Changes in Murine Tissue Concentrations of Dolichol and Dolichol Derivatives Associated with Age

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The concentrations of the three major cellular forms of dolichol (free, esterified and phosphorylated) were determined in murine liver, kidney and heart. The tissue levels of these forms of dolichol were studied in detail as a function of age. Changes in the activities of dolichyl phosphate phosphatase and dolichol kinase were also determined. In liver, the concentration of unesterified dolichol, fatty acyl dolichol and dolichyl phosphate increased markedly over a period of 6 to 25 months (four-fold, 5.5-fold and nine-fold, respectively). In kidney only, free dolichol and phosphorylated dolichol increased (approximately four-fold in each case). However, this tissue consistently showed the highest concentrations of all forms of dolichol as compared to liver and heart. In heart, both free and esterified dolichol concentrations increased (approximately 3.25-fold in each case); dolichyl phosphate levels were not determined in this tissue. In all tissues studied, the activity of the dolichyl phosphate phosphatase enzyme was considerably higher than that of the dolichol kinase enzyme. In liver, there was no evidence to suggest that either enzyme was critical in determining the relative concentrations of dolichol and dolichyl phosphate. Evidence for such a role for the kinase in the kidney was stronger. Treatment of aging mice with meclofenoxate, a drug that is reported to cause dissolution of lipofuscin, failed to prevent accumulation of dolichol and dolichyl phosphate with age. These observations suggest that not all accumulated dolichol is associated with lipofuscin. Meclofenoxate treatment had no consistent effect on the activities of the enzymes studied.
Lipids 25, 586–593 (1990).

The accumulation of lipofuscin is arguably the single most discernible cytological change that occurs in the aging cell. The formation of the lipopigment is a continuous process that commences early in life and increases with age (1). Although the presence of this pigment within aging cells has long been recognized (2), its nature remains poorly understood. It is thought to contain an autofluorescent, conjugated Schiff base that may be formed by the *in vivo* peroxidation of polyunsaturated lipids. The aldehyde product of this reaction may subsequently complex with the amino groups of proteins (3). There is strong evidence that the pigment is associated with lysosomes (4,5).

As well as its chronological accumulation, lipofuscin is reported to build up within neurons of sufferers of a number of neurodegenerative disorders. Amongst these disorders are Batten Disease (6,7) and Alzheimers' Disease (8–10).

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Abbreviations: DP, dolichyl phosphate; DTT, dithiothreitol; ED, esterified (fatty acyl) dolichol; FD, free (unesterified) dolichol; HPLC, high performance liquid chromatography; i.p., intraperitoneal; NMR, nuclear magnetic resonance; TD, total dolichol; TLC, thin-layer chromatography.

The pathogenicity of lipofuscin in both the aging cell and in disease conditions is unproven. Rats fed vitamin E-deficient diets are reported to show a decrease in learning and memory functions along with an increase in lipopigment accumulation (11); while mice receiving the drug centrophenoxine show an increase in cognitive functions with a decrease in neuronal lipofuscin (12).

Age-associated increases in dolichols were first observed in human brain (13) and have since been reported in both neural and non-neural tissues of rats (14–18), mice (19) and dogs (20). More recent evidence suggests that the active metabolite of dolichol (dolichyl phosphate) might also accumulate with age (15).

Dolichol, like lipofuscin, is reported to accumulate in both Batten Disease (14,21–23) and Alzheimers' Disease (14,24,25). Analysis of lipofuscin isolated from tissues of Batten Disease patients suggests that the pigment might contain significant amounts of dolichols that exist in the free form (13,14,24,26,27).

An association between dolichol and lipofuscin is strengthened by several observations. Lipofuscin is reported to exhibit a similar nuclear magnetic resonance (NMR) spectrum to pure dolichol (28), the induction of lipopigment granules in rat brain is accompanied by increases in brain dolichols (29,30) and, finally, tissues thought to be rich in lipofuscin such as testis and kidney (31,32) also appear to be rich in dolichol (18,19).

The drug meclofenoxate (dimethylaminoethyl *p*-chlorophenoxyacetate) has been shown to improve cognitive function in old mice (12) and in the elderly (33,34). Such improvements are accompanied by a reduction in lipofuscin accumulation in both neural (35–39) and non-neural tissues (40). Similar reductions also have been reported in cultured cells (41). Meclofenoxate may therefore offer a convenient, but hitherto unused, method to explore the relationship between lipofuscin and dolichol.

The following study is an attempt to measure the changes in concentrations of all three major forms of dolichol (free, acylated and phosphorylated) in the liver, kidney and heart of aging mice. Two of the potentially most important enzymes involved in dolichol metabolism, i.e., dolichyl phosphate phosphatase and the CTP-dependent dolichol kinase, were also studied as a function of age. The effect of lipofuscin dissolution by meclofenoxate on these parameters was noted.

MATERIALS AND METHODS

Materials. Dolichol was a gift from Kuraray Co. (Okayama, Japan), as was [1-^{14}C]dolichol (50 mCi/mL). Standard dolichol and dolichyl phosphate were purchased from Sigma Chemical Co., Ltd. (Poole, Dorset, U.K.). Meclofenoxate was a gift from Anphar Rolland Laboratories, France.

