# Depression of hepatic dolichol levels by cholesterol feeding

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**Abstract** A study was conducted to determine whether repression of 3-hydroxy-3-methylglutaryl CoA reductase by a chronic high-cholesterol diet would deplete hepatic dolichol levels. Four-week-old male C57BL/6J mice were maintained on a control diet or a diet supplemented with 5% cholesterol. Animals from both groups were killed at various times and reductase activity and levels of free dolichol, dolichyl acyl ester, dolichyl phosphate, and ubiquinone were measured. The reductase activity was reduced by 90% within 1 week and remained depressed through 56 days. Initially, the levels of the free dolichol, acyl ester, phosphoryl ester, and ubiquinone were 7, 16, 5, and 80 μg/g liver, respectively. Early increases in the concentration of dolichyl phosphate and free dolichol were similar in both the cholesterol-fed and control groups. However, in the cholesterol-fed group the concentration of dolichyl acyl esters was only 50% of that in the control group by 7 days and it remained lower throughout the experiment. Total dolichol levels were lower by about 30%. Ubiquinone levels were transiently depressed at 7 days by 33% but returned to control levels by 4 weeks. After 56 days, the control values of dolichol and dolichyl phosphate remained constant whereas the dolichyl acyl ester levels continuously increased to a value of 133 μg/g of liver by 156 days. Subcellular fractionation of livers from 4-week-old mice indicated a lysosomal distribution of dolichol and dolichyl acyl ester and a lysosomal and microsomal distribution of dolichyl phosphate. —**Kabakoff, B. D., and A. A. Kandutsch.** Depression of hepatic dolichol levels by cholesterol feeding. *J. Lipid Res.* 1987. **28:** 305–310.

**Supplementary key words** ubiquinone • HMG-CoA reductase • dolichyl phosphate • dolichyl acyl ester

It is well known that dietary cholesterol suppresses the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and cholesterol synthesis in liver. Although HMG-CoA reductase is usually considered as the regulatory enzyme for sterol biosynthesis, it also provides intermediates for the biosynthesis of dolichol and ubiquinone. Dolichol exists in cells as the free alcohol and as fatty acid and phosphate esters. The phosphorylated form is involved in the synthesis of oligosaccharide units and their subsequent transfer to proteins during the process of N-linked glycoprotein synthesis. A function for the other two forms has not been clearly demonstrated. Previous work with adult rats and rabbits (1, 2) indicated that when these animals were placed on a high-cholesterol diet there was little effect on dolichol levels under conditions where cholesterol synthesis was considerably suppressed. These authors concluded that the reductase does not regulate dolichol biosynthesis in vivo. This conclusion appears to be inconsistent with evidence that cholesterol feeding does inhibit acetate incorporation into total neutral hepatic dolichol in vitro in rats (1), rabbits (2), and mice (3). The present work was carried out to examine the effect of repressing HMG-CoA reductase levels on hepatic levels of dolichol and ubiquinone in mice fed a high-cholesterol diet from the time of weaning.

## MATERIALS AND METHODS

### Animals

Male C57BL/6J mice (4 weeks old) from the Animal Resources Department of The Jackson Laboratory were weaned on Old Guilford laboratory diet no. 911 or the same diet supplemented with 5% cholesterol.

### Materials

Ubiquinone 6, 9, and 10, cholesterol, and all reagents for marker enzyme assays were purchased from Sigma. [1-$^3$H]dolichol (3 Ci/mmol) was prepared by the method of Rupar and Carroll (4) and [1-$^3$H]dolichyl phosphate (3 Ci/mmol) was prepared by the method of Warren and Jeanloz (5). Dolichol and dolichyl phosphate standards were the generous gift of Dr. M. Mizuno, Kuraray Co., Ltd., Okayama, Japan. High pressure liquid chromatography (HPLC) grade solvents were obtained from Burdick and Jackson.

Abbreviation: HMG, 3-hydroxy-3-methylglutaryl.

