Inter-relationships between Dolichol and Sterol Synthesis in Mammalian Cell Cultures*

Michael J. James and Andrew A. Kandutsch

From The Jackson Laboratory, Bar Harbor, Maine 04609

A method is described for the measurement of [¹⁴C]-acetate incorporation into dolichol in cultured mammalian cells. In various cell types, [¹⁴C]-acetate was incorporated into dolichol at a rate 0.03 to 0.11% of the incorporation rate into sterol. When 25-hydroxycholesterol was added to L cell cultures, [¹⁴C]-acetate incorporation into both dolichol and sterol decreased in a concentration-dependent manner, although the relationship between the concentration of 25-hydroxycholesterol, and the level of inhibition differed for the two lipids. Comparison of the rates of sterol and dolichol synthesis from acetate indicated that situations may exist in which large fluctuations in sterol synthesis can occur while the rate of dolichol synthesis is only slightly affected. However, under other conditions where sterol synthesis was repressed to levels below approximately 25% of the control level, further decrease in sterol synthesis was accompanied by a proportional decline in dolichol synthesis. The results suggest that 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34), affects the rate of dolichol synthesis by altering the concentration of a substrate for an enzyme which catalyzes a rate-limiting reaction peculiar to the dolichol branch of the biosynthetic pathway. This conclusion was supported by other studies in which L cells were grown in serum-supplemented medium and 25-hydroxycholesterol was added to MOPC 104E cultures. The data indicate an interaction by which two or more biosynthetic pathways can share common intermediates and a regulatory enzyme while maintaining a large degree of regulatory independence.

Dolichols are a series of long chain polyisoprenoid alcohols found in many mammals and yeasts (1). In these organisms, the phosphate and pyrophosphate esters of dolichol function as glycosylated intermediates in the transfer of sugars to polypeptides (1, 2). Although chain length varies with their biological source, the dolichols generally consist of 16 to 22 isoprene units comprising a saturated α-residue, 2 internal trans-isoprene residues, and 13 to 19 internal cis-isoprene residues (1). Incorporation of radiolabeled mevalonate into dolichol has been demonstrated in rabbit and pig liver (3), and in normal (4), and regenerating (5), rat liver. Furthermore, incorporation of mevalonate into dolichylpyrophosphoryl oligosaccharides and dolichol has been demonstrated in rabbit and pig liver (3), and in aortic smooth muscle cells (7).

Many enzymes are common to the pathways of cholesterol and dolichol biosynthesis. In particular HMG-CoA reductase, which is a major regulatory enzyme for cholesterol biosynthesis (8), can also function in the biosynthesis of isoprene units utilized for dolichol synthesis. Although the incorporation of mevalonate into dolichol has been demonstrated many times, the influence on dolichol synthesis of regulatory changes in cholesterol synthesis via HMG-CoA reductase has been little investigated. Mills and Adamany (7) demonstrated that 25-hydroxycholesterol, which suppresses HMG-CoA reductase in cultured cells (9), inhibited the incorporation of acetate into dolichylpyrophosphoryl oligosaccharides and cholesterol by similar amounts, and suggested that HMG-CoA reductase is a rate-limiting enzyme for the biosynthesis of dolichol as well as sterol.

In this report, we describe the measurement of [¹⁴C]-acetate incorporation into dolichol and the inter-relationhip found to exist between the pathways of sterol and dolichol biosynthesis. The results suggest that HMG-CoA reductase affects the rate of dolichol synthesis by influencing the pool size of one or more sterol pathway intermediates that are also precursors of dolichol and that large fluctuations in HMG-CoA reductase activity and in the rate of cholesterol synthesis can occur simultaneously with relatively minor changes in the rate of dolichol synthesis.

EXPERIMENTAL PROCEDURES

Materials—The following compounds were obtained from the companies indicated: dolichol, dolichyl acetate, dolichyl phosphate, bovine serum albumin (essentially fatty acid free), Sigma Chemical Co., St. Louis, Mo.; [¹⁴C]-acetate acid, sodium salt (57 mCi/mmol), [12-³H]cholesterol, [¹H]dolichol, New England Nuclear Corp., Boston, Mass. Burdock and Jackson glass-distilled solvents (Muskegon, Mich.) were used for high pressure liquid chromatography.

