

# Presence of dolichol and its derivatives in human blood

P. Göran Elmberger, Peter Engfeldt, and Gustav Dallner

Division of Cellular and Neuropathology and Department of Medicine, Huddinge Hospital, Karolinska Institutet, S-141 86 Huddinge, Sweden

**Abstract** Optimal conditions for the quantitative estimation of dolichol in human plasma were determined. Because of the large amounts of other lipids present in the blood, the extraction procedure, the procedure for hydrolysis, and the HPLC procedure are of decisive importance. Human plasma contains dolichol, dolichyl esters, and dolichyl phosphate at concentrations of 41, 102, and 55 ng/g, respectively. These polyisoprenoid lipids are associated with the high density lipoprotein fraction. The relative amounts and compositions of dolichyl esters in the plasma are similar to those observed in isolated human liver microsomes and Golgi vesicles. Sixty percent of the fatty acids present are saturated and almost no long-chain polyunsaturated components are present. There is no correlation between blood dolichol content and weight, sex, dietary state, or plasma cholesterol level, but there is an inverse relationship to plasma triglyceride content. A linear increase in the total plasma dolichol content with increasing age was found. In a few pathological conditions where the level of blood cholesterol was increased, the total blood dolichol content was not affected. Apparently, dolichol is a stable lipid component of human high density lipoprotein.—**Elmberger, P. G., P. Engfeldt, and G. Dallner.** Presence of dolichol and its derivatives in human blood. *J. Lipid Res.* 1988. **29:** 1651-1662.

**Supplementary key words** dolichyl ester • dolichyl phosphate • ubiquinone • cholesterol • triglycerides • fatty acid methyl esters • human plasma • capillary gas-liquid chromatography • high performance liquid chromatography

Dolichol displays a broad distribution among tissues, cells, and intracellular organelles (1). The free alcohol and its esterified and phosphorylated forms have been found in various organs of different experimental animals. However, in all human organs the dolichol content is considerably higher than in experimental animals; in some human tissues, such as endocrine organs, this lipid is present in mg quantities (2, 3). The phosphorylated form of dolichol constitutes only a few percent of the total and this derivative plays a well-established role in glycoprotein synthesis (4). Only indirect evidence concerning the biological function(s) of dolichol and dolichyl esters is available and it suggests that membrane fluidity, permeability, and fusion processes are regulated by these compounds (5-8). Our limited knowledge about dolichol function is explained in part by

the fact that reliable procedures for the extraction, isolation, and quantitation of the free alcohol and its derivatives have become available only in recent years (9).

In animals, inducers of the ER and peroxisomes, plasticizers and carcinogens, can alter the dolichol contents of liver homogenates, microsomes, and/or lysosomes (10, 11). Data in the literature also indicate the presence of increased dolichol concentrations in Alzheimer's disease and ceroid lipofuscinosis; in the latter disease the level of dolichyl-P is also elevated (12-16). During the aging process considerable increases in the dolichol contents of certain parts of the human brain occur, whereas the level of dolichyl-P remains largely unchanged (12, 14, 15, 17-20). In a number of organs of mouse and rat the dolichol level also increases with age (21-23). Human blood also contains dolichol and a slight decrease in its amount during aging has been reported (24). Dolichol was found in the blood of the pig, the chicken (25, 26), and the mouse as well, where a slight decrease in this parameter during the subsequent 2-year aging period was observed (27).

The fate of injected dolichol has also been studied. After injection of this lipid into the tail vein of a rat, dolichol was found to be associated with HDL. In addition, under in vitro conditions, dolichol, but not dolichyl palmitate, bound exclusively to the HDL fraction of human and rat plasma (28). Subsequently, an increasing uptake into a number of organs, particularly the liver, was observed (29). In contrast, another investigation demonstrated that incubation of dolichol with human serum in vitro resulted in the recovery of this lipid in the VLDL fraction (30). When this dolichol lipoprotein preparation formed in vitro was injected into the bloodstream of rats, the free alcohol was associated at first with the VLDL fraction, but after 4 days it was found to be translocated and appeared in association with HDL (31).

Abbreviations: GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; ER, endoplasmic reticulum; dolichyl-P, dolichyl phosphate.

In this and in a following investigation, no oxidative breakdown of the  $^{14}\text{C}$ -labeled lipid was observed (32).

In the present study our aim was to establish a reproducible method for the quantitation of blood dolichol. The distribution of endogenously synthesized dolichol, dolichyl ester, and dolichyl-P, their relation to lipoproteins, their possible origin and possible changes during aging were then analyzed. A preliminary report on some of these findings has appeared (33).

## MATERIALS AND METHODS

### Subjects

The influence of various factors on the dolichol content of human plasma was investigated. Plasma samples from 35 subjects were analyzed. All the subjects were members of a group undergoing a medical check-up. Only subjects found to be healthy were selected for our study.

Blood samples were also taken from patients with poorly controlled non-ketotic diabetes mellitus and from patients with untreated hyperthyreosis, hypothyreosis, and pheochromocytoma.

Human plasma was generally sampled after a 12-hr fast. In some cases plasma was also taken after a 1-week fast and after 2 days of refeeding. The blood was collected in glass tubes containing heparin and the plasma was promptly separated at  $4^{\circ}\text{C}$  by low speed centrifugation.

This study was approved by the Ethical Committee of the hospital and informed consent was obtained from each patient.

### Animals

Rabbits were used to study the appearance of labeled dolichol in the blood after injection of precursor. These animals were anesthetized with pentothal and 1 mCi (RS)-5- $^{3}\text{H}$ mevalonolactone (30 Ci/mmol; New England Nuclear, MA) was injected into their portal veins. At various subsequent time points, blood samples were collected from the ear vein. Plasma was prepared as in the case of human samples. Rat blood was collected by puncture of the inferior vena cava.

### Preparation of experimental materials

The human liver samples, which were taken from donors of cadaveric kidneys for transplantation, were immediately homogenized and fractionated. The fractionation procedures were the same as those used earlier for rat liver (34), with the exception of the homogenization step, in which a short Turmix treatment preceded the usual Potter-Elvehjem homogenization. Marker enzyme analyses of all fractions were performed and cross-contamination was calculated as described earlier (34). These contaminations

were between 2 and 13%, i.e., about double those observed previously in corresponding fractions from rat liver.