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### Lipid extraction

At each time point, mice were killed in the morning and two sets of four pooled livers were obtained from both the cholesterol-fed and control animals. Each point in Fig. 1 represents the average of determinations from these two sets. The pools of four livers were minced and divided into three portions and weighed. [$^3$H]dolichol was added (40,000 dpm) to one portion and [$^3$H]dolichyl phosphate was added (40,000 dpm) to another as internal standards to monitor recoveries. These two portions were saponified and the nonsaponifiable fractions were used to determine total neutral dolichol and dolichyl phosphate. [$^3$H]dolichol (40,000 dpm) and ubiquinone 6 (20 $\mu$g) were added as internal standards to the third fraction of liver, and the tissue was homogenized and extracted by the method of Bligh and Dyer (6). The chloroform extract was used to quantitate the free dolichol and ubiquinone levels. The concentration of dolichyl acyl esters was determined as the difference between the total neutral dolichol and the free dolichol.

### Saponification

Saponifications were carried out at 70°C for 2 hr in 30% methanolic KOH containing butylated hydroxytoluene (25 mg/ml) and pyrogallol (25 mg/ml). The nonsaponifiable fraction was extracted in diethyl ether. For dolichyl phosphate assays, the ether fraction was rinsed once with 5% $CH_3COOH$ and evaporated under $N_2$. For total neutral dolichol assays, the ether fraction was rinsed sequentially with 5% $Na_2CO_3$, 5% $CH_3COOH$, and water before evaporation.

### Dolichyl phosphate

The nonsaponifiable fraction was dissolved in $CHCl_3$–$CH_3OH$ 2:1 and applied to a DEAE-cellulose (Whatman) column (2.2 × 3.5 cm) in the acetate form (7). The column was eluted sequentially with $CHCl_3$–$CH_3OH$ 2:1, $CHCl_3$–$CH_3COOH$ 3:1, and $CHCl_3$–$CH_3OH$ 2:1 containing 0.1 M ammonium acetate. The dolichyl phosphate, eluted with the last solvent, was washed with water to remove the ammonium acetate and evaporated to dryness in polypropylene tubes under $N_2$. The sample was then quantitated by the HPLC method of Keller et al. (8). All HPLC work was performed using a Waters model 6000A HPLC set at 2 ml/min and equipped with a Waters model U6K injector, an ISCO model 1840 variable wavelength detector, and a Spectrophysics 4100 integrator. The dolichyl phosphate was taken up in hexane–2-propanol–1.4 M $H_3PO_4$ 30:970:0.5 and applied to a $\mu$Porasil (Waters) HPLC column (0.39 × 30 cm) equilibrated with the same solvent. The dolichyl phosphate fraction ($V_E$ = 8 ml) was collected, washed with water and evaporated under $N_2$. The sample was redissolved in the column solvent of methanol–2-propanol–$H_3PO_4$ 650 ml:350 ml:2 g and applied to a $\mu$Bondapak $C_{18}$ (Waters) column (0.39 × 30 cm) equilibrated at 55°C. The mass amounts were determined by integration of the areas under the peaks of the dolichyl phosphate isoprenologs as monitored at 210 nm. Values were corrected for recovery by counting the tritiated internal standards after reverse phase chromatography in a toluene-based scintillation fluid using a Packard Tri-Carb 300C scintillation counter. The two values determined for each point did not vary more than 10% from the mean.

### Dolichol

After saponification, the total neutral dolichol fraction was treated identically to the free dolichol fraction. The samples were redissolved in $CH_3OH$–$CHCl_3$ 65:35 and chromatographed on a Lipidex-5000 (Packard) column (0.9 × 15 cm) equilibrated and developed with the same solvent mixture. The first 20 ml that was eluted was discarded and the next 30 ml was collected and evaporated in vacuo. The sample was redissolved in 0.25% 2-propanol in hexane and applied to a $\mu$Porasil column equilibrated with the same solvent. The dolichol fraction ($V_E$ = 19 ml) was collected, evaporated, redissolved in methanol–2-propanol 1:1, and chromatographed on a $\mu$Bondapak $C_{18}$ column equilibrated with the same solvent at 55°C (9) and monitored at 210 nm. Quantitation of mass amount and recovery was carried out as described for dolichyl phosphate. With one exception, the two values determined for each point did not vary more than 15% from the mean.