Cell Culture—A subline of NCTC clone 929 mouse L cells, were grown in suspension culture in Waymouth's MB 752/1 of chemically defined medium (10) supplemented with trace metals (CuSO₄.5H₂O, 5 µg/ml; ZnSO₄.7H₂O, 15 µg/ml; MnSO₄.7H₂O, 1.6 µg/ml; CoCl₂·6H₂O, 10 µg/ml; Fe(NH₄)₂(SO₄)·12H₂O, 100 µg/ml), folic acid (0.1 µg/ml), gentamicin (50 µg/ml), L-leucine (120 µg/ml), L-lysine (120 µg/ml), and L-serine (64 µg/ml). L cells, which produce desmosterol rather than cholesterol (11), were seeded into 75-cm² tissue culture flasks to which they attached and were used 24 h later. Chinese hamster lung (Dede) and ovary (CHO) cells were grown as monolayers in modified McCoY's 5a medium supplemented with 4 mg/ml of delipidated fetal bovine serum proteins as previously described (12). Murine plasmacytoma (MOPC 104E) cells were grown in Dulbecco's modified Eagle's medium (Gibco, Catalogue No. H-21) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), nonessential amino acids (Gibco, 100 µM), and glutamine (Gibco, 6 mM). All cells were grown at 37°C. Dede, CHO, and MOPC 104E cells were grown in an atmosphere of 5% CO₂, 95% air.

Incubations—Since the sterol requirements of L cells incubated with 25-hydroxycholesterol can be met by exogenously supplied cholesterol (13), 25-hydroxycholesterol and cholesterol were added si-

* This research was supported by National Institutes of Health Grant CA 02758. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
multaneously to cell cultures. Recrystallized cholesterol and vitamin E (to prevent autoxidation) were dissolved in ethanol and added to a sterile solution of 5% bovine serum albumin in L cell medium. The mixture was sonicated briefly. 25-Hydroxycholesterol dissolved in ethanol was then added to give the final concentration indicated in Fig. 3. This mixture (0.5 ml) was added to the culture medium (9.5 ml). Final concentrations were: cholesterol, 25 μg/ml; vitamin E, 0.5 μg/ml; ethanol, 0.5%; bovine serum albumin, 0.25%. The cultures were incubated with sterols and other supplements for 8 h and then [1,14C]acetate in 100 μl of ethanol was added (final concentration, 20 μCi, 350 nmol/ml) and the cells were harvested 24 h later. Protein was determined by a modification of the Lowry procedure (14).

Preparation of Nonsaponifiable Lipids—Cells were harvested by scraping, washed twice with 0.14 M NaCl, and centrifuged at 120 × g. Lipids were saponified by a modification of methods reported by Burgos et al. (15) and Keller and Adair (16). Butylated hydroxytoluene (1 mg), [1-'H]dolichol (1 × 10^6 dpm), and [1,2-'H]cholesterol (6 × 10^6 dpm), were added followed by 1 ml of ethanolic KOH (final concentration KOH, 20%; ethanol, 66%) containing 40 mg of pyrogalol. After 3 h at 80°C, 0.6 ml of water was added and nonsaponifiable lipids were extracted (twice with 10-ml volumes of freshly distilled diethyl ether containing 40 μg of vitamin E. The ether was washed twice with 0.14 M NaCl, once with 5% sodium carbonate (2 ml), and again with 3% sodium carbonate (2 ml). It was then dried with anhydrous sodium sulfate and filtered through a cellose Millipore filter (0.5 μm pore size). The solvent was evaporated under nitrogen and the residue was dissolved in 50 μl of methylene chloride for high pressure liquid chromatography.