Hemoglobin-free erythrocyte ghosts were prepared as described earlier (35). After hemolysis with a hypotonic medium, the ghosts were thoroughly washed with phosphate buffer.

For the preparation of lipoprotein fractions, fresh plasma was used. First, total plasma lipoproteins were concentrated by flotation on a gradient consisting of 8 ml plasma adjusted to  $d$  1.33 g/ml at the bottom and, above this, 31 ml 0.15 M NaCl adjusted to  $d$  1.33 g/ml. For density adjustments, solid KBr was added according to the formula given by Terpstra, Woodward, and Sanchez-Muniz (36). By centrifugation in a vertical rotor for 3 hr at 200 000  $g$ , a convex gradient was generated. The total plasma lipoprotein fraction ( $d \leq 1.25$  g/ml) was collected by removing the uppermost 2 ml and this fraction was then recentrifuged in a Beckman VTi 50 vertical rotor on a KBr gradient (37, 38). All media used in the centrifugation procedures contained 0.01% disodium EDTA and  $\text{NaN}_3$ . The three lipoprotein fractions HDL, LDL, and VLDL were identified and collected with an ISCO gradient fractionator, with monitoring of the effluent for protein at 280 nm. Nycodenz (Nyegaard), 1.50 g/ml, was used as medium for elevating the gradient. The density of the fractions was determined with a refractometer and the appropriate fractions were pooled and stored at  $-20^{\circ}\text{C}$  prior to further analysis.

### Saponification

The total content of neutral dolichol was determined by HPLC. A 2-ml aliquot of plasma was carefully weighed and supplemented with 10 ml methanol containing 2 M KOH. To this mixture, 0.5 nmol dolichol-23 was added as internal standard. The tube containing the mixture was closed and placed in a boiling-water bath for 2 hr. After cooling, 7 ml water, 2 ml methanol and 24 ml chloroform were added to attain a chloroform-methanol-water ratio of 8:4:3 (v/v) for partitioning according to Folch, Lees, and Sloane Stanley (39). The mixture was centrifuged at room temperature (to avoid precipitation of salts), and after removal of the upper phase, the lower phase was washed three times with methanol-water 1:1 containing 0.1 M KOH and 2% KCl. These washings effectively remove fatty acids. Four subsequent washes with methanol-water 1:1 were also performed. The purified extract was evaporated under nitrogen and the residue was dissolved in 150  $\mu\text{l}$  chloroform-methanol 2:1 by sonication. This solution was injected onto the HPLC column.

### HPLC procedures

For the HPLC analysis, a fully automated Waters 840 system, including a WISP autoinjector and a variable UV detector (M 481), was used. This system was complemented

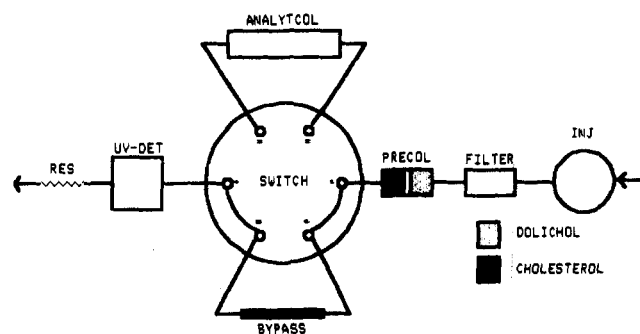
with an automated valve switch (Waters), based on a Rheodyne six-port valve (Fig. 1). The injected sample first passed a Rheodyne filter, then an MPLC precolumn (RP-8, 4.6 mm × 3.0 cm, 3- $\mu$ m particles (Brownlee Labs)). In this first phase of the chromatography, solvent A consisting of isopropanol–methanol–water 8:12:3 was used. The flow rate was 4 ml/min and for a 20-min period the eluted cholesterol was collected in a fraction collector (phase A). At this time, the switch setting was changed to direct the flow to the analytical column (ANALYTICOL, Fig. 1), which was a Hyper-sil ODS (C-18, 4.6 mm × 6.0 cm, 3- $\mu$ m particles (Hewlett Packard)). In order to minimize distortions in the baseline caused by this abrupt change, a flow restrictor was introduced into the solvent pathway after the detector. During column switching the flow rate was decreased to 0.1 ml/min and thereafter the flow rate was increased to 2 ml/min and maintained at this value throughout the analysis. The initial conditions were maintained for another 2 min in order to equilibrate the analytical column and, thereafter, a linear gradient designed to attain 100% solvent B (hexane–isopropanol–methanol 4:8:12) after 19 min was initiated. The eluate was monitored at 210 nm. The dolichyl-P present in the sample was eluted as a tailing peak with the front and did not interfere with the quantitation of dolichol.

Using the procedure described above, the dolichol content of 0.1–2.0 ml plasma could be reproducibly determined. Within this range, the response of the HPLC detector was linear. Use of the internal standard revealed the recovery to be 95% or better. The reproducibility of the total procedure was 2.7% C.V. (coefficient of variance) with a sample volume of 2 ml.

When dolichyl-P was to be quantitated, the internal standard used was 0.5 nmol dolichyl-23-P. The alkaline hydrolysis, extraction, and washing procedures were the same as those described for dolichol. After evaporation, the extract was dissolved in 10 ml chloroform–methanol 2:1 containing 0.5% ammonia. This mixture was then placed on a silica Sep Pak (Waters) and washed with 20 ml of the same solvent mixture in order to remove dolichol. The phosphorylated derivative was subsequently eluted with chloroform–methanol–water 1:1:0.3 and the eluate was evaporated. The residue was dissolved in 150  $\mu$ l chloroform–methanol 2:1 containing 20 mM H<sub>3</sub>PO<sub>4</sub>. The HPLC procedure was the same as described for dolichol, with the exception that both solvents A and B contained 20 mM H<sub>3</sub>PO<sub>4</sub>.