### Ubiquinone

A 10% aliquot of the Bligh and Dyer extract was removed before application to the Lipidex-5000 column for quantitation of ubiquinone. The aliquot was evaporated and taken up in 0.25% 2-propanol in hexane and applied to a $\mu$Porasil column. The eluate was monitored at 210 nm and the region of the eluate spanning ubiquinone 10 ($V_E$ = 9.6 ml) and ubiquinone 6 ($V_E$ = 11 ml) was collected. The sample was processed and placed on the $C_{18}$ column equilibrated with 2-propanol–methanol 1:4 at 55°C and monitored at 210 nm. The area under the ubiquinone 9 peak ($V_E$ = 11.2 ml) was integrated to determine the mass amount and the area under the ubiquinone 6 peak ($V_E$ = 6.3 ml) was measured to correct for recovery. Ubiquinone 10 was present as a very small percentage of the total ubiquinone and was not quantitated. The two values determined for each point did not vary more than 10% from the mean.

### HMG-CoA reductase

A small amount of the pooled livers in each group was set aside for the preparation of microsomes and determination of HMG-CoA reductase activity as outlined by

James and Kandutsch (3). Immediately after preparation, the microsomes were quickly frozen in liquid $N_2$ and stored until samples at all time points had been collected. The samples were then thawed and assayed for reductase activity. Protein determinations were done by the method of Lees and Paxman (10).

### Subcellular fractionation

The method of De Duve and coworkers (11) was used to prepare subcellular fractions by differential centrifugation. The five fractions prepared were the nuclear (N), the heavy mitochondrial (M), the light mitochondrial which is enriched in lysosomes (L), microsomal (P), and cytosolic (S) fractions. The N fraction was prepared with a PR-2 (IEC) refrigerated centrifuge using a 269 rotor. The remaining fractions were prepared using a Beckman L8-70 ultracentrifuge with a type 40 rotor for M and L and a type 70.1 rotor for P and S. Aliquots of each fraction were taken for protein determinations and marker enzyme assays as described below. One half of each fraction was then saponified and the nonsaponifiable component was processed as previously described to measure dolichyl phosphate and total neutral dolichol, with the exception that the total neutral dolichol was first separated from the dolichyl phosphate on the DEAE-cellulose column before Lipidex-5000 chromatography. The neutral dolichol eluted with the initial solvent system. The other half of each fraction was directly extracted and processed as described previously for free dolichol. The recoveries of free dolichol, dolichyl acyl ester, and dolichyl phosphate from the subcellular fractionation were 74%, 90%, and 96%, respectively.

### Enzyme assays

Marker enzymes assayed to monitor the distribution of lysosomes and mitochondria were N-acetyl-$\beta$-glucosaminidase (12) and malate dehydrogenase (13), respectively. Assays for malate dehydrogenase were performed with and without 0.1% Triton X-100 to determine latency as a means of monitoring the intactness of the mitochondria. The distribution of malate dehydrogenase represented in Fig. 3 corresponds to that portion that exhibits latency and hence intact mitochondria. The recoveries of the mitochondrial and lysosomal markers from the subcellular fractionation were 80% and 95%, respectively.

## RESULTS

### Effects of dietary cholesterol

In confirmation of previous work (3), **Fig. 1a** shows that HMG-CoA reductase levels were depressed by 90% within a week of cholesterol feeding and remained depressed through 56 days. Cholesterol feeding appeared to cause a slight initial increase in free dolichol but the value returned to the control level by 56 days after weaning (**Fig. 1b**). Dolichyl acyl ester concentrations in the cholesterol-fed group (**Fig. 1c**) were only 50% of that found in the controls within 1 week and remained depressed relative to the controls throughout the experiment. Dolichyl phosphate levels (**Fig. 1d**) were essentially unaffected by cholesterol feeding although the distribution of the dolichyl phosphate isoprenologs shifted toward those of lower chain length within 7 days (**Fig. 2**). A similar shift in chain length was also observed for neutral dolichol (data not shown) in agreement with the report of Tavares et al. (1). In contrast to the lower levels of total dolichol in cholesterol-fed mice, which persisted throughout the feeding period, ubiquinone levels (**Fig. 1e**) were transiently depressed (by 33%) at 1 week but recovered to control group values by 4 weeks. The adult ubiquinone levels determined here are in good agreement with previously reported values (14). The recovery to the control levels by the cholesterol-fed animals may reflect an adaptation to a more efficient utilization of ubiquinone from the diet which contained about 23 $\mu$g/g of ubiquinone 9.

