Formation of 7-dehydrocholesterol-containing membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome

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Abstract Smith-Lemli-Opitz syndrome (SLOS) is a recessive disease typified by 7-dehydrocholesterol (7DHC) accumulation and depletion of cholesterol. Because cholesterol is a primary component of detergent-resistant membrane domains ("rafts"), we examined the compatibility of 7DHC with raft formation. Liposomes containing bovine brain phosphatidylcholine, sphingomyelin, cerebrosides, and either cholesterol, 7DHC, or coprostanol (the latter being incompatible with raft formation) were prepared. 7DHC was indistinguishable from cholesterol in its ability to become incorporated into membrane rafts, as judged by physical and chemical criteria, whereas coprostanol did not form rafts. The in vivo compatibility of 7DHC with raft formation was evaluated in brains of rats treated with *trans*-1,4-*bis*(2-dichlorobenzylamino-ethyl)cyclohexane dihydrochloride (AY9944), which mimics the SLOS biochemical defect. 7DHC/cholesterol ratios in rafts and whole brains from AY9944-treated rats were similar, indicating comparable efficiency of 7DHC and cholesterol incorporation into brain rafts. In contrast, dolichol (a nonsterol isoprenoid incompatible with raft formation) was greatly depleted in brain rafts relative to whole brain. Although brain raft fractions prepared from AY9944-treated and control rats yielded similar sterol-protein ratios, their gel electrophoresis profiles exhibited multiple differences, suggesting that altered raft sterol composition perturbs raft protein content. These results are discussed in the context of the SLOS phenotype, particularly with regard to the associated central nervous system defects.—Kennedy, R. K., T. P. Arnold, and S. J. Fliesler. **Formation of 7-dehydrocholesterol-containing membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* 2004.** 45: **347–355.**

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Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive genetic disease with a carrier frequency of ~1 in 30 persons of northern and central European heritage (1). It is characterized phenotypically and functionally, in widely varying degrees, by mental retardation, microcephaly, syndactyly of the toes, and a host of other dysmorphic features and problems related to abnormal growth and development [reviewed in refs. (2–5)]. In 1994, Tint et al. (6) discovered that SLOS-affected individuals have greatly increased plasma levels of 7-dehydrocholesterol (7DHC), a penultimate precursor of cholesterol, and abnormally low levels of cholesterol. Since this seminal discovery, the genetic defect in SLOS has been linked to several mutations in Δ^7-dehydrocholesterol reductase (7DHC reductase; a product of the *DHCR7* gene), the enzyme responsible for the conversion of 7DHC to cholesterol (7). Survival correlates strongly and inversely with the level of serum cholesterol (8), but it remains uncertain whether the pathological symptoms are primarily attributable to a deficit of cholesterol or to increased 7DHC/cholesterol mole ratios, or to both (9, 10).

Cholesterol has been shown to have a variety of functions in vivo, including as a precursor to vitamin D, steroid hormones, and bile acids and as a structural component of cell membranes. With regard to its latter role, cholesterol is a known component of membrane lipid "rafts," detergent-resistant microdomains on the surface of cells and internal membranes that are enriched in cholesterol and sphingolipids relative to the bulk phase membrane lipid composition. The basis for raft structure appears to be the tendency of cholesterol to aggregate with sphingolipids,

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Abbreviations: AY9944, *trans*-1,4-*bis*(2-dichlorobenzylaminoethyl) cyclohexane dihydrochloride; CB, galactocerebroside; 7DHC, 7-dehydrocholesterol; DOL, dolichol; HRP, horseradish peroxidase; MBS, MES-buffered saline; PC, phosphatidylcholine; SCAP, SREBP cleavage-activating protein; Shh, Sonic hedgehog; SLOS, Smith-Lemli-Opitz syndrome; SM, sphingomyelin; SREBP, sterol regulatory element binding protein; SSD, sterol-sensing domain.

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forming a liquid-ordered structure [reviewed in ref. (11)]. Rafts can be isolated by taking advantage of their resistance to solubilization by nonionic detergents such as Triton X-100. Elegant studies by Schroeder et al. (12) have shown that rafts prepared in vitro from typical membrane components exhibit the detergent resistance of rafts isolated from natural membranes.

Caveolae are thought to constitute a distinct and specialized subset of membrane rafts [reviewed in refs. (13–17)]. They contain structural proteins of the caveolin family and assume different shapes depending on their functional state (13, 15). Recent studies have shown that many signal transduction pathways are initiated by the binding of hormones or other ligands to receptors localized in caveolae or rafts (14–16). In view of the important biological role of membrane rafts and the prevalence of cholesterol within these microdomains, the question arises whether or not 7DHC, the major sterol present in the tissues of SLOS patients, is compatible with raft structure. Indeed, it is plausible that the mere replacement of cholesterol with 7DHC may significantly affect various aspects of membrane biology, such as the alteration of the relative amount of membrane surface area occupied by rafts or the perturbation of protein composition or function within rafts. For example, it has been shown that the activity of inward-rectifier K^+ channels is modulated by optical isomers of cholesterol (18) and that the replacement of cholesterol with non-cholesterol sterols alters the activity of the serotonin transporter (19).