When the distributions of dolichol and dolichyl ester were studied, samples were supplemented with 0.5 nmol of both dolichol-23 and dolichyl-23 palmitate as internal standards. Without prior alkaline hydrolysis, this mixture was extracted with 20 volumes of chloroform–methanol 2:1 at room temperature for 1 hr. After filtration, the residue was reextracted with an additional 10 ml of the same solvent mixture. The pooled filtrates were adjusted to a chloroform–methanol–water ratio of 8:4:3 for partitioning and the lower phase was washed three times with methanol–water

#### A: Sample enrichment and precleaning



#### B: On column concentration and analytical chromatography

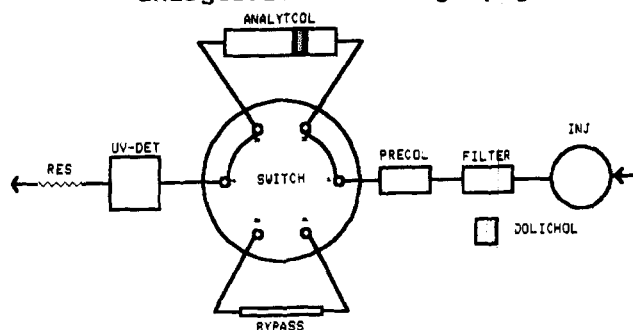


Fig. 1. Schematic presentation of the column-switching system used for analysis of plasma dolichol, dolichyl phosphate, and dolichyl esters.

1:1. After evaporation, the sample was dissolved in 400  $\mu$ l chloroform–methanol 2:1 and placed on a C18 Sep Pak. After 1 hr, when the majority of the solvent had evaporated from the Sep Pak, the sample was washed with 60 ml methanol, which removes most lipids other than dolichol and dolichyl esters. The neutral dolichols were then eluted with 20 ml chloroform–methanol 2:1 followed by 20 ml n-hexane. These eluates were pooled and evaporated and the residue was dissolved and injected onto the HPLC column. The HPLC procedure was the same as described above for dolichol, and the dolichyl esters eluting after the free alcohol were collected as a single fraction. These dolichyl esters were then subjected to alkaline hydrolysis and quantitated as the free alcohol. When alkaline hydrolysis was performed to determine the fatty acid composition, no dolichyl-23 palmitate was added.

Cholesterol and ubiquinone were quantitated by HPLC as described previously (40). The standards used were ergosterol and ubiquinone-6.

#### Determination of fatty acid compositions

The dolichyl ester fraction was isolated as described above, except that all solutions employed prior to the preparative HPLC run contained 0.15% butylated hydroxytoluene to prevent lipid peroxidation. The dolichyl ester fraction from HPLC was evaporated to dryness under ni-

trogen and dissolved in 3 ml chloroform and 3 ml methanol containing 3 M HCl in preparation for hydrolysis and methylation. The tubes were sealed under a nitrogen atmosphere and placed in an oven at 80°C for 2 hr. The methylated fatty acids were extracted with 1 ml hexane and washed with 0.1% Na<sub>2</sub>CO<sub>3</sub> and then with water. Methylated fatty acids were quantitated using a Shimadzu 6 AM gas-liquid chromatograph equipped with a 50 m × 0.25 mm fused silica column containing cyanosiloxan 60 and a flame ionization detector. A split/splitless injector was used for sample introduction and 0.5 μl was injected in the splitless mode. Hydrogen was used as the carrier gas at a linear flow rate of 0.5 m/s corresponding to 1.47 ml/min. The injector and detector temperatures were set at 230°C and the column temperature was programmed from 150 to 210°C. Chromatography was completed within 25 min. Fatty acid methyl esters were identified by comparison of their retention times with known standards.

### Chemical measurements

Protein was measured with the procedure of Lowry et al. (41) using bovine serum albumin as standard. Triglycerides were quantitated as described earlier (42).

### Standards

Dolichol-23 was isolated from bovine pituitary glands (43). Dolichyl-23 palmitate was synthesized by esterification of dolichol-23 with the corresponding acid chloride (44). Dolichol-23 was phosphorylated according to Danilov and Chojnacki (45). Ergosterol, ubiquinone-6, and methylated fatty acid esters were purchased from Sigma.

### Statistical methods

All statistical procedures were performed on a computer using the Statpac statistical software package at Karolinska Institutet. All subjects were characterized with respect to plasma levels of dolichol, cholesterol, and triglycerides, and weight, sex, nutritional state, and age were also recorded. Computation of means, ranges, standard deviations, and coefficients of variance (C.V.) were performed for each variable. When the grouped frequency distributions for all parameters were plotted, they were all seen to be approximately normally distributed. Multiple linear regression analysis was employed to characterize the relationships between plasma dolichol and all the other variables (46, 47).

TABLE 1. Hydrolysis of dolichyl esters<sup>a</sup>

Solvent Mixture	Base	Temperature	Hydrolysis of		
			Dolichyl Ester <sup>c</sup>	Protein <sup>d</sup>	Recovery <sup>b</sup>
	molar	°C	%	%	%
A. One-phase system					
M-W 2:3	1.7 KOH	100	43	100	95
M-W 4:3	0.4 KOH	70	39	85	93
M-W 5:1	1.7 NaOH	70	79	97	96
M-W 5:1	1.7 KOH	100	96	100	96
DC-M-W 1:1:0.3	1.7 KOH	100	60	100	70
T-M-W 5:10:1	1.0 KOH	100	99	81	81
H-I-W 10:10:1	1.0 KOH	100	97	78	86
M-W 2:3	1.7 KOH	100	45	100	95
1.0 mM NaDOC					
M-W 2:3	1.7 KOH	100	43	100	91
10.0 mM NaDOC					
B. Two-phase system					
DC-M-W 8:4:3	1.7 KOH	100	85	92	83

<sup>a</sup>Dolichyl-23 palmitate standard was mixed with 200 mg bovine serum albumin and different solvent mixtures as shown. Hydrolysis was performed for 1 hr on a water bath at the indicated temperatures. Sample preparation and HPLC quantitation of dolichol-23 and any remaining dolichyl-23 palmitate were performed as described in Materials and Methods. The results given are the means of three experiments. Abbreviations: H, n-hexane; T, toluene; C, chloroform; DC, dichloromethane; I, isopropanol; W, water; M, methanol.

