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# Uptake and transport of fluorescent derivatives of dolichol in human fibroblasts

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We are using fluorescent derivatives to visualize the endocytic transport of dolichol intermediates from the cell surface to the lysosome, and to estimate their rate of turnover within the lysosome. Anthroyl dolichol and anthroyl [1-$^{14}$C]dolichol were synthesized and purified by chromatography on silica and $C_{18}$ Sep-Paks followed by high-performance liquid chromatography on $C_{18}$. The successful synthesis of anthroyl polyisoprenoid alcohols was confirmed by the use of uv-visible spectrometry and by fluorescence spectrometry. The purified esters were taken up into Ham's media containing 10–30% fetal calf serum or alternatively reconstituted into phospholipid liposomes for delivery to human fibroblasts in culture. The uptake of fluorescent dolichol esters into the cell and into lysosomes was demonstrated using fluorescence microscopy. The localization of anthroyl dolichol in lysosomes was further documented by simultaneously labeling fibroblasts with anthroyl dolichol and FITC-dextran a recognized lysosomal marker. Fibroblasts generally showed several groupings (domains) of lysosomes, some were dually labeled while others were labeled exclusively with either anthroyl dolichol or FITC-dextran. Labeling with anthroyl dolichol was very slow relative to labeling of the same fibroblasts with FITC-dextran suggesting that anthroyl dolichol acts as a labeling agent for intracellular membranes, particularly those of the lysosome while the dextran fluorescence is presumably of lysosolic origin. Several types of experiments were done with anthroyl [1-$^{14}$C]dolichol to establish that the fluorescence seen in lysosomes represents anthroyl dolichol. Anthroyl dolichol appears to enter fibroblasts intact, since we were unable to recover any free [1-$^{14}$C]dolichol from total lipid extracts of (i) media used for the uptake of anthroyl dolichol or (ii) the media removed from cells labelled for 42 h. In addition, attempts to hydrolyze anthroyl [1-$^{14}$C]dolichol in vitro using whole fibroblast homogenates at pH 4.0 and 7.5 were unsuccessful, even though the fibroblasts expressed acid lipase activity using 4-methylumbelliferyl palmitate as substrate.

## Introduction

N-linked oligosaccharides are assembled on the lipid intermediate Dol-P. The first step is the formation of Dol-PP-GlcNAc from Dol-P and UDP-GlcNAc. Additional sugars are added in a stepwise fashion until Dol-PP-$GlcNAc_2Man_9Glc_3$ is obtained. This entire 14-sugar oligosaccharide is then transferred to nascent protein. This sequence of reactions presumably occurs only in microsomes.

Lysosomes have levels of dolichol some 170-fold higher than microsomes and account for 80–90% of the dolichol in the cell [1]. This distribution is interesting since (i) lysosomes do not *N*-glycosylate proteins, and (ii) the amounts of dolichol (Dol-P) in microsomes frequently appear rate-limiting for *N*-glycosylation.

Dolichol is found not only in microsomes and lysosomes, but in the plasma membrane, nuclei, mitochondria, and peroxisomes as well (reviewed in Ref. 1). This ubiquitous occurrence suggests that dolichol is a normal, and probably a functional component of all eucaryotic membrane systems.

Dolichol intermediates accummulate in the lysosomes of human and animal tissues with aging. The dolichol content of human brain increases 8-fold over a period of 62 years [2]. Dolichol, Dol-P, and dolichyl pyrophosphoryl oligosaccharides, including Dol-PP-$GlcNac_2Man_9Glc_3$, are elevated in the lysosomes of tissues of children affected with neuronal ceroid lipofuscinoses [3–5].

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Abbreviations: Dol-P, dolichyl phosphate; TPP, triphenylphosphine; DEAD, diethylazodicarboxylate; THF, tetrahydrofuran; DMSO; dimethylsulfoxide.

Dolichol may be of particular functional significance in lysosomes in view of the very high concentrations present. It is interesting that the lysosome, the major site of intracellular degradation, does not degrade dolichol [6,7].

Our objective in these studies was to obtain basic information concerning (i) the pathways for the delivery of dolichol intermediates to the lysosome, (ii) the rate of turnover of these intermediates in the lysosome, and (iii) the relationship between the dolichol content of the lysosome and its ability to carry out normal lysosomal transport, enzyme catalysis and fusion.

Such experiments require that we have the ability to manipulate the dolichol content of the lysosome, and that we are able to visualize/measure the delivery and turnover of these long chain lipids within the lysosome. As an initial step towards these goals, we have synthesized fluorescent anthroyl dolichols for use in transport and turnover studies. We demonstrate that anthroyl dolichol is readily taken up into lysosomes from serum components and liposomes by human fibroblasts in culture. Experiments with anthroyl [1-$^{14}$C]dolichol show that the ester linkage is not subject to cleavage by hydrolase/lipase activity in fibroblasts or in the culture media.

## Materials and Methods

*Chemicals and reagents*

Dolichol $C_{80-105}$ from pig liver, solanesol $C_{45}$, eicosaprenol $C_{100}$ ($\alpha$-unsaturated), L-$\alpha$-phosphatidylcholine and FITC-dextran (FD-40S) were purchased from Sigma, St. Louis, MO. [1-$^{14}$C]Dolichol (50 mCi/mmol), a homologous series of 15–24 isoprene units with 18 and 19 predominating was from the Kuraray, Okayama, Japan.

