placed on a DEAE-Sephadex column (B).

dolichol can also be transferred into hepatocytes and into microsomal membranes by incubation and can be used for studying the interaction between the lipid and the glycosyl transferases.

Identification of incorporated exogenous dolichol

Incubation of hepatocytes with liposomal dolichol resulted in incorporation of dolichol and dolichol phosphate into microsomes and also gave an increased glycosylation of the polyisoprenoid fraction. This effect alone, however, does not prove that the exogenous dolichol itself is glycosylated and one cannot exclude the possibility that the increased glycosylation is caused by stimulation of the endogenous dolichol system. In order to separate mannosylated individual dolichol species, we performed model experiments by using chemically phosphorylated dolichols, with different chain lengths, that were mannosylated enzymatically. These mannosylated dolichol phosphates were chromatographed on Lipidex-1000 and eluted in a water–methanol–*n*-butanol–chloroform system with decreasing water concentration (**Fig. 4**). This procedure proved to be very effective and a linear relationship was observed between the increasing chain length of mannosylated dolichol phosphates and the separated fraction.

Microsomes were incubated with labeled or unlabeled GDP-mannose in the presence of labeled or unlabeled dolichol phosphates with the length of 35, 55, or 75 carbons (**Fig. 5-*1*, *2*, *3***). As expected, on the basis of experiments in Fig. 4, the mannosylated lipids were separated and appeared in fractions 4, 8, and 12, respectively. Microsomes incubated with GDP[^{14}C]-mannose in the absence of exogenous dolichol-P showed labeling in fractions 13 and 14 (Fig. 5-*6*). Microsomes isolated from hepatocytes preincubated with dolichol (C35) and (C55) exhibited labeling with [^{14}C]mannose in fractions 13 and 14 to about the same extent as in microsomes that were not preincubated (Fig. 5-*4* and *5*). All the additional labeling in both cases appeared in a position that corresponds to dolichol (C35)-P-mannose and dolichol (C55)-P-mannose as indicated in Fig. 5-*1* and *2*. These experiments prove that the incorporated exogenous dolichol is situated in the membrane in an appropriate way in order to interact with mannosyl transferases.

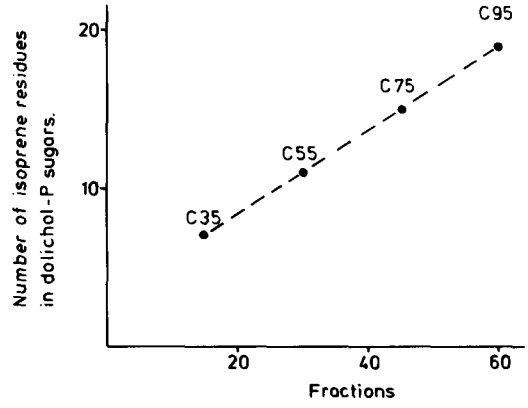

Fig. 4. Separation of different dolichol phosphate sugars on Lipidex-1000. Different dolichol monophosphates were incubated with GDP-[^{14}C]mannose in the presence of microsomes, and the chloroform–methanol 3:2 extracts were chromatographed on Lipidex-1000. The values are the results of six experiments.

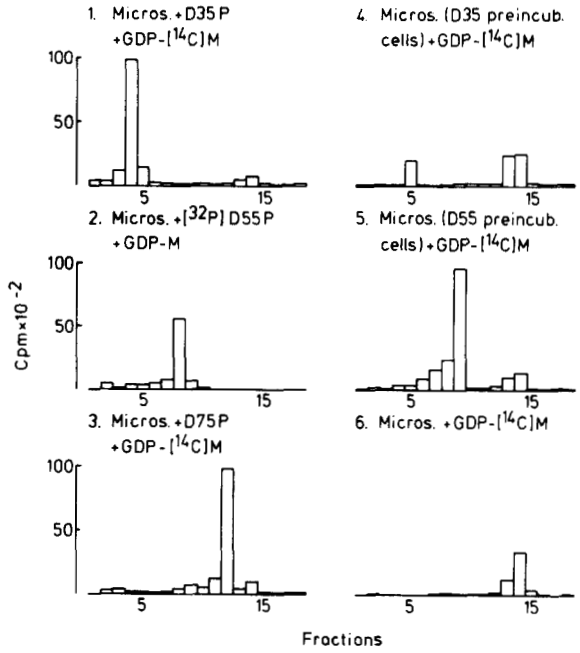

Fig. 5. Separation of mannosylated dolichol phosphates on Lipidex-1000. 1., Microsomes incubated with GDP-[^{14}C]mannose in the presence of dolichol(C35) phosphate; 2., microsomes incubated with GDP-mannose (nonlabeled) in the presence of [^{32}P]dolichol(C55) phosphate; 3., microsomes incubated with GDP-[^{14}C]mannose in the presence of dolichol(C75) phosphate; 4., microsomes from dolichol(C35) preincubated hepatocytes incubated with GDP-[^{14}C]mannose; 5., microsomes from dolichol(C55) preincubated hepatocytes incubated with GDP-[^{14}C]mannose; 6., microsomes incubated with GDP-[^{14}C]mannose. In 2., 3 nmol [^{32}P]dolichol(C55) phosphate corresponding to 700,000 cpm was added to the incubation medium. The values represent the means of four experiments.