<sup>b</sup>Recovery was calculated on the basis of the total amount of dolichol-23 and remaining dolichyl-23 palmitate as determined with an external standard.

<sup>c</sup>Hydrolysis of dolichyl ester was calculated as (amount of dolichyl-23) × 100/(amount of dolichol-23 + dolichyl-23 palmitate), as determined by HPLC after hydrolysis.

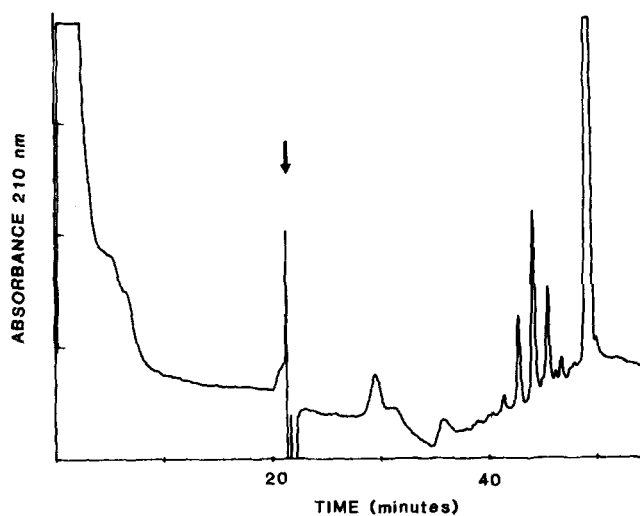
<sup>d</sup>Remaining protein was measured by weighing the precipitated and washed protein after partitioning.

## RESULTS

### Determination of dolichol

For the extraction of lipids from blood, it was found that the situation is very similar to that observed previously for tissue homogenates, where it was demonstrated that effective extraction requires an initial alkaline hydrolysis. One explanation for this finding is that tight associations of lipids with proteins are present. Since blood also contains phospholipids and triglycerides, which interfere with the separation of dolichol, alkaline hydrolysis is also a simple way to efficiently remove these components. Finally, a major part of the blood dolichol is esterified with fatty acids and, therefore, it is important to achieve complete hydrolysis of these derivatives as well. As shown in **Table 1**, alkaline hydrolysis in methanol-water 2:3 resulted in effective hydrolysis of protein, but not of dolichyl esters. When the hydrolysis mixture was completed with detergents, e.g., 1 or 10 mM sodium deoxycholate (concentrations below and above the critical micellar concentration (48)), the extent of hydrolysis of dolichyl esters was not increased. Two investigations on dolichol content in the blood, one using 0.4 M KOH in methanol-water 4:3 and the other 1.7 M NaOH in methanol-water 5:1 for hydrolysis (24, 27), have been published previously. In our hands, at a temperature of 70°C, the former procedure gave poor hydrolysis of the dolichyl ester fraction, nor was the latter fully effective. When the temperature was increased to 100°C, this latter procedure resulted in a 96% hydrolysis of the dolichyl esters in 1 hr. In our routine procedure we have employed a hydrolysis time of 2 hr, which gives complete hydrolysis of both the ester fraction and protein and an almost complete recovery of internal standard. The use of dichloromethane-methanol-water 10:10:3, a solvent mixture commonly employed in lipid chemistry, gave only partial hydrolysis and poor recovery. As shown, toluene-methanol-water and hexane-isopropanol-water were the most effective solvents for dolichyl ester hydrolysis, but because of practical difficulties in washing these light organic solvent mixtures with water (with increased losses of dolichol as a consequence) and because of the incomplete proteolysis, these mixtures are not recommended for routine use. Another approach often applied in lipid extraction, i.e., the use of dichloromethane-methanol-water 8:4:3 to give a two-phase system, was also tested. The effectiveness of hydrolysis and recovery with this procedure were also unsatisfactory. Dichloromethane was used instead of chloroform, since the former solvent is less reactive with alkali.

The separation of dolichol on HPLC is shown in **Fig. 2**. In the initial phase of the elution, cholesterol was recovered quantitatively and can be used for further investigation if desired. Twenty minutes later, after the column switch, dolichol appeared as a family of peaks containing individual members with 17 to 21 isoprene units. The main compo-



**Fig. 2.** Separation of dolichols isolated from 200  $\mu$ l human plasma by HPLC. The first peak on the left side is cholesterol. The arrow denotes the column switch. On the right side are the eluted individual dolichols.

nent contains 19 isoprene residues, whereas lower and similar amounts of 18 to 20 dolichols are present. The polyisoprenoid pattern shown in this figure was highly reproducible throughout this entire study. As also shown in the figure, no peaks were eluted after dolichol, i.e., in the region where dolichyl esters would elute if present. This indicates that complete hydrolysis of dolichyl esters was achieved under the conditions described above.

### Presence and distribution of dolichol in blood

The total dolichol content of human plasma is relatively low, i.e., ca. 0.2  $\mu$ g/g (**Table 2**). About half of these polyisoprenoids are esterified with a fatty acid. A smaller portion, 20%, is present as the free alcohol and about 30% is in phosphorylated form. The content of dolichol is comparable to that of ubiquinone, but represents quantitatively only a small part of the total blood lipid fraction, which is dominated by cholesterol, cholesteryl esters, and phospholipids.

The levels of dolichol and dolichyl ester in the plasma are 90% of those in whole blood, which is in agreement with the observation that erythrocyte ghosts have a very low polyisoprenoid content (**Table 3**). Upon separation of the lipoproteins by ultracentrifugation, dolichol was found to be associated with HDL, whereas the VLDL and LDL fractions contained little or no dolichol.

The isoprenoid content of human blood was also compared to that of the rabbit and rat (**Table 4**). Rabbit blood contains about 0.14  $\mu$ g dolichol/g in the nonsaponifiable lipid fraction, i.e., the same level as human blood, while in the rat this value is 50% lower.