It is of interest that hepatic levels of dolichyl acyl esters, and therefore total dolichol levels, increased continuously with advancing age. Since the concentrations of free dolichol and dolichyl phosphate remained essentially constant after 56 days post-weaning, the acylated proportion of the total dolichol increased from ~56% at weaning to ~72% at 156 days postweaning. These results are similar to the pattern observed in dog brain by Keller et al. (8) in which neutral dolichols increased with age while the level of dolichyl phosphate remained constant. Additionally, Sakakihara and Volpe (15, 16) showed an age-related increase in total neutral dolichol of rat and human brain.

### Subcellular distribution of hepatic dolichol

Wong, Decker, and Lennarz (17) and Eggens et al. (18) have reported a peculiar subcellular distribution for hepatic neutral dolichols in that there is a high degree of enrichment in the lysosomal fraction. These studies did not distinguish between free dolichol and dolichyl acyl esters and the animals that were used were past weaning at a time when large amounts of the neutral dolichols have begun to accumulate. To ascertain the subcellular distribution of free dolichol and its acyl esters and to determine whether lysosomal localization is apparent at an early age when smaller amounts of neutral dolichol have accumulated, subcellular fractions were obtained from livers of C57BL/6J male weanlings 4 weeks of age. The results shown in **Fig. 3** indicate that both free dolichol and its acyl ester were localized in the lysosomes, whereas dolichyl phosphate was distributed between the lysosomal and microsomal fractions. These results suggest that the lysosomal distribution of dolichol is not a result of the

accumulation of large amounts of neutral dolichol with advancing age.

## DISCUSSION

Cholesterol feeding represses hepatic HMG-CoA reductase activity by approximately 90% which, in previous studies (3), reduced hepatic dolichol biosynthesis from acetate by 49%. Since dolichol does not appear to be degraded, hepatic dolichol levels in mice fed a high-cholesterol diet were expected to increase somewhat, but not so much as in mice fed a control diet. The results of the present experiments are consistent with this prediction indicating that HMG-CoA reductase does play a regulatory role in hepatic dolichol biosynthesis. Clearly, the acylated form of dolichol was most affected by repression of the reductase. Its levels were lower by ~50% in cholesterol-fed mice resulting in a 30% decrease in total dolichol levels, correponding to a difference of ~30 μg/g liver as compared to controls. The possibility that lowered levels of dolichol found in the cholesterol-fed mice were influenced to any significant extent by changes in absorption of dietary dolichol was excluded by analysis of the feed, which indicated that there was no measurable dolichol of the chain lengths observed in mouse hepatic tissue to a detection limit of 100 ng/g of feed. Additionally, Keller, Jehle, and Adair (19) have shown that the contribution of dietary dolichol and other polyprenols to the liver dolichol pool is negligible.

It is possible that Tavares, Coolbear, and Hemming (1) did not observe a similar depression of dolichol levels with cholesterol feeding because they studied adult rats, presumably with large stores of neutral dolichol already accumulated. The relatively short, 2-week period on a 1% cholesterol diet may not have produced a noticeable decline in already large stores of dolichol. In addition, acetate incorporation into dolichol was depressed by only 27% in the rats, whereas a decrease of 49% was observed in mice under conditions similar to those used in the present study (3). Similarly, White et al. (2) did not observe a decline in hepatic dolichol levels in rabbits even when the animals were maintained on a 2% cholesterol diet for 10 weeks. The basal rates of hepatic dolichol synthesis and the extent to which acetate incorporation into dolichol was depressed in rabbits by dietary cholesterol were roughly equivalent to those we found in mice.