To investigate the ability of 7DHC to replace cholesterol in rafts, we first evaluated whether or not 7DHC was similar to cholesterol in its ability to form detergent-resistant microdomains in vitro in a model membrane system (liposomes). We then examined the ability of 7DHC to form rafts in vivo and also analyzed their lipid and protein composition, using brains from control rats and from pharmacologically altered rats in which the 7DHC/cholesterol mole ratio mimics that found in tissues from SLOS patients. Here, we demonstrate that although 7DHC is virtually identical to cholesterol in its ability to form membrane rafts both in vitro and in vivo, brain rafts containing 7DHC as their dominant sterol have altered protein composition compared with normal, cholesterol-containing rafts. These results are discussed within the context of their potential relevance to SLOS.

METHODS

Reagents and materials

Unless otherwise stated, all reagents and biochemicals were used as obtained from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine (PC), phosphatidylethanolamine, sphingomyelin (SM), and galactocerebroside (CB) all were derived from bovine brain. Cholesterol, 7DHC, and coprostanol were purchased from Avanti Polar Lipids (Alabaster, AL). All lipids were transferred to tared amber vials under dim light and were dissolved in chloroform. Vials were flushed with argon, capped with Teflon valves, and stored at −20°C. All organic solvents were HPLC grade (Burdick and Jackson, Muskegon, MI). Radiolabeled compounds were from American Radiolabeled Chemicals (St. Louis, MO). *trans*-1,4-*bis*(2-Dichlorobenzylaminoethyl)cyclohexane dihydrochloride (AY9944) was a generous gift from Wyeth-Ayerst Research (Princeton, NJ).

Preparation of liposomes

A stock solution containing PC, SM, and CB was prepared in chloroform (10 μmol/ml total lipids) in a molar ratio of 2:1:1 and was stored in the dark under argon. Aliquots of this solution were mixed with various amounts of chloroform-solubilized sterols such that the sterol varied from 0 to 33 mol% total lipid. The samples were then dried under argon in dim light. PBS (2 ml) was then added, and the samples were mixed vigorously on a vortex, incubated at 65°C for 30 min, vortexed again, and incubated for another 15 min. After cooling to room temperature, the turbidity (absorbance at 600 nm) was determined using a Shimadzu UV-160 recording spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The final concentration of lipid was 135 μM.

Isolation of rafts prepared from liposomes

Liposomes were poured into glass centrifuge tubes, treated with 10% Triton X-100 in PBS (1:9, v/v), and vigorously mixed on a vortex. The samples were then centrifuged at 10,000 *g* for 20 min (4°C). The supernatants were removed, and the pellets were dissolved in chloroform.

Lipid analysis of in vitro prepared raft fractions

Aliquots of the in vitro prepared raft pellets were applied to silica gel 60 TLC plates (EM Separations Technology, Gibbstown, NJ) and chromatographed using a two-step development procedure. The first development was with a mobile phase containing chloroform-methanol-glacial acetic acid-water (65:35:2:2, v/v/v/v); after the solvent had migrated approximately two-thirds of the way up the plate, the plates were quickly dried with a stream of nitrogen and then chromatographed in the second mobile phase (20% diethyl ether in hexane, v/v), allowing the mobile phase to reach the top of the plate. Plates were then removed, air dried, and treated with anisaldehyde spray reagent before heating with a heat gun. The stained plates were scanned using a Microtek scanner connected to an IBM-compatible computer.

Generation of increased levels of 7DHC in rat brain

Generation of the pharmacologically induced rat model of SLOS was performed essentially as described in detail previously (20). In brief, AY9944 was mixed with cholesterol-free powdered rat chow (Ralston Purina, St. Louis, MO) at a concentration of 1 mg of AY9944 per 100 g of chow. Pregnant Sprague-Dawley rats (6 days sperm positive; Harlan Bioproducts for Science, Inc., Indianapolis, IN) were fed 40 g of chow per day ad libitum throughout the time course of the experiment (gestational day 6 through postnatal day [P]28); control dams received the same chow minus AY9944. In addition, starting at P1, surviving pups from AY9944-treated dams were injected subcutaneously every other day with AY9944 (25 mg/kg) in an olive oil emulsion supplemented with a mixture of vitamins A, D_3, and E (Vital-E-A+D®, Schering-Plough Animal Health, Kenilworth, NJ; final concentrations, in International Units per milliliter, were as follows: A, 1000; D_3, 100; E, 3). One month after birth, brain tissue from treated and control animals was harvested, and the sterol profiles were analyzed by reverse-phase HPLC (20). The average 7DHC/cholesterol mole ratio of the brains used in this study was ~3:1.