[^{3}H]Dolichol was prepared by the reduction of dolichal with sodium boro-[^{3}H]hydride (5–10 Ci/mmole, Amersham International Ltd., Buckingham, U.K.) (42,43). Labelled and unlabelled dolichyl phosphate were prepared

by the method of Danilov and Chojnacki (44), with the exception that the Sephadex LH-20 desalting step was replaced by washing the $CHCl_3$ extract with 3 × 2.0 mL volumes of water to effect the removal of ammonium acetate. All other reagents, unless otherwise stated, were of analytical grade.

To minimize sample loss due to interaction with glass surfaces, all glassware was first treated with a solution of dimethyldichlorosilane (Repelcoat; BDH Chemicals Ltd., Dorset, U.K.) in accordance with the manufacturer's instructions.

Animals and treatment. A colony of 220 mice (strain MF1) was established in the animal unit of the University Hospital Medical School, Nottingham. All mice were maintained on identical, commercially prepared mouse chow and reared under identical conditions. The colony was divided into six subcolonies (SC1–SC6), each consisting of 40 individuals (20 male and 20 female), with the exception of SC1 which consisted of 20 individuals (10 male and 10 female). All members of a subcolony were born within two days of each other and were considered to be of identical age.

Members of a single subcolony were weighed and sacrificed (cervical dislocation) at predetermined ages ranging from 6 to 29 months. For 12 weeks prior to sacrifice 50% of the subcolony received daily intraperitoneal (i.p.) injections of meclofenoxate (80 mg/Kg of body weight in a final volume of 0.1 mL physiological saline). The remaining half of the subcolony received identical daily injections of physiological saline.

On sacrifice, heart, liver and kidney were removed, trimmed of excess fat, rinsed in distilled water and blotted dry. Tissues were immediately stored in sealed plastic bags at −80°C until assayed. Assays were always performed within one month of sacrifice, usually within a few days. Preliminary experiments indicated that dolichol, dolichyl phosphate and the related enzymes were stable under these conditions for up to three months.

Tissue preparation. Frozen tissue was allowed to thaw at room temperature before being "rough chopped" with scissors and homogenized at 4°C in a 0.25 M sucrose-Tris buffer (pH 7.5). Protein was determined by the method of Lowry *et al.* (45).

Assay for dolichyl phosphate phosphatase activity. The activity of dolichyl phosphate phosphatase enzyme was determined by the conversion of [^{3}H]dolichyl phosphate to [^{3}H]dolichol (46,47).

The standard reaction mixture contained 150 μM [^{3}H]dolichyl phosphate (110,000 DPM), 200 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol (DTT) and 0.5% w/v Triton X-100 in a final volume of 400 μL. The reaction was started by the addition of 350 μg of homogenate protein. Tubes were incubated at 37°C for 30 min in a shaking water bath (40 strokes/min).

The reaction was terminated by the addition of 800 μL of $CHCl_3/CH_3OH$ (2:1, v/v) which also extracted the dolichol and dolichyl phosphate. After centrifugation at 2000 rpm for 20 min, the lower $CHCl_3$ layer was retained while the upper CH_3OH/H_2O was re-extracted by the addition of 2 × 400 μL of $CHCl_3$. $CHCl_3$ layers were combined and washed with 3 × 400 μL of CH_3OH/H_2O (1:1, v/v) before being taken to dryness under a stream of nitrogen.

Assay for dolichol kinase activity. Dolichol kinase activity in tissue homogenates was assessed by measuring the enzymatic conversion of [^{3}H]dolichol to [^{3}H]-dolichyl phosphate (48).

The standard incubation mixture contained 5 μM [^{3}H]dolichol (400,000 DPM), 200 mM Tris-HCl (pH 7.5), 60 mM $MgCl_2$, 15 mM CTP, 6.25 mM NaF, 0.2 mM *p*-chloromercuriphenyl sulphonic acid (*p*-CMBS) and 0.5% (w/v) Triton X-100 in a final volume of 200 μL. Incubation conditions and the extraction of labelled substrate were identical to the methodology described for the assay of dolichyl phosphate phosphatase.

Assay for radioactivity. [^{3}H]Dolichol and [^{3}H]dolichyl phosphate were separated by thin-layer chromatography (TLC) on SG 81 silica-impregnated paper (Whatmans Ltd., U.K.) that had been pre-treated according to the method of Steiner and Lester (49).

Dried $CHCl_3$ extracts from enzyme assays were redissolved in approximately 100 μL of $CHCl_3$ and spotted along a 15 mm line on a 200 mm × 200 mm TLC sheet. Standard dolichol and dolichyl phosphate (5 μg of each) were spotted alongside of the "test" lanes. TLC plates were developed in $CHCl_3/CH_3OH/H_2O$/acetic acid (75:20:2.5:2.5, by vol) the solvent front being allowed to run 120 mm from the origin. Authentic dolichol and dolichyl phosphate were visualized by exposure to iodine vapor. "Test" lanes were protected from iodine by a glass sheet. Bands corresponding to standard dolichol (Rf= 0.92) and dolichyl phosphate (Rf=0.40) were removed and cut into small strips. Radioactivity was assessed in a scintillation counter after the addition of 10.0 mL of Scintillator 199™ (Fisons Scientific Apparatus Ltd., Leichester, U.K.).

The activity of dolichyl phosphate phosphatase was expressed as μg product formed per mg protein per min and that of dolichol kinase as ng of product per mg protein per min.

Tissue extraction of dolichol and dolichyl phosphate. Crude tissue homogenates were extracted using a modification of the method described by Folch *et al.* (50).