Anthracene-9-carboxylic acid and TPP were from Aldrich Chemical, Milwaukee, WI, and DEAD was from Fluka, Ronkonkoma, NY. The reaction solvent, (THF), was treated with $LiAlH_4$ in small amounts until the highly exothermic dehydration was complete, and then distilled at 66°C just prior to use. Polyisoprenoid alcohols were prepared for derivitization by evaporating the carrier solvent under $N_2$ at room temperature followed by lyophilization for 30 min to ensure dryness.

*Synthesis of anthroyl derivatives*

We have used the Mitsunobu reaction [8,9] to derivatize dolichol, eicosaprenol and solanesol. Solanesol is a nine isoprene 'dolichol' which we used initially as a model compound for the esterification reaction.

An aliquot (100 $\mu$l) of 200 mM anthracene-9-carboxylate and 200 mM TPP in THF was mixed with the anhydrous polyisoprenoid alcohol. After mixing an aliquot (100 $\mu$l) of 200 mM DEAD in THF was added with mixing. The reaction was allowed to proceed for 20 min at room temperature before removal of the THF by evaporation under $N_2$ at 40°C.

The residue was dissolved in 0.4 ml of benzene, loaded on a Sep-Pack Si cartridge (Waters, Bedford, MA), and anthroyl lipids were eluted in 5 ml benzene. The residue obtained after drying under $N_2$ was dissolved in 200 $\mu$l of methylene chloride and loaded on a $C_{18}$ Sep-Pak (Waters). The methylene chloride was allowed to evaporate completely from the Sep-Pak under $N_2$, after which the cartridge was washed with $2 \times 5$ ml of methanol and the anthroyl derivatives eluted in $2 \times 5$ ml of methylene chloride.

*Thin-layer chromatography*

The conversion of alcohol to anthroyl alcohol was monitored by thin layer chromatography on Silica-Gel 60H using a solvent system of hexane/diethyl ether/acetic acid (65:35:1). Compounds bearing fluorescence were revealed under ultraviolet light, while those corresponding to polyisoprenoid alcohols (or their fluorescent derivatives) could be located by exposing plates to $I_2$ vapour. To demonstrate the presence of an ester linkage (i) anthroyl dolichol (100 $\mu$g) was dissolved/suspended in 500 $\mu$l absolute ethanol by heating to 80°C and sonication. An aliquot of 200 $\mu$l of 50% (w/w) KOH in water was added slowly with stirring and this mixture was sonicated and incubated for 17 h at 105°C, and (ii) anthroyl solanesol (50 $\mu$g) was treated with 0.5 g $LiAlH_4$ in 2 ml THF for 17 h at room temperature.

Lipids (alcohol and/or anthroyl alcohol) were removed from the hydrolysis mixtures by extraction into diethyl ether ($3 \times 2$ ml) and the ether fraction was washed twice with water. The lipids were freed of solvent under $N_2$ and analyzed by thin-layer chromatography and/or HPLC.

*High-performance liquid chromatography*

To ensure that any fluorescence seen in fibroblast labeling studies is due entirely to anthroyl dolichol, a $C_{18}$ reverse phase HPLC step was included in the purification. Analyses were done on a Hewlett-Packard 1084-B dual pump HPLC with a Hewlett-Packard 1030-B variable wavelength detector set at 210 nm. Solvents and the reverse phase $C_{18}$ column (10 $\mu$m particles, Brownlee Labs, Santa Clara, CA) were maintained at 45°C for all separations.

For solanesol, the following linear gradient was used, such that methanol/isopropanol at times 0, 15, and 20 min was 90:10, 65:35, and 20:80. For the longer polyisoprenoid alcohols, the methanol to isopropanol ratio was 90:10, 65:35 and 20:80 at time equals 0, 5 and 20 min, respectively. Peaks of interest were collected and solvent was removed by rotary evaporation. The residue (anthroyl derivatives) was dissolved in

0.5–1.0 ml of chloroform/methanol (2:1) and stored in the dark at −20°C.

*UV-visible and fluorescence spectroscopy*

UV-vis spectroscopy was performed on a Hewlett-Packard 8452A diode array spectrophotometer in ethanol. The fluorescence characteristics of anthroyl polyisoprenoid alcohols were studied in DMSO on a Perkin-Elmer LS-50 luminescence spectrometer.

*Tissue culture*

Normal human fibroblasts were grown at 37°C in 5% $CO_2$ and maintained by feeding twice weekly with 10% fetal calf serum in Ham's F-10 medium with glutamine. Confluent cells were subcultured by trypsin-EDTA treatment [10].

*Fluorescence-labeling of cells*

*Uptake by serum protein / lipoprotein components.* Anthroyl dolichol was freed of chloroform and methanol by drying in a glass tube under $N_2$, followed by lyophilization for 30 min. Fetal calf serum (1–2 ml) was then added to the tube and the mixture was stirred for 3–4 hr at room temperature to allow the anthroyl dolichol to become associated with lipoprotein components. The mixture was then filtered (Millex-GV; 0.22 μm, Millipore, Bedford, MA), diluted ten-fold with Ham's medium and added to fibroblast cultures.