High Pressure Liquid Chromatography—High pressure liquid chromatography was performed using Waters Associates’ equipment, specifically two model 6000 A solvent delivery systems, U6K injector system, model 660 solvent programmer, and a Bondapak C18 reverse-phase column (4 mm × 30 cm). Two solvent systems were used: HPLC system A, 1% aqueous methanol for 32 min and then a linear gradient over 25 min from 1% aqueous methanol to 35% methylene chloride; HPLC system B, concave gradient No. 10 from 100% methanol to 100% methylene chloride over 45 min. The flow rate was always 2 ml/min and 1-ml fractions were collected, the solvent was evaporated and 5 ml of toluene-based scintillation solvent was added for determination of 14C and H activity in a Beckman LS800 liquid scintillation spectrometer. Overlap of H into the 14C counting channel was less than 0.05%.

Acetylation of Dolichol and Thin Layer Chromatography of the Acetates—The dolichol-containing fractions from HPLC system B were pooled (fractions 70 to 91), 50 μg of carrier dolichol was added, and the solvents were evaporated under nitrogen. Acetylation conditions were modified from those reported by Keller and Adair (16). Toluene (0.1 ml) containing acetic anhydride (20 μmol) was added, the mixture was placed in a reaction vial with a Teflon seal and maintained at 80°C overnight. The solvents were evaporated, 50 μl of hexane was added, and the acetylated dolichol was run on precoated silica gel plastic sheets (E. Merck) with toluene as the developing solvent. To determine 14C and D in the dolichyl acetate-containing area (RF 0.5), the band was cut from the sheet, placed in a miniscintillation vial, 0.25 ml of ethanol and 5 ml of toluene-based scintillation solution were added and radioactivity was determined.

Determination of [1,14C]Acetate Incorporation into Sterols—The sterol-containing fractions from HPLC system B were pooled (fractions 6 to 13) and the solvent was evaporated with nitrogen. Cholesterol or desmosterol (L cells) was analyzed as digi-tinin-precipitable sterols as previously described (17).

RESULTS

Separation of [14C]Dolichol—[14C]Dolichol synthesized from [1-14C]acetate by cell cultures could be largely separated from other labeled compounds by fractionating the nonsaponifiable lipids as previously described (18—20). The extract was applied to several chromatographic systems as described in the text: a, HPLC system A; b, HPLC system B; c, thin layer chromatography (toluene) after acetylation of the pooled dolichol-containing fractions (29-91) from HPLC system B; d, HPLC system A. The eluted dolichyl acetate-containing area shown in c. The H/14C ratios of the dolichyl-containing fractions are shown for each treatment. The fraction numbers used for these calculations are indicated in parentheses and base-line corrections were applied in a and d. The biosynthetically labeled lipids (——) and [1-'H]dolichol or its acetate derivative (-----) were determined as described in the text. The solvent gradients (-----) are also shown.

Fig. 1. Chromatographic separations of [14C]dolichol from L cell 14C-labeled nonsaponifiable lipids. A 150-cm 2 flask was seeded with approximately 1.5 × 10^6 cells and [14C]acetate (1 μCi) in 20 ml of medium. Fresh medium (10 ml) was added after 2 days and cells were harvested after 3 days. Nonsaponifiable lipids were extracted as described in the text except that the amount of [1-'H]dolichol added was 10 × 10^5 dpm. The extract was applied to several chromatographic systems as described in the text: a, HPLC system A; b, HPLC system B; c, thin layer chromatography (toluene) after acetylation of the pooled dolichol-containing fractions (29-91) from HPLC system B; d, HPLC system A.
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If saponifiable lipids with high pressure liquid chromatography. When L cells were used, the chromatographic conditions necessary to exclude extraneous \(^{14}\)C-labeled material from the dolichol-containing fractions (HPLC system A) caused dolichol itself to be fractionated into the individual chain length components (Fig. 1a). The coincidence of the biosynthetically \(^{14}\)C-labeled dolichol peaks with the internal standard \([^{3}\text{H}]\text{dolichol}\) peaks indicates a fair degree of purity.