For each 5.0 mL volume of homogenate (1.0 g tissue), 5.0 mL of CH_3OH was added and the mixture homogenized with 10 strokes of a motor driven Potter-Elvehjem homogenizer (medium speed setting). To allow correction of losses during extraction and isolation procedures, 1.5 ng of [1-^{14}C]dolichol and [1-^{14}C]dolichyl phosphate (110,000 DPM of each) were added. This was followed by $CHCl_3$ at the ratio of 5.0 mL per 5.0 mL of original tissue homogenate. The mixture was homogenized as previously described.

After an extraction period of 60 min at room temperature, centrifugation at 1500 rpm for 15 min gave three distinct layers. The lower $CHCl_3$ layer was removed and retained, while the upper CH_3OH/H_2O was washed with 2 × 5.0 mL volumes of $CHCl_3$. Finally the tissue pellet at the $CHCl_3/CH_3OH/H_2O$ interface was left to re-extract for 30 min in 6.0 mL of $CHCl_3/CH_3OH/H_2O$ (1:1:0.3, by vol). After centrifugation, the $CHCl_3$ layer was retained while the CH_3OH/H_2O layer was washed with 2 × 6.0 mL volumes of $CHCl_3/CH_3OH/H_2O$ (1:1:0.3, by vol).

Combined $CHCl_3$ layers from the extraction procedures were washed with 3 × 6.0-mL volumes of CH_3OH/H_2O (1:1, v/v) before being taken to dryness under a stream of nitrogen. Recovery of labelled standards at this

time was routinely in excess of 95% for dolichol and 92% for dolichyl phosphate.

Separation of dolichol and dolichyl phosphate. Dolichol and dolichyl phosphate were routinely separated by chromatography on 3.0 mL columns of DEAE cellulose (Sigma Chemical Company) equilibrated with $CHCl_3$/CH_3OH (2:1, v/v). Dry tissue extracts were re-dissolved in 0.5 mL of $CHCl_3$/CH_3OH (2:1, v/v) and applied to the column. A dolichol rich fraction was eluted with 10.0 mL of $CHCl_3$/CH_3OH (2:1, v/v), which contained 95% of labelled standard, while a dolichyl phosphate rich fraction was eluted with 20.0 mL of $CHCl_3$/CH_3OH (2:1, v/v) containing 0.25 M ammonium acetate. The recovery of dolichyl phosphate was routinely in excess of 92% (estimated by labelled standard), and the recovery of other dolichyl phosphate derivatives was assumed to be of the same order.

The dolichol-rich fraction was divided into two equal aliquots, one for the assessment of free dolichol and the other for assessment of total dolichol (free and ester forms). Both aliquots were taken to dryness under nitrogen.

The dolichyl phosphate-rich fraction from the ion exchange column was washed with 3 × 3.0 mL volumes of water to remove ammonium acetate and taken to dryness under nitrogen.

Saponification. The dry aliquot for total dolichol assessment was redissolved in 3.0 mL of cyclohexane. The saponification step involved a 45 min boiling period in a sealed tube in the presence of 1.0 mL of pyrogallol (1% in CH_3OH) and 2.0 mL of KOH (60%) in CH_3CH_2OH/H_2O (1:1, v/v). Dolichol was extracted from the mixture by the addition of 3.0 mL of freshly redistilled diethyl ether and 2.0 mL of water. After vortex mixing and centrifugation (1700 rpm for 5 min), the top ether layer was removed and retained. The remaining tube contents were re-extracted with a 3.0-mL volume of diethyl ether. Combined ether extracts were taken to dryness under nitrogen.

Sample purification. Dry aliquots for determination of total, free and phosphorylated dolichol were redissolved in 0.5 mL of propanol/methanol (20:80, v/v) and applied to Sep-pak RP C_{18} cartridges (Waters Associates, U.K.). Elution of the cartridges with propanol/methanol (20:80, v/v) (8.0 mL) removed polar contaminants from the sample while reduction in the polarity of the eluent to 80:20 (v/v) propanol/methanol (16.0 mL) permitted elution of a dolichol-rich fraction. For the elution of dolichyl phosphate, 0.1% ortho-phosphoric acid was added to the mobile phase.

Dolichol- and dolichyl phosphate-rich fractions (85% and 70% recovery, respectively, of labelled standard) from Sep-pak were taken to dryness under nitrogen and stored at −80°C for high performance liquid chromatography (HPLC) analysis.

High performance liquid chromatography. HPLC quantitation of dolichol and dolichyl phosphate was performed on an Ultrasphere ODS RP C_{18} column (5 μm; 4.6 mm × 250 mm) fitted with a precolumn of identical packing material (5 μm; 4.6 mm × 40 mm) (Beckman Ltd., U.K.). Column temperature was maintained at 24°C by a jacket and controller (Conair Churchill). Detection was at 210 nm on a Kratos SF 773 UV detector coupled to a SP 4270 integrator (both of Spectra Physics Ltd., Hertshire, U.K.). A flow rate of 1.0 mL/min was achieved using a single SP 8700 isocratic pump (also of Spectra Physics Ltd.).

Dry samples for HPLC were redissolved in 100 μL of HPLC mobile phase, and 10 μL was injected onto the column via a Reodyne 7125 injection system. The standard mobile phase for the elution of dolichol and dolichyl phosphate was propan-2-ol/methanol/hexane (40:40:20, by vol) (all HPLC grade) containing 0.1% ortho-phosphoric acid (19).