*Uptake from phospholipid liposomes.* Egg lecithin (L-α-phosphatidylcholine) and anthroyl dolichol were dissolved in chloroform/methanol (2:1) at a total lipid concentration of 1.5–2.0 mg/ml with anthroyl dolichol making up 25% of that total. Aliquots of this mixture in 16 × 150 mm glass test tubes were dried under $N_2$, and any residual solvent was removed by lyophilization for 30 min. Serum free Ham's F-10 medium was added to the dried lipids to give a final anthroyl dolichol concentration of 0.3–0.5 mg/ml, and the tubes were then incubated at room temperature for 30 min. after which the lipids were resuspended in media by mixing on a Vortex mixer for 3–5 min followed by sonication at 4°C (on ice) for 15 min with a tapered microtip set at 60–80% of maximum (Artek, Farmingdale, NY). The liposomes generated were of a uniform and small size (diameter 0.1–0.5 μm). When required, liposomes were washed by centrifugation at 34000–50000 × *g* for 30 min. The immediate use of the liposome suspension is preferred although it may in some cases be stored at 4°C for up to 48 h.

*Incubation of fibroblasts with anthroyl dolichol containing liposomes.* Fibroblasts were grown on cover slips in slant tubes in F-10 medium with 10% fetal calf serum. At 24 h prior to labeling, the media was removed and replaced with F-10 media without calf serum after first washing the cells several times with the serum depleted medium. The medium was replaced with the anthroyl dolichol containing liposome suspension and incubated for the indicated time periods. At the end of the incubation the fibroblasts were washed intensively with phosphate buffered saline in order to remove free liposomes.

*Co-labeling of fibroblasts with anthroyl dolichol and FITC-dextran.* Some fibroblasts were labeled with both anthroyl dolichol and FITC-dextran in order to demonstrate co-localization of the two fluorophores within the lysosomes. Liposome suspensions (total lipid 1 mg/ml; anthroyl dolichol 0.3–0.5 mg/ml) were prepared as described previously. Fibroblasts were incubated in F-10 medium lacking calf serum for 24 h and then incubated in the liposome suspension for an additional 24 h at which time FITC-dextran was added at 1.0–1.5 mg/ml final concentration from a 30 mg/ml stock which had been dialyzed and filtered. After an additional 24 h of incubation the subconfluent monolayers of fibroblasts were examined for uptake of the two fluorophores.

*Fluorescence microscopy.* Fibroblasts were examined for fluorescence using a Zeiss fluorescence microscope equipped with a Zeiss 35 mm camera. Excitation was at 360 nm and 470 nm, and emission at 460 nm and 515 nm for anthroyl dolichol and FITC-dextran, respectively. Neutral density filters (factor 0.56 to 1.03) can be used to avoid bleaching of the fluorescence from anthroyl dolichol. Autofluorescence which is either absent or very low in fibroblasts in newly established cultures can be significant in fibroblasts that have been in culture for extended periods of time. Although barrier filters can be used to reduce or eliminate the background autofluorescence we prefer the use of low passage cells which show little or no autofluorescence.

*Assay for anthroyl-dolichol hydrolase activity.* Enzymatic hydrolysis of anthroyl [1-$^{14}$C]dolichol was measured using the conditions of the acid lipase assay described by Patrick et al. [11]. We have also measured acid lipase activity with 4-methylumbelliferyl palmitate as substrate with the same enzyme preparations.

Assays were carried out at pH 4.0 (lysosomal activity) and pH 7.5 (background/non-lysosomal activity) at 37°C for 1 h. The 300 μl assay contained 33 mM buffer (glycine-HCl, pH 4.0, or Tris-HCl, pH 7.5), 275 μg normal human fibroblast homogenate (on the basis of protein), and substrate, $10^4$ dpm anthroyl [1-$^{14}$C]dolichol (specific activity 50 mCi/mmol), or 1 mM 4-methylumbelliferyl palmitate.

*Processing / quantitation of assays.* Assays with anthroyl-[1-$^{14}$C]dolichol substrate were stopped by adding 6 ml of chloroform-methanol (2:1). This whole mixture was washed first with 0.9 ml water, then with 2.4 ml of chloroform/methanol/water (3:47:48), and taken to dryness under $N_2$ [12]. Residual material was then taken up in a few drops of chloroform/methanol (2:1) and run on Silica Gel H 60 thin layer chromatographic

plates in a solvent system of hexane/diethyl ether/glacial acetic acid (65:35:1). Compounds containing radioactivity were located by exposure to Fuji X-Ray film for 48 h.

Assays with 4-methylumbelliferyl palmitate as substrate were terminated by addition of 3.0 ml of a pH 10.7 solution containing 0.2 M glycine, 0.125 M $Na_2CO_3$, and 0.1 M NaCl. Fluorescence due to free 4-methylumbelliferone was quantitated on a Perkin-Elmer fluorescence spectrometer (excitation 365 nm; emission 450 nm).

## Results

*Synthesis of derivatives*

Thin-layer chromatography was used as a quick method for monitoring the success of the reaction. Fig. 1a, lane 1, shows an aliquot of a reacted mixture containing 0.5 mg dolichol prior to purification; as well as lane 2, an aliquot of a dolichol standard, photographed under ultraviolet light. A fluorescent spot with the mobility of dolichyl fatty acyl esters is clearly evident. Fig. 1b (mirror image of 1a), shows the same plate after it was stained with $I_2$. It clearly shows lipid staining of both the dolichol standard (lane 1) and the mobile fluorescent spot (lane 2; corresponding to Fig. 1a, lane 1).

The reaction went to completion over a wide range of concentrations of dolichol (50 ng–500 $\mu$g) when the concentration of the other reagents was held constant. The method is therefore also useful for the quantitation of minute amounts of dolichol in biological samples by derivatization prior to HPLC combined with fluorescence detection. The chemistry utilized here works equally well for very short dolichols (i.e., solanesol; nine isoprenes), and for polyprenols (i.e., eicosaprenol) in which the $\alpha$-isoprene is not saturated. No hydrolysis of anthroyl dolichol was observed in standard saponification mixtures containing about 20% ethanol. However, when the ethanol content was increased to 70% (v/v) anthroyl dolichol was totally converted to dolichol by saponification. The lack of hydrolysis at 20% ethanol is probably due to the very limited solubility of the anthroyl esters in this mixture. Treatment of anthroyl solanesol with $LiAlH_4$ in THF (in which the presumed esters are soluble); also resulted in a complete conversion of anthroyl alcohol to alcohol.