The ratio of \([^{3}\text{H}]\text{dolichol}\) to \([^{14}\text{C}]\text{dolichol}\) estimated to be in the combined peaks after subtracting base-line levels of the isotopes was 47. The \(^{14}\text{H}/^{14}\text{C}\) ratio of the single dolichol band separated with HPLC system B (Fig. 1b, fractions 79 to 91) was lower than that obtained with HPLC system A. However, after acetylation followed by thin layer chromatography using toluene as the developing solvent (Fig. 1c), the area containing dolichyl acetate (RF 0.5) had a \(^{3}\text{H}/^{14}\text{C}\) ratio of 48. Rechromatography of the recovered dolichyl acetate on silica gel films with toluene/ethyl acetate (85:15) as the solvent system (RF 0.72) or with cyclohexane/toluene (1:1) (RF 0.22), did not alter the ratio of \(^{3}\text{H}\) to \(^{14}\text{C}\).

Dolichyl acetate, recovered after thin layer chromatography with toluene as the solvent, was rechromatographed using HPLC system A in which the individual chain length dolichyl acetates were also resolved (Fig. 1d). Although the amount of \([^{14}\text{C}]\text{dolichol}\) remaining after the previous chromatographic treatments is now small by comparison with the \(^{14}\text{C}\) background of the HPLC column, the \(^{14}\text{H}/^{14}\text{C}\) ratio of the total dolichyl acetate was again unchanged when base-line corrections were applied (Fig. 1d). The appreciable background levels in Fig. 1d apparently result from low level contamination of the instrument and column by previous injections of highly labeled lipids. The background level could be reduced but not eliminated by extensive washing of the column with various solvents.

Since the \([^{3}\text{H}]\text{dolichol}\) used in this study was prepared from pig liver dolichol, the predominant isoprenologue was dolichol-19 (18). Data from several sources (not shown) in addition to those in Fig. 1a indicate the dolichol-19/18 ratio for \([^{3}\text{H}]\text{dolichol}\) is 1.9. In L cells however, more \([^{14}\text{C}]\text{acetate}\) was incorporated into dolichol-18 than into dolichol-19 and the dolichol-19/18 ratio is 0.9. In rat liver, dolichol-18 is also present in slightly larger amounts than dolichol-19 (4).

In at least one other cell type, MOPC 104E, relatively large amounts of contaminating \(^{14}\text{C}\)-labeled material were present in the dolichol-containing fractions separated by HPLC system A. Furthermore, even when L cells were used, the large amount of \(^{14}\text{C}\)-labeled material loaded onto the HPLC column resulted in a relatively high background of \(^{14}\text{C}\) radioactivity (Fig. 1a). Therefore, the procedure used routinely to assay \([^{14}\text{C}]\text{dolichol}\) involved preliminary HPLC (system B), acetylation of the recovered dolichyl acetate, followed by thin layer chromatography of the acetates and assay of the dolichyl acetate band as illustrated in Fig. 2. Values for \([^{14}\text{C}]\text{sterols}\) and \([^{14}\text{C}]\text{acetate}\) were corrected for recoveries which averaged 46% and 36%, respectively, based upon assays of the respective \(^{14}\text{C}\)-labeled standards. The standard errors of the mean values for triplicate determinations of rates of dolichol and cholesterol synthesis in a pool of L cells from culture flasks (3 x 150 cm\(^2\)) incubated with \([^{14}\text{C}]\text{acetate}\) for 24 h were 3.3% and 2.2%, respectively.

Preliminary experiments indicated that \([^{14}\text{C}]\text{acetate}\) incorporation into sterol increased linearly with time for at least 24 h in L cells and this was the usual incubation period used in this study.