It is probable that dolichol in blood originates mainly from the liver. There is a tenfold difference in dolichol content in the livers of the rat and rabbit, on the one hand, and humans, on the other. Thus, it is apparent that the

TABLE 2. Lipid composition of human plasma

Fraction	Lipid Concentration	
	molar	± C. V. <sup>a</sup>
Dolichol	$31.5 \times 10^{-9}$	± 21
Dolichyl ester	$78.5 \times 10^{-9}$	± 29
Dolichyl phosphate	$42.3 \times 10^{-9}$	± 11
Ubiquinone (CoQ <sub>10</sub> )	$0.73 \times 10^{-6}$	± 18
Cholesterol	$5.58 \times 10^{-3}$	± 22
Cholesteryl ester	$4.45 \times 10^{-3}$	± 29
Triglycerides	$1.42 \times 10^{-3}$	± 53
Phospholipids	$2.84 \times 10^{-3}$	± 19

<sup>a</sup>Values given are means ± coefficients of variance. Thirty five separate samples were analyzed. The reproducibility of repeated analyses from a single blood sample was 3%.

level in the blood is independent of the hepatic polyisoprenoid concentration.

### In vivo labeling

The lipoproteins of the blood are synthesized in the liver and the assembly of the lipoprotein structure occurs in the Golgi system (49, 50). The transport mechanism, including discharge of the particles into the blood, has been studied in detail (51–53). Since endogenously synthesized dolichol is associated with HDL in blood, we decided to study the possible involvement of the liver in the synthesis and secretion of blood dolichol using an in vivo approach. We injected [<sup>3</sup>H]mevalonolactone, which is known to effectively label cholesterol secreted from the liver, into the portal vein of rabbits. In these experiments the appearance of newly synthesized dolichol in the peripheral venous blood was monitored (Fig. 3). During the first 20 min after injection, the specific labeling of blood dolichol was very low, in agreement with observations that such a time period is required for the transport of blood proteins in the ER-Golgi system. Between 20 and 40 min postinjection, there was a rapid increase in dolichol labeling, reaching a plateau after 1 hr. This labeling pattern is in agreement with the idea that blood dolichol is synthesized in and discharged from the liver.

TABLE 3. Distribution of dolichol in human blood

Fraction	Total Neutral Dolichol <sup>a</sup>	
	ng/g ± C. V. <sup>b</sup>	% of total
Whole blood	161 ± 24	100
Plasma	143 ± 21	89
VLDL		0
LDL		12
HDL		75
Erythrocyte ghosts	16 ± 11	10
Serum	137 ± 19	85

<sup>a</sup>Total neutral dolichol was determined as dolichol after hydrolysis.

<sup>b</sup>Values represent means ± coefficients of variance of six separate samples.

TABLE 4. Dolichol content in the blood plasma and liver of various species

Species	Plasma Dolichol	Liver Dolichol <sup>a</sup>
	μg/g ± C. V. <sup>b</sup>	
Human	0.143 ± 21	456 ± 18
Rabbit	0.137 ± 12	38.5 ± 15
Rat	0.059 ± 13	45.8 ± 18

<sup>a</sup>Analysis of dolichol in liver homogenates was performed as described for plasma.

<sup>b</sup>Values represent means ± coefficients of variance of six determinations.

### Dolichyl ester

A considerable portion of the dolichol in rat liver was found to be esterified with a fatty acid (34). In our routine measurements of plasma dolichol, extractions were performed after a complete alkaline hydrolysis, so that all extracted polyisoprenoids were present as free alcohols. In order to study the dolichyl ester fraction of blood, Folch extraction of plasma was performed without prior hydrolysis. Esterified dolichols were then quantitated on reversed phase HPLC (Table 5). As much as 71% of the total blood dolichol appeared to be esterified with a fatty acid.

Since blood dolichol is thought to originate from the liver, it was of interest to subfractionate fresh human liver and analyze the relative levels of dolichyl ester and free dolichol in various subcellular compartments. The level of total neutral dolichol (dolichol + dolichyl ester) in the plasma is only 1.4 pmol/mg protein, which is much lower than that present in the liver or in subfractions of this organ. Human liver has considerably higher concentrations of dolichol than those found in experimental animals, but the intracellular distribution of dolichol in the liver of these animals is similar. At the site of synthesis in the endoplasmic reticulum, the concentration is lowest, whereas in the mitochondrial-lysosomal fraction and Golgi vesicles the total neutral dolichol content is 8–10 times higher. The amount of dolichol in the cytosolic fraction is surprisingly high and may reflect transport between intracellular membranes.

About half of the dolichol in the total homogenate is esterified, which is also the case for the high-speed supernatant. The mitochondrial-lysosomal fraction exhibits a relatively low level of esterification, i.e., about 25%. Both in microsomes and in Golgi vesicles approximately 70% of the dolichol is present in esterified form. This latter finding is interesting in the light of the hypothesis that dolichol transport follows the ER-Golgi-blood route, since the level of esterification is in fact about the same in these three compartments.

The fatty acid content of the plasma dolichol fraction was analyzed by capillary gas-liquid chromatography (Table 6). The two major fractions, each constituting about 30% of

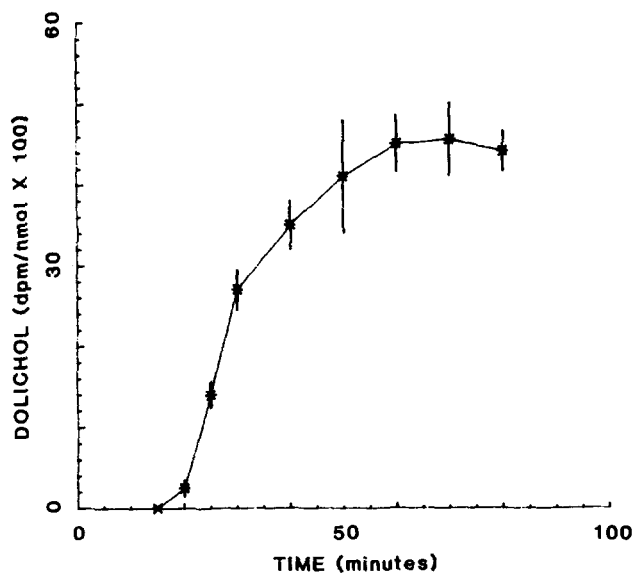


Fig. 3. In vivo labeling of blood dolichol in the rabbit. Four rabbits were injected with 5 mCi [ $^3\text{H}$ ]mevalonolactone each intraportally and blood samples were taken from the ear vein at various time points for determination of radioactivity in dolichol. Values are means  $\pm$  SD.

the total, were the fully saturated components palmitate and stearate. Among the unsaturated fatty acids, 16:1, 18:2, and, in particular, 18:1 were present; but surprisingly the polyunsaturated long-chain fatty acids 20:4 and 22:6 were almost completely absent. For comparison, the dolichyl esters of the liver homogenate and subfractions were also investigated. The main differences between liver homogenate and plasma in this respect were the relatively high amount of 18:0 and low amount of 18:2 in the plasma. The mitochondria, Golgi vesicles, and high-speed supernatant fraction from liver displayed fatty acid patterns very similar to that of the homogenate. The microsomes exhibited a higher relative concentration of 16:0 and a lower concentration of 18:1 and 18:2. The dolichyl ester fraction of rat liver homogenate was also analyzed and no major differences were observed in comparison with the human values.