*High-performance liquid chromatography*

HPLC was used as a final purification step for anthroyl polyisoprenoid alcohols to ensure removal of trace amounts of fluorescence not associated with lipid carrier. Fig. 2a shows a chromatogram of unreacted dolichols (major homologues 18 and 19 isoprenes) having retention times between 15 and 20 min. On conversion to the corresponding anthroyl derivatives (Fig. 2b), the retention times shifted to 20–25 min. Similar shifts in retention were obtained when solanesol and eicosaprenol were converted to fluorescent derivatives.

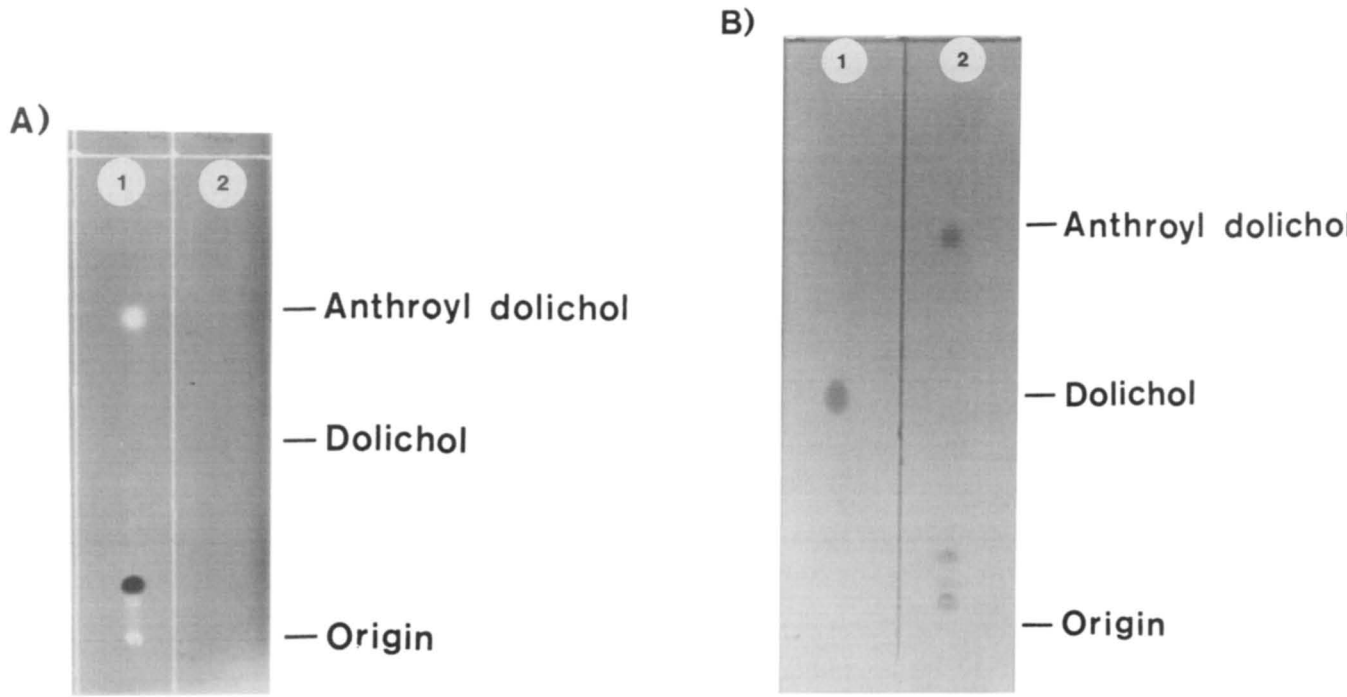



Fig. 1. Thin-layer chromatography of reaction mixture in which anthroyl dolichol was produced. Anthroyl dolichol formed was detected by (A) fluorescence, or (B) by $I_2$ staining.

*Absorbance and fluorescence characteristics of anthroyl derivatives*

UV-visible and fluorescence spectra were measured in ethanol and DMSO, respectively. Anthroyl solanesol was chosen (as a representative anthroyl polyisoprenoid alcohol) because of its greater solubility (than anthroyl dolichol) in these solvents.

UV-visible spectra are shown in Fig. 3a(i) for solanesol and 3a(ii) for anthroyl solanesol. Solanesol shows a single peak at 206 nm (double bonds) while the anthroyl derivative has an additional peak at 254 nm. Solanesol and dolichol derivatives generate similar spectra although the 206 to 254 ratio would be influenced by the number of isoprene units in the lipid moiety.