Preparation of raft fractions from rat brain

The procedure followed was similar to that of Martens et al. (21) with minor modifications. Rat brain (~0.6 g) was homoge-

nized in 10 ml of MES-buffered saline (MBS; 25 mM MES, pH 6.5, and 0.15 M NaCl) containing 1% (v/v) Triton X-100, 1 mM PMSF, and 2 μg/ml each pepstatin and leupeptin using 18 strokes of a loose-fitting Dounce homogenizer. This homogenate was centrifuged at 3,000 rpm at 4°C for 15 min. The pellet was resuspended in 5 ml of MBS and stored at −20°C. A solution of 75% (w/v) sucrose in MBS was added to the supernatant to adjust the final sucrose concentration to 40%. This solution was then transferred to a 17-ml centrifuge tube (Ultra-Clear, Beckman, Fullerton, CA) and overlaid sequentially with 3 ml each of 30, 25, and 5% (w/v) sucrose (all in MBS). The gradient was centrifuged overnight at 30,000 rpm in a Beckman SW 28.1 rotor at 4°C. After centrifugation, the bottom portion of the gradient containing the 40% sucrose was collected and stored frozen. The raft fraction, floating above the 25% sucrose layer, was collected, diluted 10-fold with MBS saline, and centrifuged for 1 h at 30,000 rpm in a Beckman Ti 50.2 rotor at 4°C. The supernatant was discarded, and the pellet, containing the purified rafts, was resuspended in 1 ml of MBS and stored frozen (−20°C).

Analysis of raft fractions

Brain membrane rafts are enriched in specific lipids (e.g., cholesterol, gangliosides) and proteins relative to the bulk membrane phase and other cellular compartments and organelles. To assess the purity of the brain raft preparations, equal amounts of protein (0.66 μg) from each of the fractions generated during the raft preparation were applied to a nitrocellulose membrane and subjected to dot blot analysis for GM1 ganglioside [a specific lipid enriched in rafts (11)] by probing with horseradish peroxidase (HRP)-conjugated cholera toxin (List Biological Laboratories, Inc., Campbell, CA), which binds specifically to GM1 ganglioside. In parallel, one-dimensional SDS-PAGE analysis was performed, using 20 μg of protein from each of the fractions, with subsequent Western blot analysis for both a known raft resident protein (CD90, or Thy-1) and a protein not associated with rafts (Na^+,K^+-ATPase) (22). The electrophoresis was carried out with a Laemmli-type system (23), using precast 4–20% acrylamide gradient gels (Gradipore, Inc., Hawthorne, NY). Western blot analysis was performed using standard procedures (24). A monospecific antibody against CD90 (MCA47R) was obtained from Serotec (Raleigh, NC). Antibody for Na^+,K^+-ATPase (MA3-929) was obtained from ABR-Affinity BioReagents (Golden, CO). Dilutions of primary and secondary antibodies, as well as HRP-based chemiluminescent detection reagents (enhanced chemiluminescence; Amersham Biosciences Corp., Piscataway, NJ), were prepared and used according to the manufacturer's recommended protocol. As shown in **Fig. 1**, GM1 ganglioside was clearly enriched in the raft fraction compared with the other fractions tested. CD90 was detected only in the raft fraction, whereas Na^+,K^+-ATPase was present in the initial supernatant and at the bottom of the sucrose gradient, but was absent from the raft fraction. Enrichment of cholesterol in rafts, compared with the parent membrane homogenate, also was assessed using reverse-phase HPLC (see below and **Table 1**).

Two-dimensional gel electrophoresis of brain homogenate and raft fractions (100 μg of protein per sample) was performed by a fee-for-service laboratory (Kendrick Labs, Madison, WI) according to the method of O'Farrell (25). Analysis was performed on two independent preparations of both homogenates and raft fractions from both control and AY9944-treated rats. In brief, isoelectric focusing was carried out in glass tubes (inner diameter 2.0 mm) using 2% 3.5–10.0 ampholines (Amersham Biosciences) for 9,600 V h. An internal standard of tropomyosin (50 ng) was added to each sample. This protein migrates as a doublet with a lower polypeptide spot of molecular weight 33,000 and isoelectric point 5.2; an arrowhead on the stained gels (see Fig. 6) marks its position. The pH gradient indicated above the gel was determined with a surface pH electrode. After equilibration for 10 min in buffer [10% (v/v) glycerol, 50 mM DTT, 2.3% (w/v) SDS, and 62.5 mM Tris-Cl, pH 6.8], each tube gel was affixed to the top of a "stacking gel" that was on top of a 10% acrylamide slab gel (0.75 mm thickness). SDS-PAGE was performed for ~4 h at 12.5 mA/gel. Molecular weight standards were added to the agarose that sealed the tube gel to the slab gel; these standards appeared along the basic (right-hand) edge of the silver-stained gel. Gels were dried between sheets of cellophane and scanned on a flat-bed scanner.