Relative Incorporation of \([^{14}\text{C}]\text{Acetate}\) into Dolichol and Sterol in Various Cell Types—Among several cell types investigated, L cells incorporated \([^{14}\text{C}]\text{acetate}\) into dolichol at the highest rate (Table I). L cells also had a high rate of sterol synthesis by comparison with the other cell types in Table I. However, in all cell types investigated, the rate of incorporation of \([^{14}\text{C}]\text{acetate}\) into dolichol was extremely low compared with the incorporation rate into cholesterol (Table I). While differences in \([^{14}\text{C}]\text{Acetate}\) incorporation into sterol and dolichol may reflect basic differences between cell types, culture conditions may also influence the rates. For example, L cells were cultured in chemically defined medium, whereas Chinese hamster lung and ovary cell lines were cultured in the presence of delipidated fetal bovine serum, and the plasmacytoma line was cultured in the presence of whole horse serum. As shown in Table II, the addition of delipidated fetal bovine serum to L cell culture medium increased the rate of \([^{14}\text{C}]\text{Acetate}\) incorporation into desmosterol by 72% and slightly decreased (17%) its incorporation into dolichol. By contrast, supplementing L cell medium with 15% whole fetal bovine serum resulted in a decrease in \([^{14}\text{C}]\text{Acetate}\) incorporation into desmosterol (42%) and into dolichol (24%), (Table II).

Effect of 25-Hydroxycholesterol—25-Hydroxycholesterol inhibited \([^{14}\text{C}]\text{Acetate}\) incorporation into both sterol and dolichol in a dose-dependent manner in L cells (Fig. 3). However, the relationships between the levels of inhibition and inhibitor

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**Table I**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Sterol</th>
<th>Dolichol</th>
<th>Cells/flask</th>
<th>Dolichol/sterol</th>
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<tr>
<td>Dede</td>
<td>170,900</td>
<td>189</td>
<td>9.2 x 10^6</td>
<td>0.11</td>
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<tr>
<td>CHO</td>
<td>75,857</td>
<td>41</td>
<td>4 x 10^6</td>
<td>0.05</td>
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<tr>
<td>MOPC 104E</td>
<td>756,670</td>
<td>187</td>
<td>7.9 x 10^6</td>
<td>0.03</td>
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<tr>
<td>L cells</td>
<td>1,421,100</td>
<td>655</td>
<td>0.6 x 10^6</td>
<td>0.05</td>
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</tbody>
</table>

**Table II**

<table>
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<th>Supplement</th>
<th>Protein*</th>
<th>Dolichol</th>
<th>Desmosterol</th>
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<tbody>
<tr>
<td>None</td>
<td>4.5</td>
<td>328</td>
<td>928,417</td>
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<tr>
<td>Delipidated FBS</td>
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<td>273</td>
<td>1,598,049</td>
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<tr>
<td>10% FBS</td>
<td>4.9</td>
<td>243</td>
<td>723,238</td>
</tr>
<tr>
<td>15% FBS</td>
<td>4.8</td>
<td>248</td>
<td>536,326</td>
</tr>
</tbody>
</table>

*Cells were harvested by scraping, washed 3 times with 0.14 M NaCl (10 ml), and an aliquot taken for protein determination.

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These results indicate the existence in L cells of a well-defined and interesting inter-relationship between dolichol and sterol synthesis. HMG-CoA reductase which is a major regulatory enzyme for cholesterol synthesis is also in the pathway of dolichol synthesis. Current evidence suggests that the branch point for dolichol synthesis occurs at the level of farnesyl pyrophosphate and involves isopentenyl pyrophosphate as another substrate. Gough and Hemming (4) used double-labeled stereoisomers of mevalonic acid to demonstrate that rat liver dolichols possess three biogenetically trans-isoprene units, the remainder being biogenetically cis-residues. They suggested that these polyisoprenes are biosynthesized from all-trans-farnesyl pyrophosphate by the cis additions of isopentenyl pyrophosphate (4). The incorporation of [14C]-labeled isopentenyl pyrophosphate into dolichyl phosphate has since been demonstrated to occur in subcellular fractions of rabbit and chicken livers in vitro (19). Incorporation of [14C]-labeled isopentenyl pyrophosphate into a compound with chromatographic and chemical properties compatible with 2,3-dehydrodolichyl phosphate has been demonstrated in a particulate preparation from hen oviduct and in this system, exogenous farnesyl pyrophosphate was necessary for maximal incorporation of [14C]-labeled isopentenyl pyrophosphate (20). The first reaction unique to dolichol synthesis may therefore be the cis addition of isopentenyl pyrophosphate to farnesyl phosphate as suggested by Gough and Hemming (4).