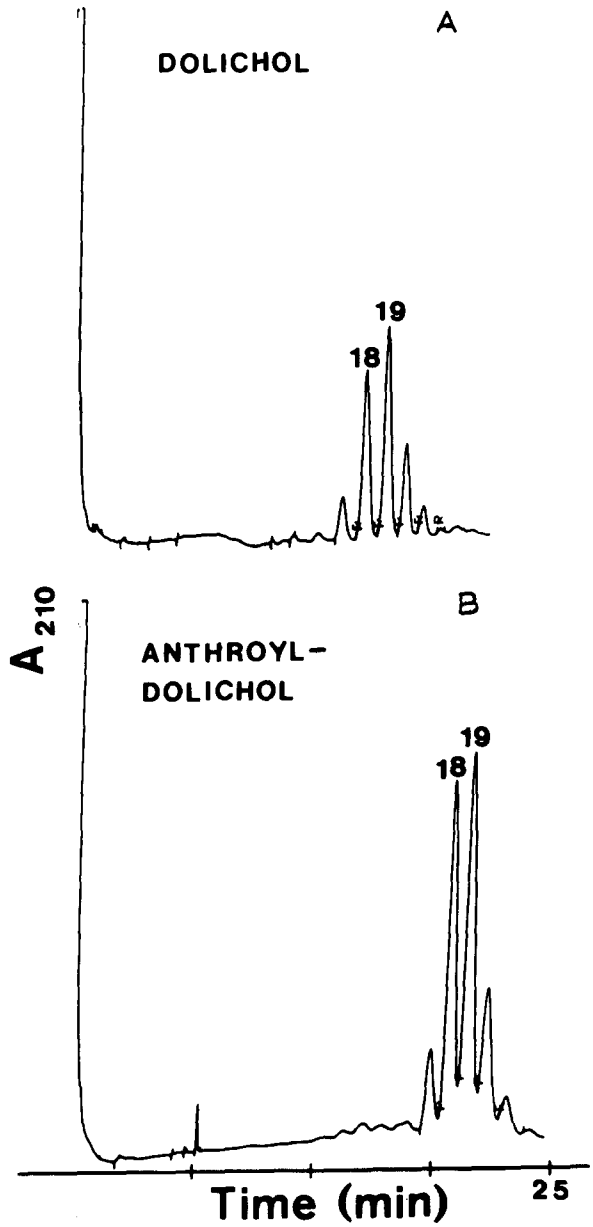



Fig. 2. $C_{18}$-reverse phase high-performance liquid chromatography of (a) dolichol and (b) anthroyl dolichol.

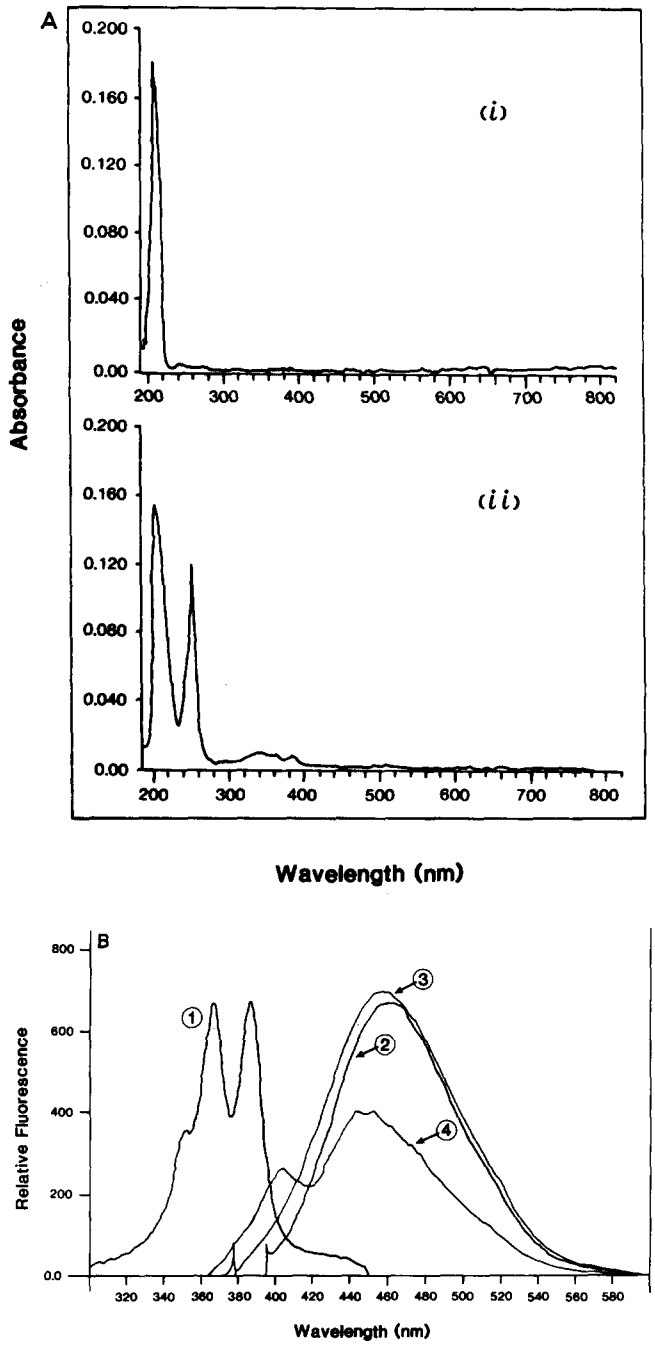
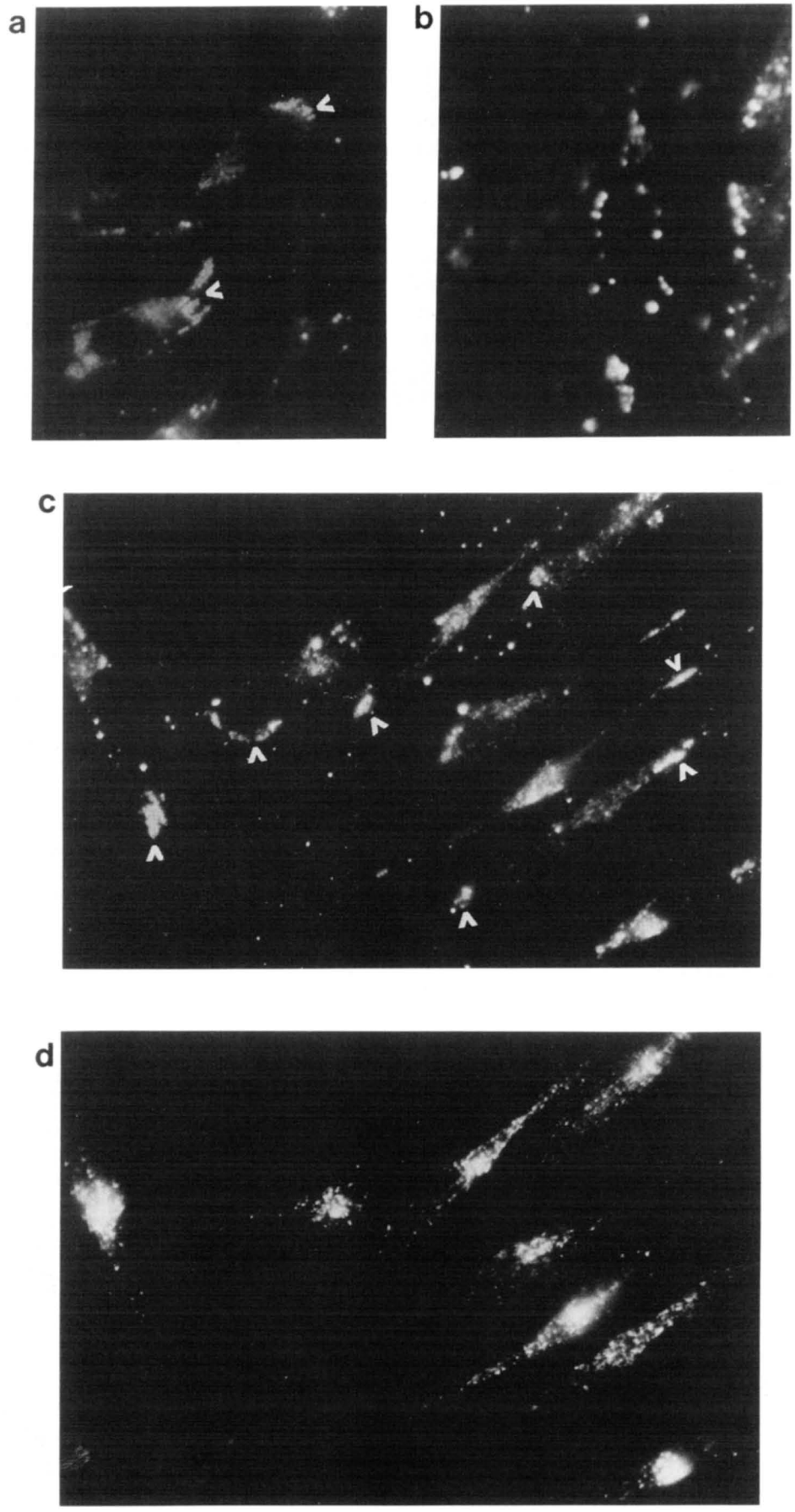