Dolichol/dolichyl phosphate, 17-21, were recovered after each HPLC run and assayed for radioactivity to assess the overall recovery of labelled standards (usually 60-80% for dolichol and 40-50% for dolichyl phosphate). The peak areas of sample dolichol/dolichyl phosphate (isoprenologs 18-20) were calculated by "valley to valley" integration and the concentration in the sample calculated from double log plots of standard dolichol (1-28 μg/10 μL) and dolichyl phosphate (0.05-12.35 μg/10 μL).

Dolichol and dolichyl phosphate concentrations were expressed as μg/g wet weight of tissue.

RESULTS

Figure 1 shows the body weight and liver weight of animals sacrificed at different ages. It demonstrates a steady rise in body weight until 20 months of age, followed by a small fall by 29 months (12% of maximum for males, 5% for females). Throughout this period males were heavier than females, as expected. Mean liver weights fluctuated only very slightly, showing a weak peak at 20 months. Treatment with meclofenoxate for 12 weeks prior to sacrifice caused no marked difference in total body weight or liver weight. Body weight of females older than 11 months was slightly lower than in controls. The weights of kidneys and hearts (data not shown) varied very little during this period.

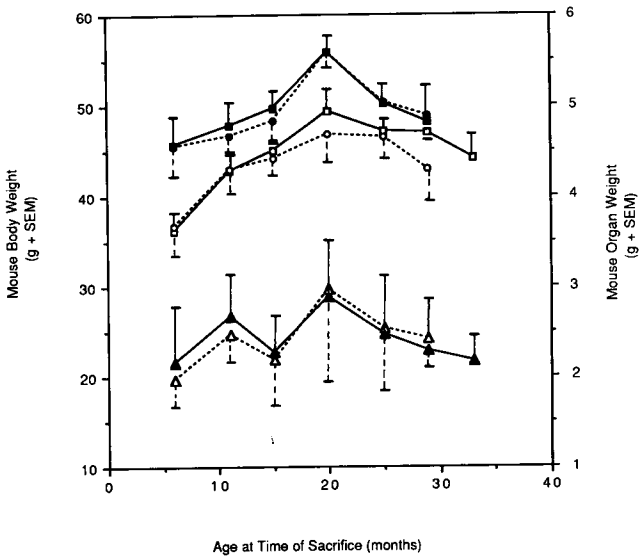


FIG. 1. Mean body weight and liver weight of male and female mice at the time of sacrifice. Body weight: —■—, control males; -●-, meclofenoxate males; —□—, control females; -○-, meclofenoxate females; liver weight: —▲—, control mice; and -△-, meclofenoxate mice.

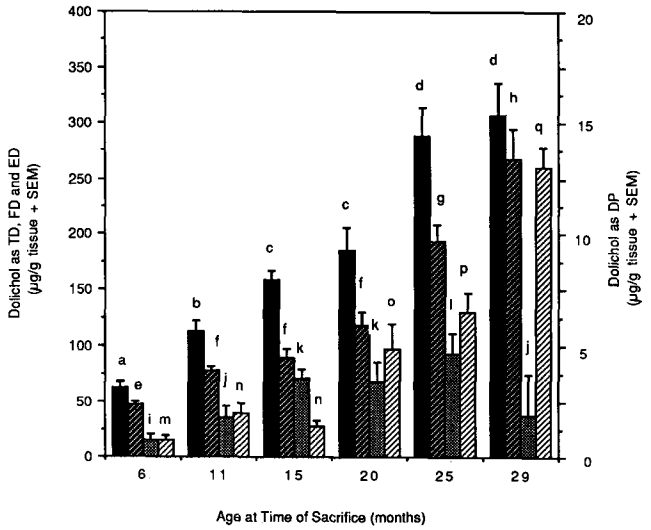
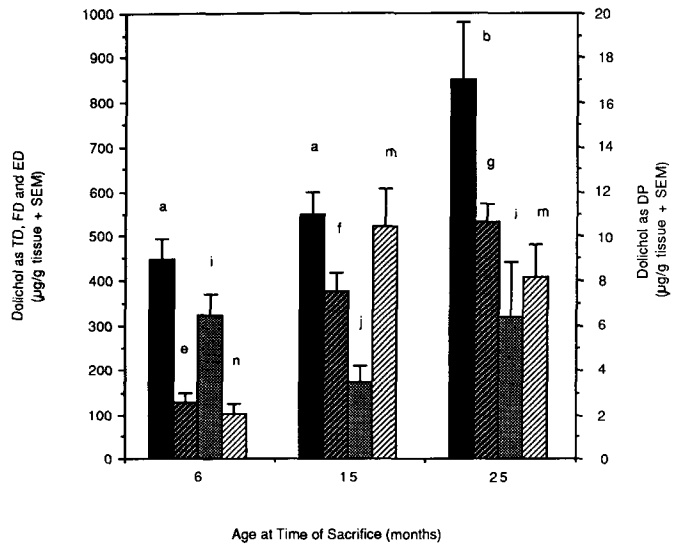


FIG. 2. Variation with age of the concentration of total dolichol (TD), of free (unesterified) dolichol (FD), of esterified (fatty acyl) dolichol (ED) and of dolichyl phosphate (DP) in liver of control mice. The standard error of the mean of three determinations on the liver of each of 3–8 mice at each time point is given. Statistically significant differences ($P < 0.05$) between the mean values are indicated by different letters, the series a to d, e to h, i to l and m to q relating respectively to total dolichol (solid bar), free dolichol (white diagonal lined bar), fatty acyl dolichol (grey bar) and dolichyl phosphate (black diagonal lined bar). Thus the same letter against two mean values indicates that differences between them are not statistically significant.