25-Hydroxycholesterol specifically suppresses HMG-CoA reductase activity in cell cultures and the decline in enzyme activity is directly paralleled by a decline in sterol synthesis (9). The present data indicate that 25-hydroxycholesterol also inhibited the incorporation of [14C]acetate into dolichol. However, the rate of dolichol synthesis was less affected than was the rate of sterol synthesis at low concentrations of the inhibitor. This distinction between the sensitivities of the dolichol and sterol biosynthetic pathways to the dose of 25-hydroxycholesterol is explained if a diminution in the level of HMG-CoA reductase activity reduces the concentration of a substrate for an enzyme which catalyzes the rate-limiting reaction in the dolichol branch of the biosynthetic pathway. In support of this scheme, a plot of the rate of dolichol synthesis against increasing rates of sterol synthesis gave a curve characteristic of first order saturation kinetics with the rate of dolichol synthesis approaching a maximum as the rate of sterol synthesis increased to levels higher than 25% of the uninhibited value (Fig. 4). Assuming that the rate of cholesterol synthesis is representative of the concentration of the intermediate pool in question, the relationship shown to exist between these two pathways by this curve is consistent with a model whereby 25-hydroxycholesterol acts to limit the concentration of an intermediate required for dolichol synthesis. This explanation implies that the rate-limiting enzyme in the dolichol pathway is saturated by a lower level of intermediates (possibly farnesyl pyrophosphate and/or isopentenyl pyrophosphate) than is required to saturate the next rate-limiting enzyme past the branch-point in the sterol synthesis pathway.

Some support of this interpretation of the data comes from studies by Gold and Olson (21) of the analogous pathway for formation of the polyisoprenyl side chain of coenzyme Q. These investigators found that the incorporation of various concentrations of [2-14C]mevalonate into coenzyme Q by rat liver slices indicated an apparent $K_m$ of 1 mM for cholesterol biosynthesis and 0.2 mM for coenzyme Q biosynthesis. Therefore,
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they suggested that "one enzyme in the pathway for CoQ formation is more easily saturated with substrate (or intermediate) than are the enzymes in the pathway for cholesterol biosynthesis."

An alternative model to explain the differential effects of 25-hydroxycholesterol on dolichol and sterol synthesis involves the existence of essentially separate biosynthetic pathways, one being more resistant to the diol than the other. In this regard, Momose and Rudney (22) have reported evidence that the inner membrane of rat liver mitochondria contains a system for synthesizing polyisoprenylphosphate derivatives from isopentenyl pyrophosphate. However, there is no evidence for the existence of a separate HMG-CoA reductase in mammalian mitochondria which might have a sensitivity to 25-hydroxycholesterol different from that of the microsomal reductase.

On the basis of results which showed that 25-hydroxycholesterol inhibited the incorporation of acetate into the dolichol moiety of dolichylpyrophosphoryl oligosaccharides by 91% and into cholesterol by 82% in bovine aortic smooth muscle cells, Mills and Adamany suggested that HMG-CoA reductase may function as a rate-controlling enzyme in the biosynthesis of dolichols as well as sterols (7). However, their results derive from studies with cells cultured in medium containing 10% fetal bovine serum whereas the incorporation rate into sterol was increased only 10% (Table II). Furthermore, when 25-hydroxycholesterol was added to MOPC 104E cells grown in medium supplemented with delipidated horse serum, cholesterol and dolichol synthesis would be of physiological value since their end products have quite dissimilar functions. It is possible that agents which specifically regulate dolichol synthesis will be found. However, the results of the present study indicate a system by which a branching biosynthetic pathway can share common intermediates and a common regulatory enzyme while maintaining a large degree of regulatory independence.

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Inter-relationships between dolichol and sterol synthesis in mammalian cell cultures.
M J James and A A Kandutsch


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