Fig. 3. Absorbance and fluorescence characteristics of anthroyl derivatives. (A) UV-visible spectra of (i) solanesol and (ii) anthroyl solanesol in ethanol. Concentrations were; solanesol 0.9 $\mu$M, anthroyl solanesol, 0.9 $\mu$M. (B) Fluorescence spectra for anthroyl solanesol in DMSO. Concentration 0.9 $\mu$M. Labeling: 1, excitation spectrum for emission at 460 nm; 2–4, emission spectra for excitation at 385.6, 366.1 and 350.6 nm, respectively.

Fig. 3(b) shows a corrected fluorescence excitation spectrum (line 1) for emission at 460 nm for anthroyl solanesol. Excitation maxima were seen at 385.6 nm and 366.1 with a shoulder at 350.6 nm. Lines 2–4 in


a
b
c
d

this figure show emission spectra for excitation at each of these respective wavelengths.

*Fluorescence-labeling of fibroblasts*

Fig. 4a shows a field of fibroblasts exposed to anthroyl dolichol liposome containing media for approx. 24 h. The darkened nuclei and the outlines of individual cells are visible. Fluorescent material has clearly been taken up by the fibroblasts and it has been localized to and concentrated within numerous small vesicles (lysosomes/endosomes) present primarily in the perinuclear space. The distribution seen here is similar to that seen in human fibroblasts and 3T3-K fibroblasts stained with acridine orange, a fluorophore specific for the acidic environment of the lysosome (C.A. Rupar, unpublished data; L. Jiang, unpublished data). Similar patterns of lysosome distribution are seen in the laser scanning images obtained from murine and human fibroblasts labeled with FITC-dextrans [13].

Fig. 4(b) reveals contact and adhesion between individual liposomes and the plasma membrane of fibroblasts. Contact is observed within 1–2 h of adding anthroyl dolichol liposome containing medium to the cultured fibroblasts and may indicate the initiation of a series of fusion processes.

In order to confirm the observation that anthroyl dolichol is deposited in the lysosomes of fibroblasts we have attempted to demonstrate the co-localization of fluorescence labeled dolichol and FITC-dextran. Fig. 4(c) demonstrates a localization of anthroyl dolichol in lysosomal domains located at the ends of fibroblasts as well as in the perinuclear spaces after 60 h of labeling.