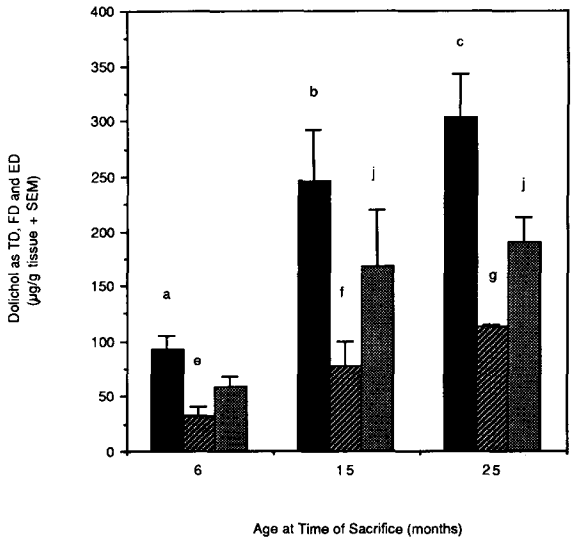


FIG. 3. Variation with age of the concentration of dolichol and its derivatives in kidney (a) and heart (b) of control mice. Details are as given in Figure 2.

At all ages, no statistically significant differences (Student *t*-test, $p=0.05$) between male and female mice with respect to the concentrations of free, esterified and phosphorylated dolichol and enzyme activity were observed. Meclofenoxate did not preferentially influence dolichol concentrations or enzyme activity of one sex (data not shown).

The concentration in liver of unesterified (free) dolichol and its fatty acid ester at regular time points during the life of mice up to 29 months is shown in Figure 2. A steady increase in total and free dolichol of five- to six-fold occurred during this period. A similar increase in the concentration of fatty acid ester of dolichol occurred up to the age of 25 months, after which a fall was apparent. Figures 3a and 3b demonstrate similar large increases in the free and total dolichol of kidney and heart over the 6 to 25 month period, with the proportion of ester varying. Kidney was the richest source of both free dolichol and its fatty acyl derivative at all time points studied. Generally, the proportion of the fatty acid ester was highest in heart and lowest in liver.

Changes in concentration of dolichyl phosphate in liver and kidney are summarized in Figures 2 and 3a, respectively. Heart tissue yielded insufficient dolichyl phosphate (less than 0.1 μg/g tissue) for a reliable assay. The results showed a 15-fold increase in hepatic dolichyl phosphate, most of this occurring between 15 and 29 months of age. In kidney, a five-fold increase was observed between 6 and 15 months of age and the concentration remained equally high over the next 10 months. Kidney was a richer source of dolichyl phosphate than was liver at 6, 15 and 25 months.

The activity of dolichyl phosphate phosphatase was assayed and the results are reported in Figure 4. Although the activity in liver fell slightly in early adult life, it then rose to reach the highest level at 29 months, being approximately double that observed at 6 months. In kidney at 6 months, the activity was about five times that in liver of the same age but it then changed very little over the next 19 months. A moderate increase in activity occurred in heart such that, although at six months it was just over half of that in liver, by 25 months both tissues showed very similar activities.

Dolichol kinase activity (Fig. 5) in kidney peaked at 15 months of age, and at this stage was much higher than in liver and heart. Differences between the tissues were less marked at 6 and 25 months.

The concentrations of dolichol and fatty acyl dolichol in livers of animals treated with meclofenoxate for 12 weeks prior to sacrifice are recorded in Figure 6.

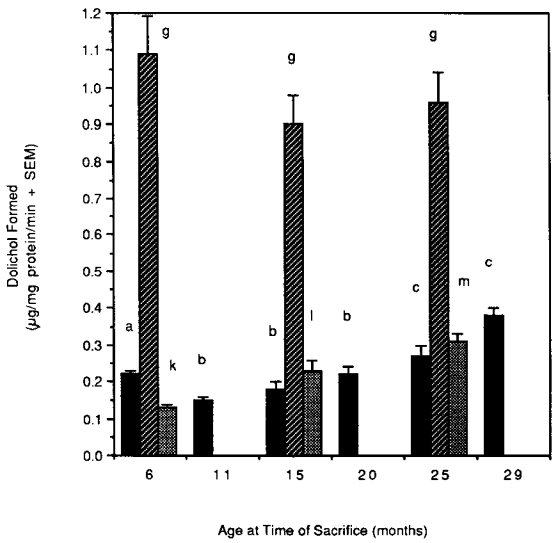


FIG. 4. Variation with age of the activity of dolichyl phosphate phosphatase in liver, kidney and heart of control mice. Statistically significant differences ($P < 0.05$) between the mean values are indicated by different letters, the series a to f for liver (solid bar), g to j for kidney (black diagonal lined bar) and k to m for heart (grey bar). Thus the same letter against two mean values indicates that the differences between them are not statistically significant.