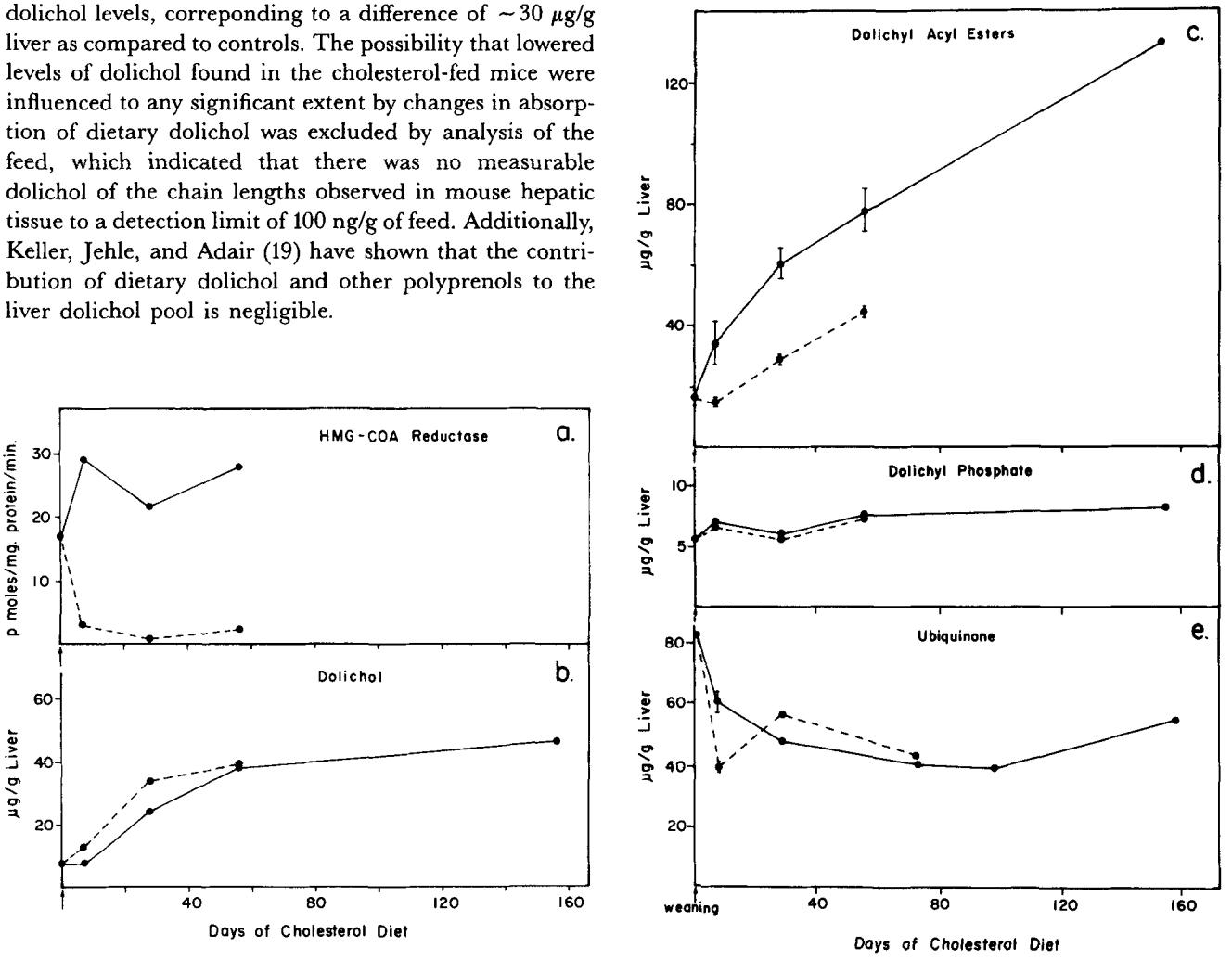


**Fig. 1.** Levels of HMG-CoA reductase (a), dolichol (b), dolichyl acyl ester (c), dolichyl phosphate (d), and ubiquinone 9 (e) versus age past weaning for C57BL/6J male mice fed a 5% cholesterol diet (- - - -) and control mice on a normal diet (——). Ranges of values are indicated for those points significantly affected by cholesterol feeding.