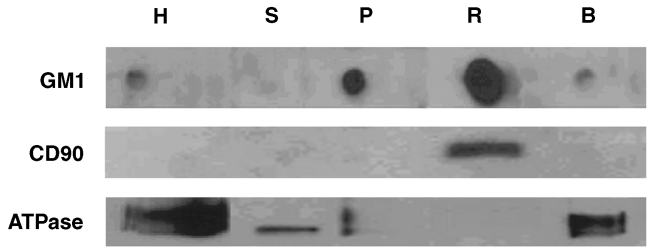

Fig. 1. Rat brain lipid rafts are enriched in GM1 ganglioside and CD90, but not Na^+,K^+-ATPase. Aliquots (0.66 μg of protein) of each fraction were blotted onto nitrocellulose membranes and then probed with horseradish peroxidase (HRP)-conjugated cholera toxin (enhanced chemiluminescence detection). B, bottom of gradient; H, initial homogenate; P, pellet from initial centrifugation; R, purified raft fraction; S, supernatant from initial centrifugation. Note the enhanced labeling of the raft fraction with HRP-conjugated cholera toxin relative to the other fractions. In parallel, each fraction (20 μg of protein) was subjected to one-dimensional SDS-PAGE and Western blot analysis, probing with antibodies specific for either CD90 or Na^+,K^+-ATPase (enhanced chemiluminescence detection). Note the selective detection of CD90 in the raft fraction and the absence of detectable Na^+,K^+-ATPase in that same fraction.

Other methods

The protein content of subcellular fractions was determined using BCA reagent (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. Lipids were extracted using the method of Bligh and Dyer (26). The organic phase was analyzed for phosphorus according to Ames (27), and aliquots were subjected to TLC as described above. Cholesterol, 7DHC, and dolichol (DOL) were also assayed in the lipid extract. Aliquots were subjected to saponification and extraction and analyzed by reverse-phase HPLC according to Keller (28).

RESULTS AND DISCUSSION

7DHC is compatible with lipid raft formation

To compare the ability of 7DHC, relative to that of cholesterol, to incorporate into rafts, we prepared liposomes in 2 ml of PBS containing brain PC, SM, and CB in the molar ratio 2:1:1 and added either no sterol or increasing amounts of cholesterol, 7DHC, or coprostanol. In these experiments, the amount of total lipid remained constant (270 nmol). The yield of solubilized lipid was invariant as measured by light absorbance at 600 nm (~0.15 absorbance units). After the addition of Triton X-100 to give a final concentration of 1.0% (v/v), the absorbance of the samples at 600 nm was again determined. **Figure 2** shows

TABLE 1. Composition of homogenate and raft fractions from control and AY9944-treated rat brains with regard to total protein, cholesterol, 7DHC, and DOL content, as described in Methods

Sample	Fraction	Cholesterol	7DHC	Ratio 7DHC/Cholesterol	DOL	Ratio DOL/Cholesterol
		μg/mg protein			*μg/mg protein*	
Control No. 1	Homogenate	94.4	ND	–	0.17	1.8
	Rafts	456			0.075	0.16
Control No. 2	Homogenate	100	ND	–	–	
	Rafts	605			–	
AY9944 No. 1	Homogenate	44.3	132	3.0	–	
	Rafts	132	401	3.0	–	
AY9944 No. 2	Homogenate	18	52	2.9	–	
	Rafts	141	384	2.7	–	

AY9944, *trans*-1,4-*bis*(2-dichlorobenzylaminoethyl)cyclohexane dihydrochloride; 7DHC, 7-dehydrocholesterol; DOL, dolichol; ND, not detectable. Note the marked enrichment of sterol in raft fractions compared with their parent homogenates.

that the absorbance for the liposomes containing cholesterol and 7DHC increased in a similar manner as the mol% increased to 33% (i.e., a typical sterol concentration in mammalian plasma membranes). In contrast, liposomes prepared with coprostanol, a sterol previously shown to be incompatible with raft formation (29), did not retain any turbidity after detergent addition. Raft formation was also monitored by the ability to form detergent-insoluble lipid pellets after centrifugation. In a separate experiment, liposomes containing various amounts of cholesterol, 7DHC, or coprostanol were treated with Triton X-100 (final concentration 1%) and centrifuged. The detergent-resistant pellets were dissolved in chloroform, and aliquots were subjected to TLC analysis (see Methods). As shown in **Fig. 3**, the addition of increasing amounts of either cholesterol or 7DHC resulted in the formation of detergent-insoluble raft pellets containing SM, CB, and PC. In contrast, no lipid pellets were obtained with liposomes that contained coprostanol (data not shown).