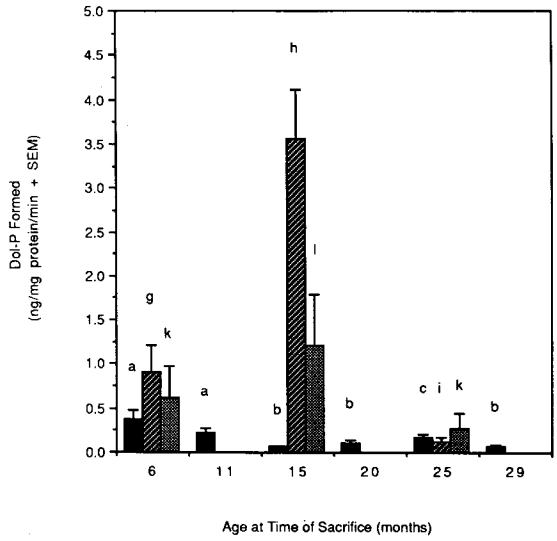


FIG. 5. Variation with age of the activity of dolichol kinase in liver, kidney and heart of control mice. Statistical data are as detailed in Figure 4.

Comparisons with Figure 2 reveal that in the livers of young adult mice (six-months-old), the treatment caused a marked increased (2.5-fold) in the concentration of both free and esterified dolichol. Over the next 14 months, the differences became less, having almost disappeared at 20 and 25 months of age. However, an increase seen at 29 months in untreated animals was prevented by treatment with meclofenoxate. The increase in the amount of fatty acyl dolichol was maintained until the 25th month, varying between 1.2 and 2.5 times the control figure, but then fell and became lower than the control figure.

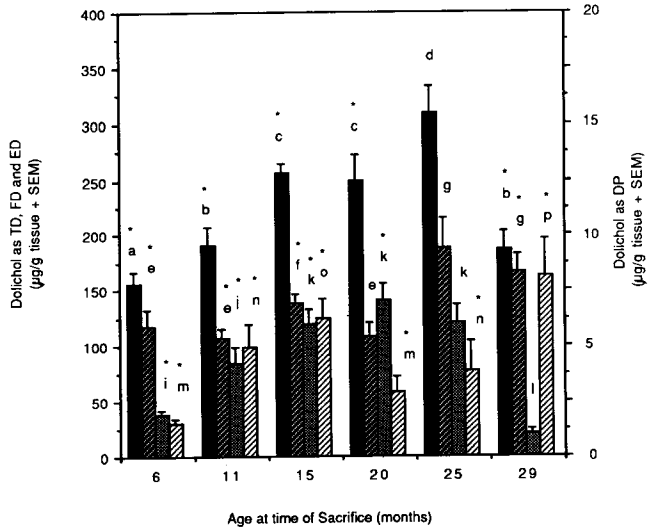


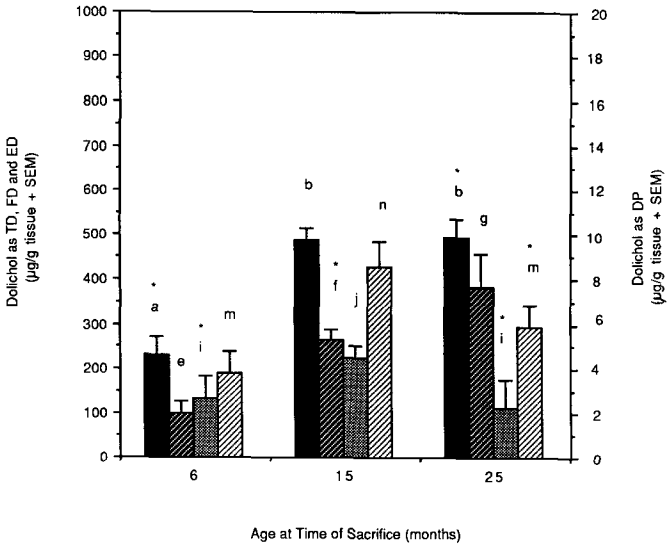
FIG. 6. Variation with age of the concentration of dolichol and its derivatives in liver of mice treated with meclofenoxate. Details are as given in Figure 2. The presence of an asterisk (*) denotes a significantly different value ($P < 0.05$) from controls (i.e., a comparison with Figure 2.)

In kidney (Fig. 7a), meclofenoxate caused a moderate fall in dolichol concentrations in older mice (compare with Fig. 3a), but its effect on fatty acyl dolichol was much more dramatic, causing a decrease to less than a half of that at both 6 and 25 months. In heart (Fig. 7b), the most significant effect of meclofenoxate treatment was at 15 months, when both free and esterified dolichol were markedly lower than in controls (Fig. 3b). Meclofenoxate caused a smaller response to aging of dolichyl phosphate levels in liver (Fig. 6) at 20 months and beyond, but an increase was not prevented. Similarly, in kidney (Fig. 7a), the increases were much less marked.

With regard to dolichyl phosphate phosphatase in liver, meclofenoxate stimulated an increase in activity at all ages, with that at 25 months being particularly marked (almost three-fold, see Fig. 8). In kidney and heart, differences caused by the treatment were small, becoming negligible in older mice. Although meclofenoxate reduced the size of the peak of kidney dolichol kinase activity at 15 months, by 25 months its effect was stimulatory. Figure 9 shows that the treatment caused a fall in kinase activity in heart, especially in younger mice, in contrast to liver, where after six months meclofenoxate consistently stimulated a small increase in dolichol kinase activity.