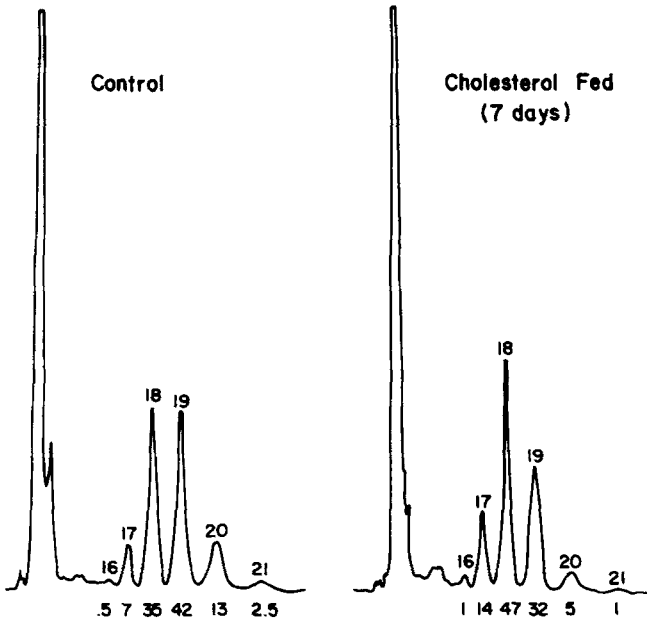


**Fig. 2.** HPLC profile of hepatic dolichyl phosphate from normal and cholesterol-fed mice as chromatographed on a μBondapak $C_{18}$ column processed as described in Methods. The number above the peak represents the number of isoprene units and the number below represents its percentage contribution to the total dolichyl phosphate.

However, the adult rabbits had already accumulated massive amounts of neutral dolichol (15–20 ± 4 mg/g), three orders of magnitude higher than we measured in the weanling mice. Therefore, the effect of cholesterol that we observed in mice would not be detected in these rabbits.

It should also be noted that although the reductase activity was severely repressed (90%), enough intermediates were generated by the remaining activity to support a limited production of dolichol (3) and probably production of some ubiquinone. Due to this, and the large stores of dolichyl acyl ester and free alcohol, even chronic repression of reductase activity by cholesterol feeding had no effect on the levels of dolichyl phosphoryl esters other than to shift the distribution of isoprenologs to lower chain lengths. The changes observed would not be expected to