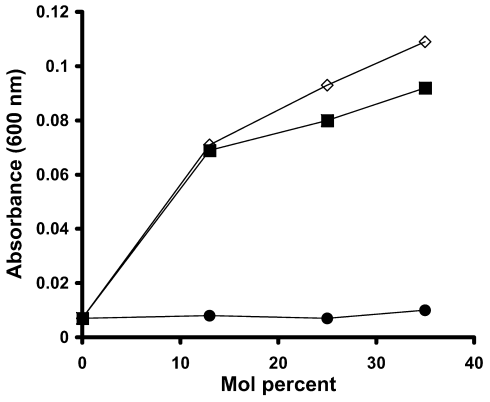

Fig. 2. 7-Dehydrocholesterol (7DHC; closed squares) and cholesterol (open diamonds), but not coprostanol (closed circles), incorporate into lipid rafts in vitro. Liposomes were prepared as described in Methods with increasing amounts of 7DHC, cholesterol, or coprostanol while maintaining a constant amount of 270 nmol of total lipid. Triton X-100 was added to a final concentration of 1% (v/v), and the turbidity was determined by spectrophotometry (absorbance at 600 nm).

We next prepared liposomes containing either cholesterol or 7DHC and then exposed them to increasing temperature while monitoring their turbidity at 600 nm. As shown in **Fig. 4**, the stability of liposomes prepared with 7DHC was very similar to that of liposomes prepared with cholesterol. While this work was in preparation, Xu et al. (30) carried out studies on the ability of sterols other than cholesterol to promote detergent-insoluble domains in vitro. Using a fluorescence-based method and the criterion of detergent resistance, they concluded that 7DHC and ergosterol (both of which contain a Δ^7 double bond

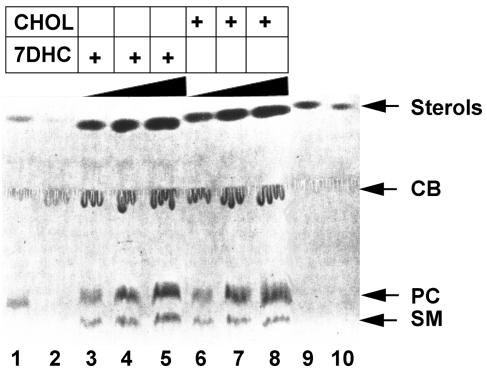

Fig. 3. Comparison of the ability of 7DHC and cholesterol to form detergent-resistant membrane domains (rafts). Sterols at concentrations of 13, 25, and 33 mol% were added to a standard mixture of lipids containing brain phosphatidylcholine (PC), sphingomyelin (SM), and galactocerebroside (CB) in chloroform (see Methods). The samples were dried, sonicated in 2 ml of PBS, and treated with Triton X-100 [final concentration 1% (v/v)]. After centrifugation, the resulting pellets were suspended in chloroform and subjected to silica TLC with a two-step development procedure (see Methods). Note that addition of either 7DHC or cholesterol results in the formation of detergent-resistant, sedimentable rafts, with sterols as well as PC, SM, and CB detected in the pellets. No pellets were obtained when coprostanol was used as the only sterol (not shown). Lane 1, lipid mixture containing brain PC, CB, and SM; lane 2, lipid mixture to which Triton X-100 was added (final concentration 1%) before centrifugation; lanes 3–5, lipid mixture plus 13, 25, and 33 mol% 7DHC, respectively, plus Triton X-100; lanes 6–8, lipid mixture plus 13, 25, and 33 mol% cholesterol, respectively, plus Triton X-100; lane 9, cholesterol standard; lane 10, 7DHC standard.

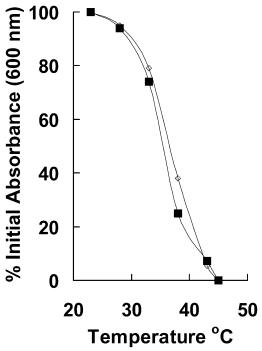

Fig. 4. Comparison of the thermal stability of lipid rafts prepared with cholesterol (open diamonds) and 7DHC (closed squares). Liposomes containing PC, SM, CM, and either 7DHC or cholesterol were prepared as described in Methods and adjusted with Triton X-100 to a final concentration of 1% (v/v). Samples were then placed in a water-jacketed cuvette and subjected to a temperature gradient while monitoring absorbance at 600 nm.