The labeled lysosomes were clearly visible as bright blue coloured structures of uniform size (0.3–0.4$\mu$) at a density of 20–50/fibroblast. The fluorescence intensity of these structures was lower than observed for anthroyl dolichol containing liposomes of the same size due to an apparent dilution and redistribution of liposomal lipids into cellular and intracellular membranes. The fluorescence due to anthroyl dolichol could be bleached quite rapidly as the intensity of the exciting light was increased.

Fig. 4(d) demonstrates the subcellular distribution of fluorescence due to FITC-dextrans incorporated into the same fibroblasts using excitation at 460 nm. The overall distribution pattern of the green coloured FITC fluorescence (emission 519 nm) seen here (for the same field of cells as Fig. 4(c)) was similar to but not identical to the pattern observed with anthroyl dolichol. Fibroblast lysosomes appeared in some cases in certain distinct and separate localization domains depending on whether they incorporated anthroyl dolichol or FITC-dextran, although other groups of lysosomes clearly show an overlap of the two fluorescences. This co-localization provides direct evidence that anthroyl dolichol is taken up by fibroblasts and concentrated within their lysosomes. Cells labeled with media or liposomes (each without anthroyl dolichol) as controls for background fluorescence demonstrated no fluorescence under the standard conditions for visualization.

Although FITC-dextran is known to be delivered to the soluble interior of the lysosome by fluid phase pinocytosis and intracellular translocation, nothing is known concerning the mechanisms involved in the uptake and intracellular transport of dolichol intermediates. Based on physical properties it is almost certain that anthroyl dolichol is a labeling agent of biological membranes (it is totally insoluble in aqueous solutions), and on this basis we believe that dolichol delivered to lysosomes is in the lysosomal membrane.

To eliminate the inhibitory effect of serum on anthroyl dolichol uptake which is perhaps due to dolichol binding to serum lipoproteins [14], and to minimize the possible exchange between exogenous and endogenous dolichol, the cultured fibroblasts were subjected to a 12–24 h incubation in calf serum free Ham's medium before replacing with anthroyl dolichol containing liposome suspension in serum free medium.

*Hydrolysis of anthroyl dolichol*

We were unable to demonstrate release of [1-$^{14}$C]dolichol from anthroyl [1-$^{14}$C]dolichol in in-vitro incubations in which human fibroblast homogenate was

Fig. 4. Fluorescence microscopy of human fibroblasts. (a) Fibroblasts were incubated with a suspension of liposomes containing anthroyl dolichol and PC in F-10 medium for approximately 24 h. Final concentration of anthroyl dolichol in the media was 100 $\mu$M. Excitation and emission wavelengths were as indicated in Materials and Methods. Blue coloured fluorescent domains indicated by arrows contain 10–25 individual lysosomes having an average size of 0.3–0.4 $\mu$m (diameter). (b) Fibroblasts were exposed to anthroyl dolichol suspension for 1 h, and then were examined by fluorescence microscopy. The liposomes appear to have initiated contact and attachment/adhesion processes with the membranes as evidenced by the apparent attachment of fluorescent liposomes to the cell surfaces. (c,d) Double labeling with anthroyl dolichol and FITC-dextran. Fibroblasts were first incubated in the suspension of liposomes in F-10 medium for 48 h at a concentration of 100 $\mu$M anthroyl dolichol. After 24 h, stock FITC-dextran was added to the same medium to a final concentration of 1 mg/ml. Incubation was continued for an additional 24 h after which the cells were examined under the fluorescence microscope at excitation 360 nm (anthroyl dolichol) and 460 nm (FITC-dextran). The same field of fibroblasts shows different fluorescence patterns for anthroyl dolichol (c) and FITC-dextran (d). The two fluorescences do overlap in some lysosome domains. Arrows in Fig. 4c indicate lysosomal domains labeled only with anthroyl dolichol.

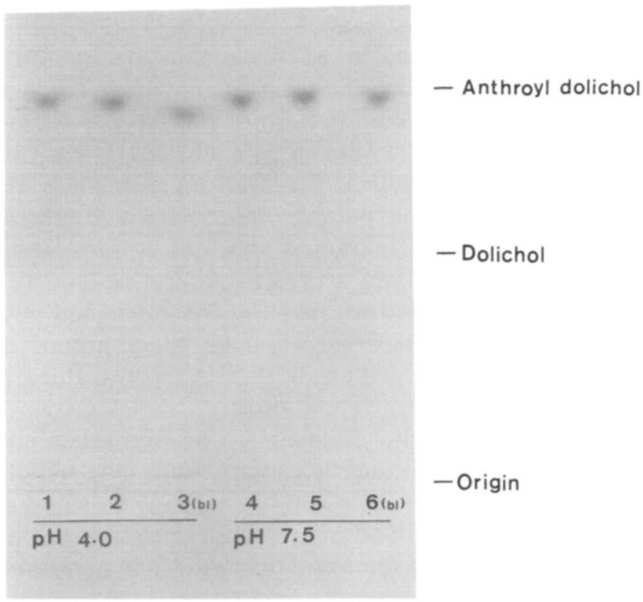



Fig. 5. Assay for in-vitro anthroyl [1-$^{14}$C]dolichol hydrolase activity. Assays were carried out at pH 4.0 for 60 min (lanes 1 and 2: duplicate assays; lane 3: blank), and pH 7.5 (lanes 4 and 5: duplicate assays; lane 6: blank) and processed for thin-layer chromatography as indicated in the text.

used as an enzyme source. This is shown in Fig. 5 using conditions that favour (pH 4.0) and do not favour (pH 7.5) the expression of lysosomal acid hydrolase activity.