DISCUSSION

The finding that kidney is the richest source of dolichol in mice is in keeping with the observations of Pullarkat *et al.* (19) and is in contrast to rat and human tissues in which liver yields the most (51–54). Mouse kidney is also a richer source of lipofuscin than liver and heart (31,32,53,54). The actual concentration of total dolichol in heart and liver of six-month-old mice was very close (within 2%) to that reported by Pullarkat *et al.* (19) for a different strain of mice whereas in kidney the yield was 33% higher. Comparisons of older mice suggest that



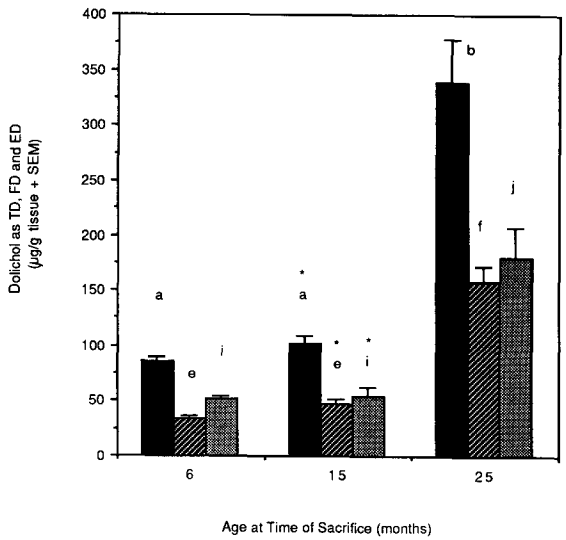


FIG. 7. Variation with age of the activity of dolichyl phosphate phosphatiase in liver, kidney and heart of mice treated with meclofenoxate. Statistical data are as detailed in Figure 4. The presence of an asterisk (*) denotes a significantly different value (P <0.05) from controls (i.e., a comparison with Figure 4.)

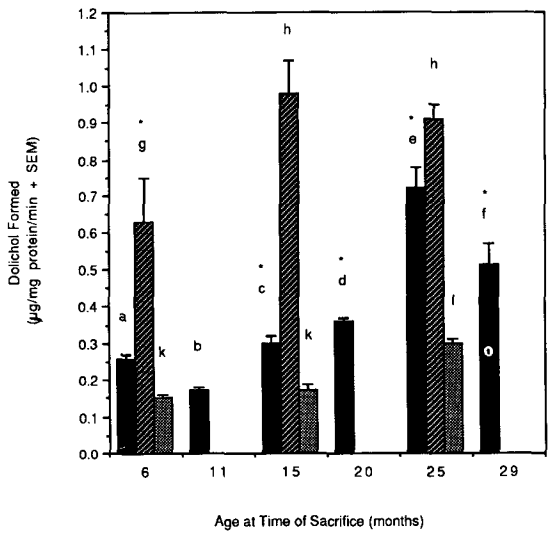


FIG. 8. Variation with age of the activity of dolichyl phosphate phosphatase in liver, kidney and heart of mice treated with meclofenoxate. Statistical data are as detailed in Figure 4. The presence of an asterisk (*) denotes a significantly different avlue (P <0.05) from controls (i.e., a comparison with Figure 4.)

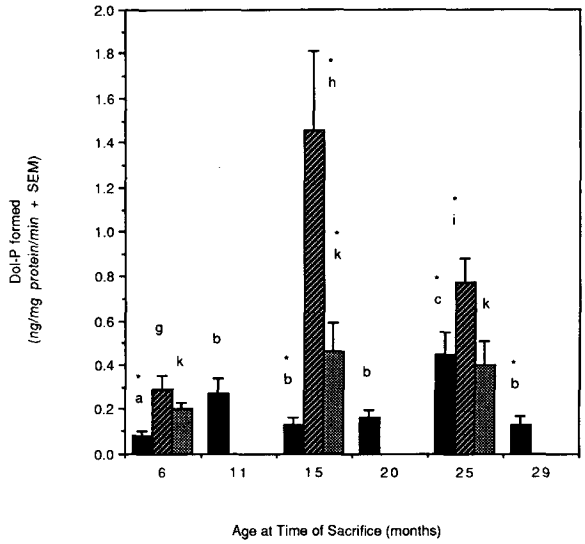


FIG. 9. Variation with age of the activity of dolichol kinase in liver, kidney and heart of mice treated with meclofenoxate. Statistical data are as detailed in Figure 4. The presence of an asterisk (*) denotes a significantly different value (P<0.05) from controls (i.e., a comparison with Fig. 5.)

Pullarkat's strain, while showing significant increases in concentration of dolichol with age, responded less markedly, especially in kidney and liver, than did the Nottingham strain. A more recent report by Crick and Rip (55) support our observations of increasing dolichol concentrations in murine tissues with age. However, the authors also report a decrease in the concentration of liver dolichyl phosphate in mice aged between 1 and 24 months. The reason for this difference between the two studies is not clear, but it may be due, at least in part, to the different methodology employed and/or biological variation between the strains of mice studied.

There are no published results against which to compare directly the assays of fatty acyl dolichol reported for mice in Figures 2, 3a and 3b. The proportion of ester varies markedly from species to species for any single tissue and from tissue to tissue in any single species (54,56-60). Figure 2 shows that in liver the fraction of dolichol that is esterified remains fairly constant at about 65% of the total dolichol over the 25 months studied. Kidney started with an equally high level at six months, but then remained at a concentration lower than the liver over the next 19 months.