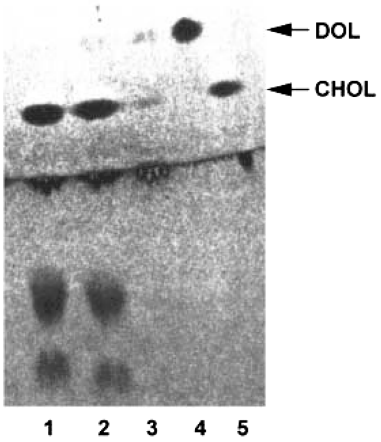

Fig. 5. Dolichol (DOL) does not promote the formation of, or incorporate into, detergent-resistant membrane domains in vitro. Liposomes were prepared with a lipid mixture (see Fig. 2 legend) to which various additions were made (see below), treated with Triton X-100 (final concentration 1%), and centrifuged, and the resulting pellets were subjected to TLC (see Methods). Lane 1, lipid mixture containing 33 mol% cholesterol; lane 2, lipid mixture containing 33 mol% cholesterol plus 3 mol% DOL; lane 3, lipid mixture containing 33 mol% DOL; lane 4, DOL standard; lane 5, cholesterol standard.

in ring B of the sterol nucleus) support the formation of ordered sphingolipid/sterol domains in vitro; in fact, they found that 7DHC promotes raft formation even better than does cholesterol. Thus, although different methods were used, our findings are in good agreement with those of Xu et al. (30). In addition, using Langmuir-Blodgett methodology with PC-sterol monolayer films on an air-aqueous interface, it has been shown (31) that 7DHC is comparable to cholesterol with regard to pressure-area isotherms at 10, 20, and 30 mol% sterol, with 7DHC exhibiting only slightly greater molecular area than cholesterol. This similarity in physical behavior between 7DHC and cholesterol is consistent with the present results.

Nonsterol isoprenoids are not compatible with lipid raft formation

DOL, a long-chain, nonsteroidal isoprenoid alcohol that is more than twice as long as a typical biological membrane is wide, has been claimed to form hexagonal II phase domains in membranes (32). To determine if DOL is compatible with raft formation, we added DOL to a PC-SM-CB liposome preparation at a final concentration of 33 mol%, as described above (see Methods). As shown in **Fig. 5**, DOL at this concentration did not result in the formation of rafts; rather, DOL was unable to enter rafts that were induced by cholesterol incorporation. It should be noted that because the molecular weight of DOL is approximately three times that of cholesterol, 3 mol% DOL would have been detectable easily in the TLC system used if it had been present in the pellets. We also tested whether squalene, a 30-carbon hydrocarbon precursor of sterols, could induce the formation of rafts in PC-SM-CB mixtures. As with DOL, no rafts were formed when squalene was added at 33 mol% (data not shown). In addition, when [^{14}C]squalene was added to a liposome preparation that contained 33 mol% cholesterol followed by treatment with 1% Triton X-100 and centrifugation, the vast majority (88%) of the squalene remained in the supernatant. In contrast, >85% of the cholesterol was found in the pellet. From these data, we conclude that the isoprene chain per se is not sufficient to promote the formation of lipid rafts; rather, only isoprenoids containing a cyclized sterol nucleus are compatible with raft formation. This conclusion is consistent with other studies that have shown that few proteins isolated from the membrane raft fraction of biological tissues are isoprenylated (33).

Analysis of rafts in a rat model of SLOS

To determine if 7DHC is compatible with the formation of membrane rafts in biological tissues, we analyzed brains from AY9944-treated rats with regard to their content of rafts and compared them with brains from age-matched control rats. This rodent model has been shown to yield 7DHC/cholesterol mole ratios in the same range as those found in tissue specimens from human SLOS patients (20). Brain rafts were prepared by sucrose gradient ultracentrifugation after Triton X-100 treatment and then diluted, and the detergent-insoluble pellets were collected by ultracentrifugation. Aliquots of resuspended raft pellets were extracted with chloroform-methanol solutions and analyzed for their lipid composition (see Methods). There was no detectable difference in the pattern of polar lipids as revealed by TLC (data not shown). Analysis of the neutral lipid fraction by reverse-phase HPLC in duplicate samples (Table 1) indicated that, for samples isolated from brains of AY9944-treated rats, the 7DHC/cholesterol mole ratio was the same in the raft fraction as in the homogenate; also, the 7DHC/protein and cholesterol/protein ratios were increased comparably in rafts relative to their parent homogenates. These data indicate that there is no preferential exclusion of 7DHC from rafts compared with cholesterol, consistent with the in vitro data de-

scribed above. The increased sterol-protein ratio is consistent with the well-described enrichment of sterols in membrane rafts relative to the membrane bulk phase. The fact that the relative enrichment of sterol in the control rafts (average 5.44-fold) is comparable to that of rafts from AY9944-treated rat brains (average 7.85-fold) further indicates that 7DHC is similar to cholesterol in its ability to support raft formation.

In addition to analyzing for sterols, we also assayed for DOL in one of the normal raft samples and the homogenate. As shown in Table 1, the DOL/cholesterol mole ratio was substantially lower in the raft fraction compared with the homogenate. This in vivo finding is fully consistent with our in vitro results described above, indicating that DOL tends to be excluded from membrane rafts.