Intracellular phosphorylation of dolichol

A part of the dolichol transferred to the hepatocytes is phosphorylated, indicating that an effective mechanism for this process must exist. A CTP-specific kinase is described in liver and brain that effectively phosphorylates dolichol (20, 21). By using different subcellular fractions, we were able to investigate phosphorylation of C55 polyprenols with [γ-^{32}P]CTP. Phosphorylation of endogenous dolichol was at a low level and α-unsaturated C55 was less active as a substrate (**Table 7**). Microsomes phosphorylate dolichol (C55) effectively but this reaction is practically absent in Golgi and outer and inner mitochondrial membranes.

The transverse topology of microsomal membranes may be studied by using proteolysis to probe the outer surface of the intact vesicle (13). The proteolytic treatment used did not interfere with membrane permeability which remained intact (**Table 8**). The intramicrosomal water, which is the measure of the water space in the microsomal vesicles not available for macromolecules (22), was unchanged after the combined trypsin + pronase treatment. Nucleoside diphosphatase, a luminal enzyme of the liver microsomes, was not liberated by proteolysis. Mannose-6-phosphate did not penetrate microsomal membranes and therefore it exhibited a high degree of latency. The latency was not decreased after proteolysis. Thus, these experiments demonstrate that the proteolytic treatment employed interferes only with the outer, cytoplasmic surface of the intact microsomes and consequently, may be used to probe the transverse distribution of membrane enzymes. In the presence of deoxycholate in low concentration, the permeability of the vesicles increases for macromolecules and proteases can be used to probe the inner surface (23). The results in Table 8 show that combined trypsin plus pronase treatment, not only in the presence of deoxycholate but also in its absence, abolished the phosphorylating capacity of the particles. Thus the CTP-kinase reactions appear to have a single intracellular lo-

TABLE 7. Phosphorylation of dolichol in the presence of [γ-^{32}P]CTP[a]

Fraction	Incubation Time	Addition	Polyprenol-P
	min		*pmol / mg protein*
Microsomes	5	none	0.4 ± 0.03
Microsomes	5	C_{55} (α-unsaturated)	2.7 ± 0.33
Microsomes	2.5	α-diH-C_{55}	5.9 ± 0.51
Microsomes	5	α-diH-C_{55}	11.0 ± 0.92
Golgi membranes	5	α-diH-C_{55}	1.2 ± 0.15
Outer mitochondrial membranes	5	α-diH-C_{55}	0.2 ± 0.02
Inner mitochondrial membranes	5	α-diH-C_{55}	0.3 ± 0.02

[a] α-Saturated and unsaturated C55-polyprenols were incubated with [γ-^{32}P]CTP and microsomes in the presence of 0.05% Triton X-100 and the phosphorylated polyprenol was isolated by chromatography. Dolichol(C55) was also incubated with [γ-^{32}P]CTP and isolated Golgi, outer mitochondrial, and inner mitochondrial membranes.

The values are means ± S.E.M. (n = 6).

TABLE 8. In vitro phosphorylation of dolichol(C55) by microsomal membrane preparations in the presence of [γ-^{32}P]CTP[a]

Pretreatment of Microsomes	Intramicrosomal Water	Nucleoside Diphosphatase	Mannose-6-Phosphatase			Dolichol-P
			+ Triton	− Triton	Latency	
	μl/mg dry weight	*μmol P_i/min per mg phospholipid*	*μmol P_i/min per mg phospholipid*		%	*pmol/mg protein*[b]
None	1.25	1.62	0.85	0.05	94	57.3 ± 5.0
Trypsin + Pronase	1.23	1.59	0.70	0.07	90	1.8 ± 0.2
Deoxycholate	0.39	0.16	0.64	0.59	8	33.1 ± 3.2
Trypsin + Pronase + Deoxycholate	0.38	0.14	0.09	0.08		0

[a] Isolated intact microsomal vesicles were subjected to proteolytic treatment which solubilized 20% of protein from the outer surface. The permeability for macromolecules was increased by including 0.05% deoxycholate in the incubation medium which made it possible for the proteolytic enzymes to enter into the lumen of the microsomal vesicles.