Sakakihara and Volpe (16) observed that in rat brain the fraction of dolichol that was esterified remained fairly constant during development and aging. This relationship is similar to that of cholesterol and its fatty acid esters, which led the authors to suggest that dolichyl ester may be a convenient storage form of dolichol. It has been

reported (57) that in rat liver the concentration of free dolichol remains fairly constant, but that of ester increases during early stages of growth (weanling to 5 months of age). All such observations suggest that at all stages of development and aging, most tissues contain a substantial fraction of their dolichol in the fatty acyl form.

The proportion of dolichol recovered as phosphate was routinly less than 4% in all three murine tissues studied. Most authors using similar extraction procedures report a low figure, e.g., in rat liver 2%, 4% and 10% (51,61) and in human liver 4% (53). It has been argued by Keller *et al.* (62) that these figures are low due to inadequate extraction procedures. By direct saponification of tissue followed by ether extraction, Keller's group reports higher concentrations of dolichyl phosphate in rat tissues. Indeed, they suggest that in several tissues the concentration exceeds that of non-phosphorylated dolichol. Preliminary attempts by the authors to repeat the extraction procedure of Keller *et al.* (62) on murine tissues failed to detect higher concentrations of dolichyl phosphate than are shown in Figures 2 and 3a.

The concentration of dolichyl phosphate in rat tissues during the first few months of life is reported to remain fairly constant (15,20). Keller's group also reports (20) that in two normal English setters, aged 13 and 45 months, the concentration of dolichyl phosphate was essentially the same. On the other hand, the results reported in Figures 2 and 3a, which represent the first systematic investigation of dolichyl phosphate concentrations in a group of aging animals demonstrate very clearly marked increases in both liver and kidney as aging proceeds.

The enzyme studies (Figs. 4 and 5) show a much higher dolichyl phosphate phosphatase than dolichol kinase activity at all ages and in all three tissues studied. The range of values in murine liver 0.2–0.38 μg product/mg protein/min) is intermediate between values reported for rat liver by Boscoboinik *et al.* (63) and Rip *et al.* (47), i.e., 0.16 and 1.70 μg product/mg protein/min, respectively. In heart, a moderate increase in phosphatase activity between 6 and 25 months echoes the increased dolichol content observed over the same period. However, the 1.7-fold increase in hepatic phosphatase between 6 and 29 months, although substantial, is considerably less than the change in concentration of dolichol over the same period. Similarly, in kidney the increase with age in the concentration of dolichol is not matched by a corresponding increase in phosphatase activity. The liver and kidney results are consistent with the view expressed by Ekström *et al.* (64,65) that the formation of dolichol may not be solely by dephosphorylation of dolichyl phosphate. However, further information on the rate of degradation of dolichol and on its rate of *de novo* synthesis in these tissues is essential if this view is to be established further.

Comparison of Figures 2 and 5 shows little or no correlation of changes in the activity of hepatic dolichyl kinase with changes in the concentration of its product dolichyl phosphate or its substrate dolichol. This may be partly the result of the location of at least some of the putative substrate and product in separate subcellular pools that are not available to the enzyme. It may also suggest that the concentrations of dolichol and dolichyl phosphate are also strongly influenced by the rate of their *de novo* synthesis and/or catabolism. Whatever the reasons, it is clear that the results in this paper provide no support for the view that the activities of dolichol kinase and dolichyl phosphate phosphatase dictate the tissue concentrations of dolichol and dolichyl phosphate in mouse liver.

In kidney, the kinase is much more active than in liver, in keeping with the higher concentration of dolichyl phosphate in the former tissue. Also, the large increase in activity at 15 months as compared with that at 6 months correlates well with a large peak in the concentration of dolichyl phosphate at the later age. However, the big drop in enzyme activity at 25 months of age is accompanied by a relatively small fall in the concentration of dolichyl phosphate. Thus, although the evidence from kidney is more consistent with the kinase having a critical role in the control of dolichyl phosphate levels, it is clearly not the whole story.

There was no correlation between murine heart dolichol content and the activity of the kinase in this tissue. This, coupled with the failure to detect dolichyl phosphate, leaves the importance of the role of this enzyme in this tissue obscure.

The effect of meclofenoxate treatment on liver dolichol was biphasic. In mice before 20 months of age, it resulted in elevated levels with respect to controls, but in older mice (29 months of age) it brought levels of dolichol and dolichyl ester below those of controls. This latter phase was seen in kidney at all times studied, as well as in heart at 15 months of age, and is consistent with an association of dolichol and its ester with lipofuscin. The meclofenoxate treatment adopted is reported to be sufficient to cause dissolution of most of the lipofuscin present (38,39). However, appreciable quantities of dolichol still accumulate in the presence of meclofenoxate. This suggests that not all of the accumulated dolichol in these tissues is associated with lipofuscin.

The response of dolichyl phosphate concentrations in liver and kidney to meclofenoxate treatment was similar in showing an increase (over controls) in younger mice but attenuating the age-related increase observed in older control mice. Whether or not this fall in very old mice results from (or in) the corresponding latter fall in dolichol, or if it has any important consequences for the rate and extent of protein N-glycosylation is uncertain and requires further investigation.

Neither dolichyl phosphate phosphatase nor dolichol kinase showed a consistent response to meclofenoxate treatment. Correlations of these responses with changes in dolichol and dolichyl phosphate concentrations were poor.

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