To determine if the protein composition was affected by the AY9944 treatment, we carried out two-dimensional PAGE-isoelectric focusing analysis of brain raft fractions from two control and two AY9944-treated rats. Silver staining of these gels confirmed that the replicate samples for a given treatment regimen had essentially identical protein patterns (data not shown). However, the protein profiles of the raft samples from AY9944-treated rat brains (**Fig. 6B**) were significantly different from those of the control samples (Fig. 6A). For example, focusing on one rectangular region of each gel (Fig. 6, insets), two protein spots (arrows 2 and 3) are clearly present in the control gel (inset in A) but are absent in the gel corresponding to the AY9944-treated specimen (inset in B). Conversely, protein components that are nearly absent (arrow 1) or relatively lightly stained (arrow 4) in the control gel are much more intensely stained in the gel of the drug-treated specimen. Preliminary densitometric analysis of these gels has indicated that there are multiple proteins, of as yet undetermined identity, whose levels differ by at least 3- to 5-fold when comparing control brain rafts and rafts from AY9944-treated rats. A detailed analysis of the raft proteome in AY9944-treated and control adult rat brains is currently in progress, but is beyond the scope of the present report. In this regard, it should be noted that the proteome of lipid rafts prepared from HeLa cells was reported recently (34) and was shown to be highly enriched in signaling molecules relative to the total membrane fraction.

There are several possible explanations for the difference in protein composition in rafts from brains of control versus AY9944-treated animals. One possibility is that AY9944 itself somehow alters gene expression directly, independent of its ability to alter sterol composition. To determine if the administration of AY9944 at the current dose altered gene expression in rat brain, we also carried out two-dimensional PAGE-isoelectric focusing analysis of the homogenate fractions from control and AY9944-treated rat brains. As shown in **Fig. 7**, there were no gross differences apparent in the two-dimensional protein profiles of whole brain homogenates as a function of drug treatment. As expected, the general protein pattern of whole brain homogenates was different from and more complex than that of the rafts. Although we cannot rule out subtle differences in protein levels, these results suggest that there is no gross alteration of gene expression brought about by the AY9944 treatment.

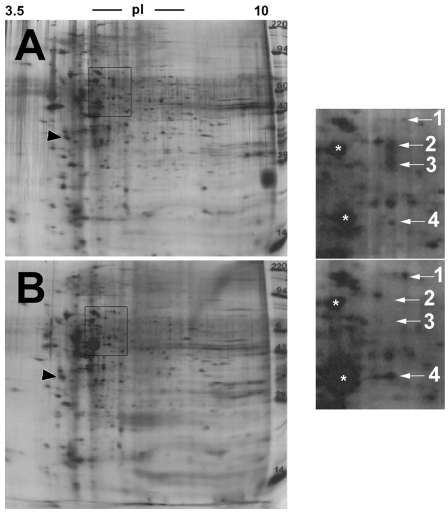

Fig. 6. Two-dimensional gel electrophoresis profiles of brain raft proteins from control rats (A) and *trans*-1,4-*bis*(2-dichlorobenzylaminoethyl)cyclohexane dihydrochloride (AY9944)-treated rats (B). Comparable protein loads (100 μg of total protein) were applied to each gel. After silver staining, gel images were digitized using a flatbed scanner and subjected to computer-aided image analysis. The arrowheads denote the internal protein standard (tropomyosin, 50 ng). The panels at right show higher magnification views of selected regions of the gel images (insets in A and B), illustrating specific protein component differences (denoted with numbered arrows) between rafts from AY9944-treated rats (B) versus control rats (A). Spots denoted with asterisks are geographic markers to aid in comparison between gel images. See Methods for details.

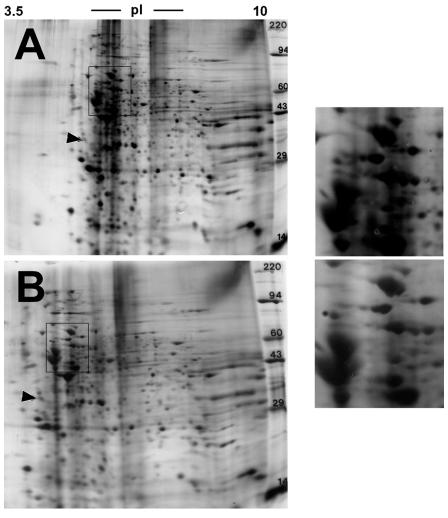

Fig. 7. Two-dimensional gel electrophoresis profiles of whole brain homogenate proteins from control rats (A) and AY9944-treated rats (B). See Fig. 6 legend for details. The panels at right show higher magnification views of selected regions of the gel images (insets in A and B) corresponding to the same regions highlighted in Fig. 6. Note the lack of gross differences between the gel images as a function of AY9944 treatment.