[b] The pretreated microsomal membranes were incubated with [γ-^{32}P]CTP as in Table 7.

The values are means ± S.E.M. (n = 6) in the case of dolichol-P. Other measurements are duplicates.

cation that is on the cytoplasmic surface of the endoplasmic reticulum.

DISCUSSION

The aim of this investigation was to establish a pathway for enrichment of microsomal membranes with dolichol without using detergent or procedures that change the structure of the membranes. Incorporation of dolichol into egg lecithin liposomes proved to be an effective way to transfer individual polyprenols to isolated hepatocytes, a procedure also suitable for study of the intracellular movement of these lipids between membrane compartments.

The interaction of the liposomes with hepatocytes is an interesting phenomenon, but the mode of interaction is not quite clear. The liposomal membrane may be engulfed in larger or smaller pieces as a type of endocytosis or it may be dissolved in the plasma membrane and further transport can occur from this location to other intracellular compartments. This uptake mechanism appears to be a valuable tool for transfer of not only dolichol but also various lipid-soluble substances from the extracellular to the intracellular water compartment of the hepatocyte without using agents, such as detergents, interfering with the structural makeup.

The presence of dolichol and also of dolichol phosphate in the soluble cytoplasm raises the question of whether the lipid is present in carrier protein-bound form. Different kinds of lipid carrier proteins such as phospholipid exchange protein (24), sterol carrier proteins (25), and retinol binding protein (26) have been identified in the liver cytoplasm. The existence of a dolichol transporting protein is perhaps a necessity of a normal cellular function. It is demonstrated in this study that the CTP-mediated dolichol kinase is present exclusively in microsomes. The biosynthetic pathway which results in the product dolichol pyrophosphate is postulated to be localized to the outer mitochondrial membrane (27) and the enzymes that dephosphorylate dolichol pyrophosphate and dolichol phosphate are enriched in lysosomes and plasma membranes (28). Since dolichol, in different forms, is present in all or most intracellular membranes, an effective mechanism should exist for redistribution of the phosphorylated and nonphosphorylated forms.

The liposomal model for dolichol uptake may be the physiological pathway for cellular uptake of dolichol, not only in the liver but also in other organs. Dolichol is present in all tissues so far investigated but we do not know if its biosynthesis occurs in all tissues. If the biosynthesis is restricted to a few tissues, one has to anticipate transport of dolichol through the blood to different organs and uptake by a mechanism that may be similar to that which is demonstrated here by hepatocytes. Previously, Keenan, Fischer, and Kruszek (29) found that intravenously injected dolichol was associated exclusively with high density lipoprotein. The possibility was also raised that dietary dolichol is taken up by different organs and utilized like the dolichol synthesized endogenously (28).

The uptake and the distribution of dolichol in the intracellular membranes create an interesting situation, since it allows individual membranes to be enriched in a single type of dolichol. Since these specific dolichols now are partly in a phosphorylated form (see Table 4), they participate in the glycosylation process as demonstrated in this study. Consequently, we have access to a unique system for future study of the intramembranous distribution of dolichols with different residues and for analysis of their relation to various neighbouring structures. We do not yet know the exact intramembranous distribution of the exogenous dolichol and we

have no evidence that the distribution is identical with that of the endogenous counterpart, but the exogenous dolichol is active in that part of glycoprotein synthesis which occurs in liver microsomes and therefore the procedure appears to be suitable for studying dolichol function.

It has been suggested previously that the different dolichols specifically interact with individual sugars and this specificity could be a regulatory factor in glycoprotein synthesis (30). In this study we found differences in the interaction of sugar nucleotides with microsomes enriched in the two different types of dolichols. In the future it will be necessary to isolate and systematically characterize the different dolichols, use them in liposomal transfer experiments, and test the interaction of isolated microsomes with various sugar nucleotides.

The dynamic nature of dolichol in the hepatocyte is reflected not only by the broad distribution of the free dolichol but also by the general appearance of the phosphorylated form. The microsomal kinase, which is specific for dolichol is the only enzyme in the cell that can activate the α-saturated alcohol. The fact that the enzyme is on the cytoplasmic surface is probably of functional importance as it may elicit a rapid interaction of the activated lipid intermediate with carrier proteins for further transport to other intracellular membranes. Such a mechanism is operating with the majority of the phospholipids in the hepatocytes. These are synthesized on the outer surface of the endoplasmic reticulum and to a large extent transported to other membranes (31). The rapid distribution and the presence of dolichol phosphate in the intracellular membranes appear to be important factors in regulation of glycoprotein synthesis. In our system, the increase of dolichol phosphate in microsomes resulted in an increase of in vitro protein glycosylation.

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