### Variations in dolichol content in a human control population

The cholesterol and triglyceride contents of the blood are known to be dependent on a large number of physiological and pathological factors. In order to identify parameters that may contribute to variations in the blood dolichol level, we investigated the influence of age, sex, weight, food supply, and plasma levels of triglycerides and cholesterol. In order to evaluate the influence of these various conditions on blood dolichol content, multiple linear regression analysis was performed. The multiple correlation coefficients were calculated as described in Materials and Methods. In principle, if this coefficient is close to +1 or -1, there is a good linear relationship between dolichol level and the parameters investigated. If the value is close to zero, there is no such relationship. As seen in Table 7, this coefficient was 0.79 for age and -0.52 for the plasma triglyceride content. Thus, there is at least some relationship between blood dolichol content and these factors. In the case of sex and weight, the relationship to blood polyisoprenoid content was very weak and when the possible effects of food supply or plasma cholesterol content were considered, no relationships could be observed.

In light of these considerations, one can present the results of the regression analysis in the following equation:

$$\text{PLASMADOL (ng/g)} = 55.1 + 1.32 \times \text{AGE (yr)} - 13.9 \times \text{TG (mM)} + 3.18 \times \text{CHOL (mM)} - 2.73 \text{ (if } \sigma \text{)}$$

The multiple coefficient of determination  $R^2$ , which is an estimate of the proportion of the variability of the dependent variable (plasma dolichol) that can be attributed to variation of the independent variables, is high, i.e., 0.77. Thus, 77% of the observed variation in plasma dolichol content can be associated with the predictors age, sex, and contents of triglycerides and cholesterol in plasma. Conversely, the formula could be used to calculate the expected level of plasma dolichol in a healthy individual.

The level of total blood dolichol in individuals between

TABLE 5. Dolichol and dolichyl ester levels in human blood plasma and subcellular fractions from human liver<sup>a</sup>

Fraction	Dolichol		Dolichyl Ester	
	<i>pmol/mg protein</i>		<i>pmol/mg protein</i>	% of total
Plasma	0.41 $\pm$ 20		1.03 $\pm$ 30	71
Homogenate	1562 $\pm$ 17		1331 $\pm$ 34	46
Mitochondria <sup>b</sup>	2684 $\pm$ 25		895 $\pm$ 37	25
Microsomes	89 $\pm$ 12		282 $\pm$ 15	72
Golgi vesicles	677 $\pm$ 15		2143 $\pm$ 16	76
Supernatant	284 $\pm$ 17		206 $\pm$ 29	42

<sup>a</sup>Values represent means of four different livers  $\pm$  C.V.

<sup>b</sup>Mitochondrial-lysosomal fraction.

TABLE 6. Fatty acid compositions of dolichyl esters in blood plasma and hepatic subcellular fractions<sup>a</sup>

Fraction	Fatty Acid								
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:6
	<i>% of total</i>								
Human									
Plasma	3.5	31.0	9.1	28.1	16.4	6.9	2.0	1.8	1.5
Liver									
Homogenate	2.2	27.7	11.1	7.1	22.1	22.9	2.7	2.3	1.9
Mitochondria <sup>b</sup>	2.7	30.3	6.8	11.1	27.1	16.8	2.3	1.6	1.4
Microsomes	1.7	50.2	10.4	19.0	9.5	4.6	1.6	1.7	1.3
Golgi vesicles	2.5	23.2	5.1	16.2	32.5	14.5	2.1	2.2	1.8
Supernatant	2.5	28.7	6.8	13.4	29.2	11.5	3.4	2.2	2.3
Rat									
Liver homogenate	2.4	29.0	4.1	15.1	30.8	9.5	3.1	3.7	1.3

<sup>a</sup>Values represent means of four different analyses.

<sup>b</sup>Mitochondrial-lysosomal fraction.

the ages of 20 and 60 is shown in Fig. 4. The linear regression line displays a moderate increase with age, from the value of 105 ng/g at 20 to 175 ng/g at 60 years of age. This figure shows the individual values measured and it is apparent that the total variation in blood dolichol content is relatively limited.

One blood parameter that appears to have some association with the dolichol level is the triglyceride content. In this case the blood triglyceride content showed a weak negative correlation with the dolichol content (Fig. 5).

#### Blood dolichol content in certain human pathological states

In a number of human diseases the level of blood lipids is altered to a significant extent. We have selected patients with four such conditions, i.e., with diagnoses of diabetes mellitus, hypothyreosis, thyrotoxicosis, and pheochromocytoma (Fig. 6). A plot of the dolichol values as a function of age and comparison with the regression line and its 95% tolerance limits obtained for healthy individuals suggest that total dolichol content in plasma does not deviate significantly from the normal level in these diseases.

### DISCUSSION

The presence and nature of dolichol in various organs of both experimental animals and humans have been relatively well investigated, whereas blood dolichol has not been studied in detail. It has, however, been shown that injected dolichol is bound to the HDL fraction of blood and is not extensively metabolized.