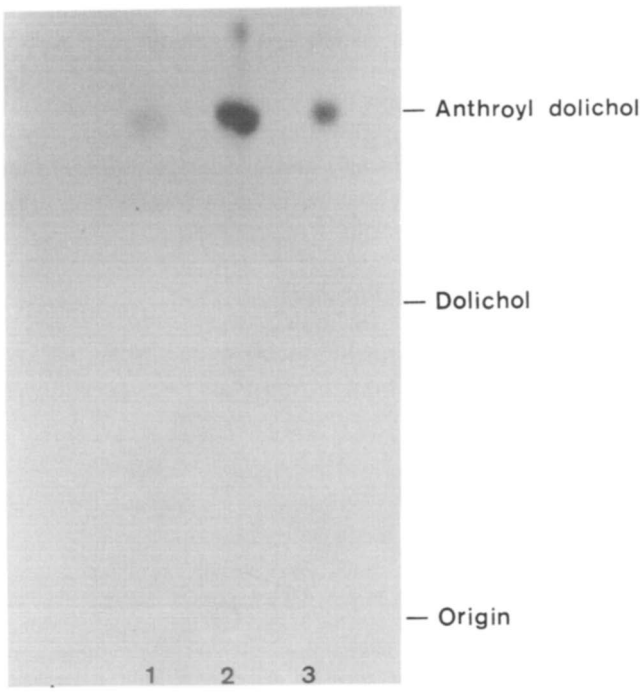



Fig. 6. Lack of conversion of anthroyl [1-$^{14}$C]dolichol to [1-$^{14}$C]dolichol in vivo. Total lipid extracts from: lane 1, the anthroyl [1-$^{14}$C]dolichol fetal calf serum mixture (2600 dpm); lane 2: media from cells labeled with anthroyl [1-$^{14}$C]dolichol for 42 h (25000 dpm) were examined by thin-layer chromatography and compared to authentic anthroyl [1-$^{14}$C]dolichol (lane 3, 7200 dpm).

TABLE I

*Anthroyl-dolichol hydrolase and acid lipase activities of human fibroblasts*

The values are the average of triplicate determinations which agreed to within 10%.

| | Substrate | | | |
|---|---|---|---|---|
| | anthroyl [1-$^{14}$C]dolichol | | 4-MU-palmitate | |
| | pH 4.0 | pH 7.5 | pH 4.0 | pH 7.5 |
| Activity [a] | 0 | 0 | 190 | 12.5 |

[a] nmol product/h per mg fibroblast protein.

Hydrolysis of anthroyl dolichol could not be demonstrated in vivo, either. Fibroblasts were fed with media containing anthroyl [1-$^{14}$C]dolichol ($1.4 \cdot 10^6$ dpm/ml media) for 42 h. Aliquots of the anthroyl [1-$^{14}$C]dolichol fetal calf serum mixture stirred 4 h, and media removed from fibroblasts labeled 42 h did not contain any [1-$^{14}$C]dolichol when examined by thin-layer chromatography and autoradiography after total lipid extraction (Fig. 6, lane 1,2).

The human fibroblasts did however express lysosomal acid lipase activity when the same homogenates were assayed with 4-methylumbelliferyl palmitate as substrate (Table I). This observation suggests that the lysosomal acid hydrolases (at pH 4.0) do not recognize anthroyl dolichol as a substrate.

## Discussion

The purpose of this study was to develop methods that could be used to investigate (i) the mechanism of uptake of dolichol intermediates into cells, (ii) the mechanisms involved in their intracellular transport and deposition into lysosomes, and (iii) the rate of turnover of these intermediates within the lysosome.

We have demonstrated the quantitative conversion of solanesol, dolichol and eicosaprenol to their corresponding anthroyl derivatives over a wide range of substrate concentrations and have developed methods for their purification and quantitation. The chemistry was equally effective using dolichol ($\alpha$-isoprene saturated) or eicosaprenol ($\alpha$-isoprene unsaturated indicating a lack of specificity for the stereochemistry of the $\alpha$-isoprene).

In-vitro and in-vivo experiments using anthroyl [1-$^{14}$C]dolichol showed very clearly that the ester linkage is not cleaved by any enzyme(s) present in fetal calf serum, tissue culture medium, or the human fibroblasts themselves under the conditions of our experiments. This finding indicates that the fluorescence distribution we see in lysosomes is due to lipid linked anthracene and not due to anthracene-9-carboxylate (the expected product of esterase activity on anthroyl dolichol).

The apparent lack of catabolism of these fluorescent derivatives by fibroblasts makes them an attractive choice for studies on the mechanism of dolichol accumulation in the lysosome. It is highly likely that (based on solubility and polarity considerations) anthroyl dolichol is a membrane labeling agent, and as such, labels the intracellular membranes including the lysosomal membrane, rather than the cytosol or lysosol.

Lysosomal localization has been documented by showing co-localization of anthroyl dolichol with FITC-dextran – an agent known to label lysosomes. There are interesting differences in labeling patterns seen with anthroyl dolichol and FITC-dextran which probably relate at least partially to the polarity (water solubility) and kinetics of uptake of the labeling agents, although the possibility that lysosome populations are specifically labeled with anthroyl dolichol and/or FITC-dextran because of functional or compositional differences in the lysosomes themselves cannot be discounted and remains an interesting possibility.

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