A second possible reason for the difference in raft protein composition brought about by AY9944 is that by changing the cellular sterol composition, the activities of proteins that are involved in (for example) signal transduction, gene expression, posttranslational modification, protein turnover, or trafficking are altered. Possible candidates are those proteins that possess sterol-sensing domains (SSDs) (35), which include proteins involved in sterol-regulated transcription [i.e., sterol regulatory element binding protein (SREBP), SREBP cleavage-activating protein (SCAP) (36)], cholesterol trafficking (37), and cholesterol-dependent patterning in early development (38). There are no reports documenting how 7DHC might interact with the SREBP-SCAP system, and, as mentioned above, we saw no major change in protein expression as a function of AY9944 treatment. Related to feedback control of sterol biosynthesis, a recent study claims that 7DHC specifically induces the turnover of HMG-CoA reductase in the *DHCR*7-null mouse (39). Regarding sterol trafficking, Wassif et al. (40) have reported that SLOS fibroblasts failed to degrade LDL at a normal rate and that these cells exhibited formation of lysosomal inclusions similar to those seen in Niemann-Pick type C (NPC) cells, in which cholesterol trafficking from the lysosome is impaired. These workers proposed that 7DHC may directly or indirectly inhibit the function of the NPC1 protein through its SSD and that 7DHC may perturb the function of other SSD-containing proteins in SLOS. With regard to development, cholesterol is covalently attached to Sonic hedgehog (Shh) (41), a signaling protein involved in embryogenesis, and cholesterol has been shown to be involved in Shh trafficking and signal transduction (42, 43). In the context of the current results, because AY9944 was administered at doses below those that cause teratology, and because the animals exhibited no gross morphological defects with the exception of reduced body weight, it is unlikely that the differences in raft protein composition are connected to the Shh-related requirement for cholesterol in development. It is noteworthy in this regard that Wassif et al. (44), using a "knock-out" mouse model of SLOS, reported that the response of frontal cortex neurons to the neurotransmitter glutamate was significantly impaired. However, they could not detect any alteration in the expression of glutamate receptor subunits.

The altered cholesterol synthesis brought about by AY9944 may also affect the levels of neurosteroids, a possibility raised by Wassif et al. (44) in the study mentioned above. Given the broad range of functions attributable to these compounds (45), i.e., modulation of γ-aminobutyrate-A and *N*-methyl-D-aspartate function, modulation of sigma receptor function, regulation of myelinization, neuroprotection, and growth of axons and dendrites, it is reasonable to consider that alteration of their metabolism could affect either the levels or activities of specific proteins in the central nervous system.

Another possible reason for the altered raft protein patterns is that the increased levels of 7DHC do not affect gene expression per se but rather change the milieu of the raft to such an extent that some proteins are not incorporated at their normal levels (either less than or greater than normal). Examination of this possibility will require identifying the different proteins and monitoring their presence in the various subcellular fractions using immunochemical methods.

Taken together, these results indicate that 7DHC can substitute for cholesterol with regard to its ability to support raft membrane domain formation in vivo. The overall yield of raft protein and the general polar lipid profile were comparable in brain rafts isolated from both control and AY9944-treated rats, despite the marked difference in sterol composition of those brain specimens. It is worthy of note that the steady-state level of 7DHC in normal adult rat brain is below the level of detection by our methods (<1 μg/g wet weight). Nevertheless, the protein pattern in brain rafts, but not brain homogenates, from AY9944-treated animals was clearly different from that of controls. It is possible that one or more of the protein profile differences observed on our two-dimensional gels of rat brains may be relevant to some of the neurological defects associated with SLOS. Also, there may be more subtle differences not detectable using the staining methods applied here. Future studies will be directed at unequivocal identification of those proteins that exhibit significantly different levels in brain rafts in AY9944-treated versus control samples. From the function of these proteins, if known, it may be possible to gain further insights into the molecular cause of the observed SLOS defects.

Finally, the current findings may have broader relevance within the context of processes underlying other diseases that profoundly affect the nervous system. For example, the generation of amyloid-β, the peptide considered to be primarily responsible for the formation of plaques associated with Alzheimer's disease (46), occurs via the cleavage of amyloid precursor protein by two secretases, β and γ. The β-secretase is thought to occur in lipid rafts, and it has been proposed that reduction of brain cholesterol in lipid rafts shifts the processing of amyloid precursor protein to the nonamyloidogenic α pathway (47). Hence, amyloidogenic processing of the Alzheimer amyloid-β precursor protein appears to integrally involve lipid rafts. Refolo et al. (48) have reported that administration of BM15.766, a compound pharmacologically similar to AY9944 in that it inhibits 7DHC reductase and causes the accumulation of 7DHC, reduces levels of amyloid-β in a mouse model of Alzheimer's disease. Our data suggest that it is unlikely that BM15.766 acts by altering the physical properties of the neural raft, because 7DHC appears to be able to replace cholesterol in the formation of lipid rafts. However, as shown by our two-dimensional gels, the presence of 7DHC can affect the protein composition of the raft. In this regard, it should prove interesting to determine if the presence of β-secretase is altered in the brain raft membranes from control and AY9944-treated animals.

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