Studies on blood dolichol are complicated. The blood contains relatively high concentrations of various other

lipids, in contrast to its low content of dolichol, which causes problems during separation and quantitation. For example, the blood content of cholesterol exceeds the polyisoprenoid level by as much as 25,000 times. In addition, a strong association between dolichol compounds and blood proteins requires an effective extraction procedure. Because of the low amounts of dolichol present, recovery will be poor if the isolation procedure involves many steps. Present HPLC techniques demonstrate high sensitivity and, consequently, the HPLC detection itself does not cause any problems. Steps where excessive losses of dolichol may occur include transfers between test tubes and purification on open column systems. With the sample preparation scheme described in the Materials and Methods, only one test tube and one transfer step is employed prior to HPLC. An advantage with the column switching technique used is that the large amounts of other lipids present act as carriers for dolichol throughout the sample preparation. After loading a large amount of the lipid sample on the precolumn, cholesterol

TABLE 7. Correlation between the plasma dolichol content and various parameters in a control population

Parameter	R (from Multiple Linear Regression)
Age	0.79
Sex <sup>a</sup>	0.16
Weight	0.23
Plasma triglycerides	-0.52
Plasma cholesterol	0.01
Caloric intake	0.02

<sup>a</sup>Dolichol amounts were slightly lower in males.



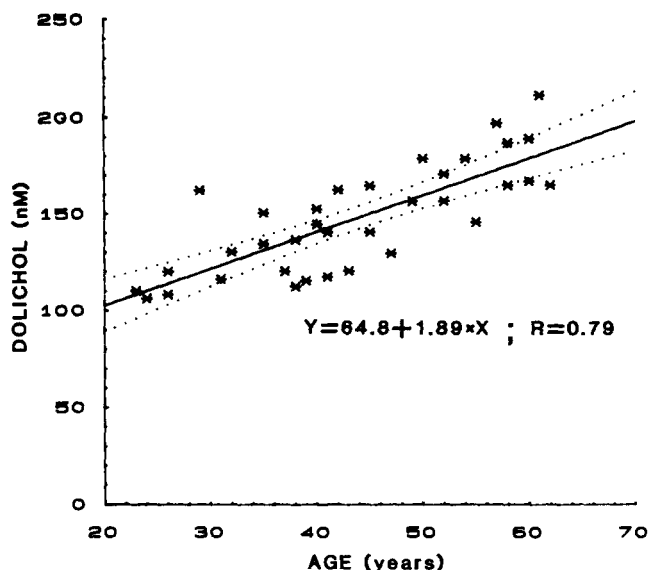


Fig. 4. Increase in dolichol levels with age. The individual values ( $n = 35$ ) are plotted in a scattergram. The regression line, its 95% confidence limits, and its equation are shown on the figure. Student's  $t$ -test of  $R$  indicates a positive linear correlation between age and dolichol content in the plasma. ( $t = 7.40^{***}$ ;  $d.f. = 33$ ).

is eluted under initial conditions and shunted directly to the detector. The dolichol fraction remains on the precolumn, but because of lipid overloading of the precolumn, the individual isoprenoid peaks are subject to serious broadening. In order to restore this lost chromatographic efficiency and to increase the sensitivity of the analysis, great care has been taken to choose an appropriate combination of precolumn and analytical column. With the combination chosen here, the dolichol fraction is eluted from the precolumn under solvent conditions permitting on-column concentration to take place on the top of the analytical column. Under these conditions it was possible to achieve a 95% recovery of dolichol with a relatively simple and time-saving procedure. In preliminary experiments we also have found this procedure to be useful and easily applicable for the analysis of the dolichol or dolichyl-P content in small tissue samples.

Dolichol is present in human plasma at a constant level, but in much lower concentration than the two major lipids of the blood, cholesterol and triglycerides. Only about 20% of this dolichol is present as the free alcohol, 52% is esterified with a fatty acid and the rest, i.e., 28%, is phosphorylated. All these forms are associated with the HDL fraction, at least under normal physiological conditions. The isoprenoid composition of plasma dolichol is the same as that found for human liver and as described previously for other human tissues (3). In addition, the fatty acids of dolichyl esters have a composition similar to that of tissue dolichyl esters (34) and distinct from the fatty acid compositions of phospholipids (54). The dolichol pattern is charac-

terized by a high degree of saturation and almost total absence of polyunsaturated long-chain fatty acids.

The question naturally arises as to the origin of the dolichol in blood. A large portion of other blood lipids is synthesized in the liver and secreted from the hepatocytes through the ER-Golgi system. In order to address this problem we performed a subfractionation of fresh human liver to investigate its lipid content. Because of the limited availability of human livers, a very precise subfractionation, as performed with rat liver, was not possible. However, the subfractions obtained represent reasonably well-isolated individual organelles and may therefore be used to obtain information about the organization of human hepatocytes.

As in rat liver (55), the low concentration of polyisoprenoid compounds in human liver microsomes is in contrast to the high concentrations in Golgi vesicles and in the mitochondrial-lysosomal fraction. Most interestingly, about 70% of the neutral dolichol fraction in the microsomes and the Golgi vesicles, as well as in plasma, is in esterified form. This observation is consistent with the idea that the esterified form is primarily secreted to the blood from the liver ER-Golgi system. It has been proposed that the fatty acid moiety of the phospholipids is utilized as a signal for targeting these lipids to other locations (56), which also appears to be the case for dolichyl esters (34). This may also be the reason for the esterification of polyisoprenoids destined for the blood. However, the fatty acid composition of blood dolichyl ester is different from that of the Golgi system. For this reason, this hypothesis should require further investigation in the future.