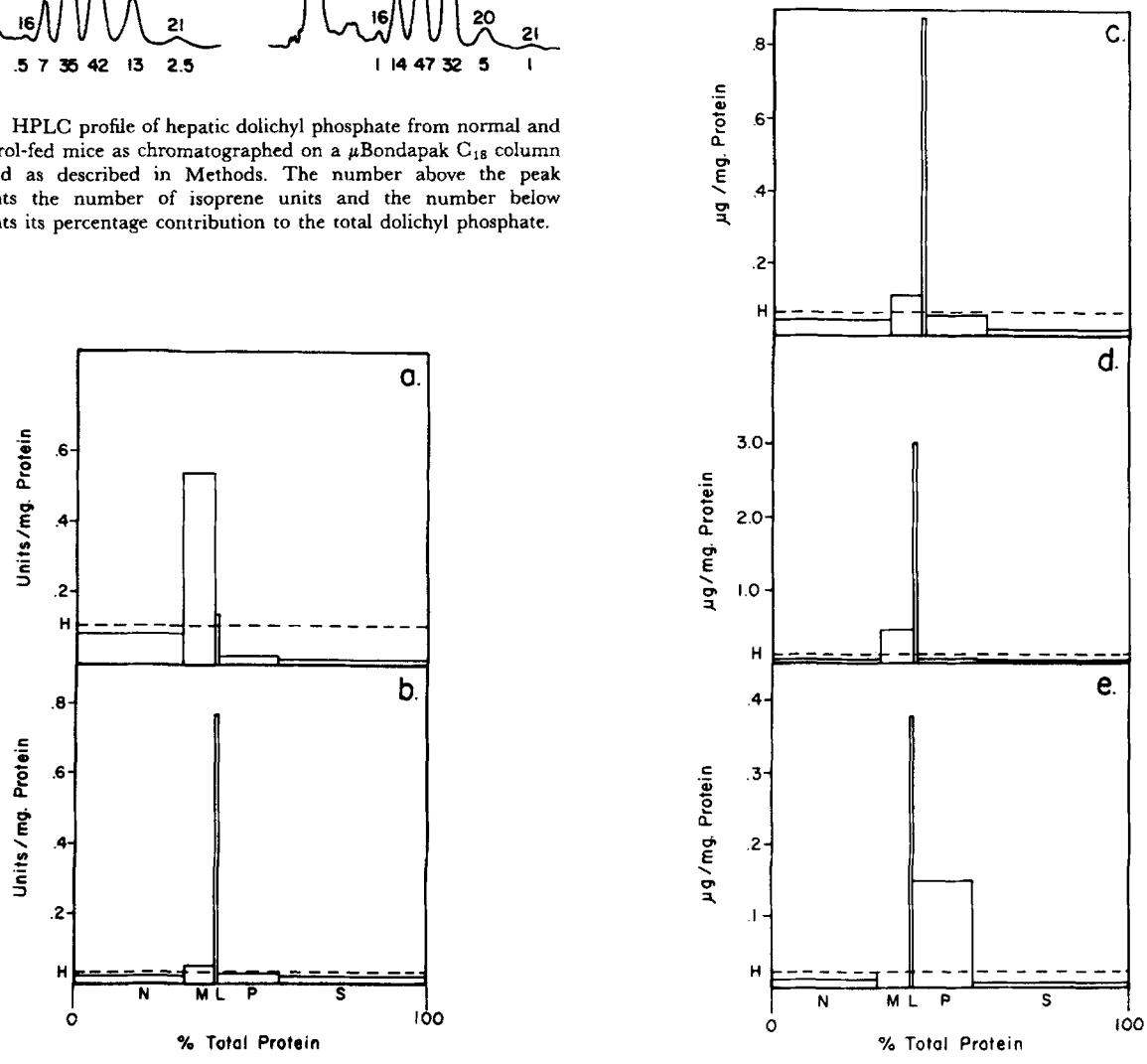


**Fig. 3.** Subcellular distribution of latent malate dehydrogenase (a), N-acetyl-β-glucosaminidase (b), dolichol (c), dolichyl acyl ester (d), and dolichyl phosphate (e). The y-axis represents the specific activity of each component and the x-axis represents the percent of the total protein each subcellular fraction represents. Enzyme assays and subcellular fractionation were carried out as described in Methods to obtain the nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and cytosolic (S) fractions. The dashed line corresponds to the specific activity in the whole homogenate (H).

interfere with N-linked glycoprotein synthesis. Previous work indicates that treatment for 2 weeks with a high-cholesterol diet has no effect on mannose incorporation into serum glycoproteins (3).

The only known function for dolichol is the role of dolichyl phosphoryl ester in N-linked glycoprotein synthesis and the site of its action is the endoplasmic reticulum. The microsomal enrichment of dolichyl phosphate in the present work and that of others (18) is consistent with that function. In contrast, the neutral dolichols are directed to the lysosome, even at an early age, where their physiological role is poorly understood. Our results show that dolichyl acyl esters in mouse liver increased continuously with age, and Keller et al. (8) have shown similar increases in total neutral dolichol in dog brain. In contrast, the concentration of dolichyl phosphate was closely regulated in both organs. The fact that, relative to controls, levels of acyl esters diminished in the cholesterol-fed mice while those of free and phosphorylated dolichol were maintained can be explained in two different ways. One possibility is that the dolichyl acyl ester is a storage form of dolichol which is in a steady-state equilibrium with free dolichol. When dolichol precursors are limited, ester hydrolysis would increase relative to ester formation to yield more free dolichol for phosphorylation. On the other hand, because there is no known degradative pathway for dolichol, it is possible that the dolichyl acyl ester formation is an essentially irreversible process in vivo, the sole function of which is the sequestration of dolichol in lysosomes to protect against its possible harmful interactions with cellular membranes. When dolichol precursors are limited, the activity of the acyl transferase involved in ester formation would decrease, thus directing the limited amounts of newly formed dolichol towards the maintenance of the free and phosphorylated dolichol pools. In either case, it seems that the continuous accumulation of highly nonpolar lipids to such a large extent might at some point become detrimental to the cells and thus ultimately play a role in the general process of cell aging and death.

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