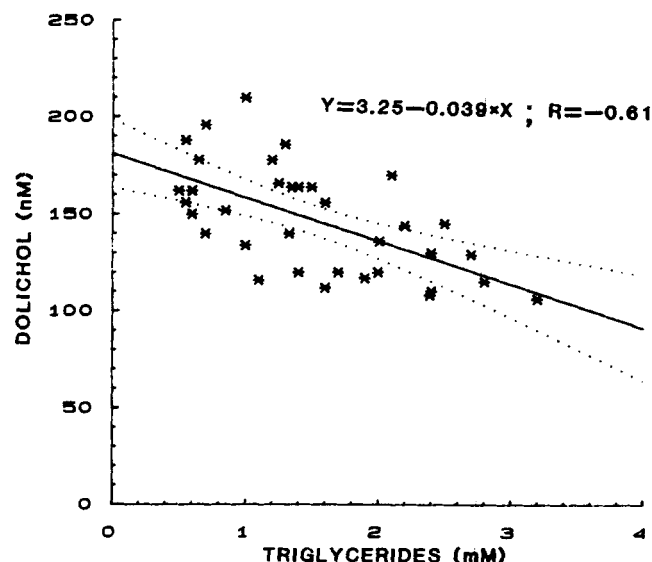
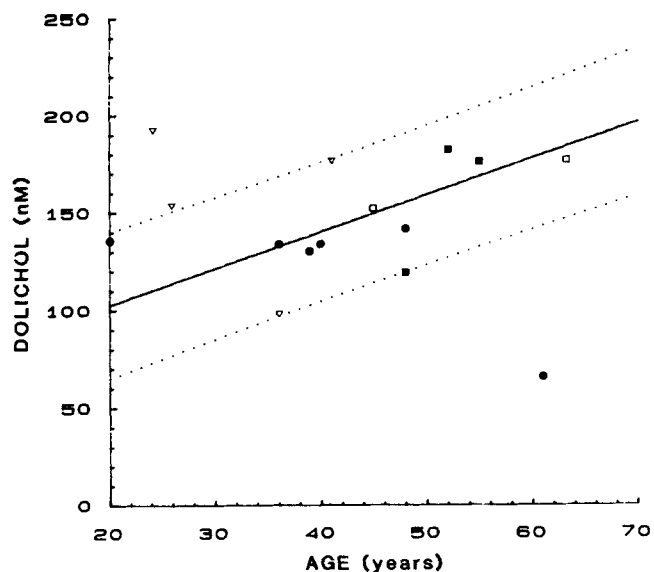


Fig. 5. Relationship between the levels of dolichol and triglyceride content in human plasma. The regression line and its equation are shown in the figure. Student's  $t$ -test of  $R$  indicates a negative relationship between the contents of dolichol and triglyceride in human plasma. ( $t = 3.22^{**}$ ;  $d.f. = 33$ ).



**Fig. 6.** Dolichol levels in various human pathological conditions. The individual points show the dolichol contents in the plasma of patients with diabetes mellitus (●), hypothyreosis (■), thyreotoxicosis (▽), and pheochromocytoma (□). Values are plotted against age. The regression line for the control population and its 95% tolerance limits are shown for comparison.

The composition of blood and liver dolichyl esters and our *in vivo* labeling experiments in rabbits are in agreement with the proposal that blood dolichol is synthesized in the liver. On the other hand, this type of data is circumstantial and definitive evidence can only be provided by further tests, e.g., studies with perfused livers. Our investigations have demonstrated that between different species the liver dolichol content may vary to a large extent, while variations in the blood levels of this lipid are much more limited. It is probable that the level of dolichol in the blood is not simply a reflection of liver dolichol content, representing some sort of "leakage," but is regulated by strict mechanisms, as is the case for other blood lipids.

The level of dolichol in blood is very constant under normal conditions. In our experiments we found a moderate linear increase in blood dolichol content with aging. This increase is in agreement with observations that the content of dolichol, but not dolichyl-P, in human brain increases extensively during aging (20). Recent investigations performed on the organs of individuals between the ages of 2 and 80 have shown that in the aging process all human tissues accumulate dolichol (A. Kalén, personal communication).

In a previous investigation it was found that the dolichol content of human blood decreases to a moderate extent during aging (24). However, in this study, a hydrolysis procedure that did not affect the majority of the dolichyl esters was utilized and the quantitation of dolichol by HPLC was performed using a fluorescent derivative of this lipid. For these reasons the results of this previous investigation cannot be directly compared with ours.

We found a negative correlation between the triglyceride and dolichol contents in human blood. This is remarkable, since it is seemingly in disagreement with the constant level of the polyisoprenoid lipid in blood. It is possible, however, that this phenomenon does not reflect changes in dolichol itself, since the HDL content of plasma is known to be inversely correlated to triglyceride content (57). Thus, the negative correlation observed may be the result of variations in lipoprotein levels.

We have also analyzed a number of conditions under which the blood cholesterol level is known to be altered. In these cases, however, no alteration in blood dolichol content could be observed. These findings, on the other hand, do not exclude the possibility that under certain pathological conditions the amount of this lipid in blood may change.

At present, the functional significance of the presence of dolichol in the blood remains unexplained. Since dolichol is present in all tissues, cells, and membranes, it is not surprising that this lipid is also present in the blood. While the function of the free alcohol has not yet been established, its phosphorylated counterpart serves as an obligatory intermediate in the biosynthesis of N-glycosidically linked oligosaccharide chains in glycoproteins. There are glycosyl transferases in the blood (58) and, even though it is improbable that these enzymes utilize dolichyl-P in some way, this question has not yet been studied.

In comparison with cholesterol, the dolichol concentration in the blood is very low. There are several possible explanations for this difference. In a previous investigation it was found that all tissues investigated in an experimental animal were capable of both dolichol and cholesterol synthesis (40). The cholesterol synthesis was, however, much more unevenly distributed among the tissues, i.e., liver dominated, than the ubiquitous dolichol synthesis. Different organs require very different amounts of cholesterol, e.g., as substrate for hormone or bile acid synthesis. This may necessitate transport of this lipid in the blood from one organ to another. Furthermore, it is well established that the intracellular cholesterol pool is composed partly of endogenously synthesized cholesterol and partly of dietary cholesterol (59). Distribution of the latter occurs by transport in the blood. In the case of dolichol, it was found that longer polyisoprenoids are not at all taken up from the diet, whereas shorter polyisoprenoids are taken up, but only to a very limited extent (60, 61). Therefore, it appears that the high cholesterol content in the blood may be explained partly by the dietary uptake and partly by the necessity of external supply in some tissues. Since dolichol is probably not used as a substrate in metabolic processes, the endogenous production within the cell might be sufficient for basal structural requirements.

Finally, the amount of a lipid is not the only reliable measure of its importance in life processes. The appearance, the turnover, the breakdown, and uptake are all factors which are of importance in this respect. It will be an im-

portant task to study further the biosynthetic appearance, turnover, and uptake of blood dolichol in the process of searching for its functional importance. ■■

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