Fluorescence and Multiphoton Imaging Resolve Unique Structural Forms of Sterol in Membranes of Living Cells*

Avery L. McIntosh, Adalberto M. Gallegos, Barbara P. Atshaves, Stephen M. Storey, Deepak Kannoju, and Friedhelm Schroeder‡

From the Department of Physiology and Pharmacology, Texas A & M University, Texas Veterinary Medical Center, College Station, Texas 77843-4466

Received for publication, June 3, 2002, and in revised form, November 19, 2002 Published, JBC Papers in Press, November 26, 2002, DOI 10.1074/jbc.M205472200

Although cholesterol is an essential component of mammalian membranes, resolution of cholesterol organization in membranes and organelles (i.e. lysosomes) of living cells is hampered by the paucity of nondestruc-
tive, nonperturbing methods providing real time structural information. Advantage was taken of the fact that the emission maxima of a naturally occurring fluorescent sterol (dehydroergosterol) were resolvable into two structural forms, monomeric (356 and 375 nm) and crystalline (403 and 426 nm). Model membranes (sterol:phospholipid ratios in the physiological range, e.g. 0.5–1.0), subcellular membrane fractions (plasma membranes, lysosomal membranes, microsomes, and mitochondrial membranes), and lipid rafts/caveolae (plasma membranes, cholesterol-rich microdomain purified by a non-detergent method) contained primarily monomeric sterol and only small quantities (i.e. 1–5%) of the crystalline form. In contrast, the majority of sterol in isolated lysosomes was crystalline. However, addition of sterol carrier protein-2 in vitro significantly reduced the proportion of crystalline dehydroergosterol in the isolated lysosomes. Multiphoton laser scanning microscopy (MPLSM) of living L-cell fibroblasts cultured with dehy-
droergosterol for the first time provided real time images showing the presence of monomeric sterol in plasma membranes, as well as other intracellular mem-
brane structures of living cells. Furthermore, MPLSM confirmed that crystalline sterol colocalized in highest amounts with LysoTracker Green, a lysosomal marker dye. Although crystalline sterol was also detected in the cytoplasm, the extralysosomal crystalline sterol did not colocalize with BODIPY FL C5-ceramide, a Golgi marker, and crystals were not associated with the cell surface membrane. These noninvasive, nonperturbing methods demonstrated for the first time that multiple structural forms of sterol normally occurred within membranes, membrane microdomains (lipid rafts/caveolae), and intracellular organelles of living cells, both in vitro and visualized in real time by MPLSM.

Cholesterol is essential for optimal membrane transport, receptor-effector coupling, cell recognition, and other eukary-

* This work was supported in part by United States Public Health Service Grant GM31651 from the National Institutes of Health. 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.
† To whom correspondence should be addressed: Dept. of Physiology and Pharmacology, Texas A & M University, TVMC, College Station, TX 77843-4466. Tel.: 979-862-1433; Fax: 979-862-4929; E-mail: Fschroeder@cvm.tamu.edu.

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 8, Issue of February 21, pp. 6384–6403, 2003

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 8, Issue of February 21, pp. 6384–6403, 2003

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 8, Issue of February 21, pp. 6384–6403, 2003

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 8, Issue of February 21, pp. 6384–6403, 2003

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
hydroergosterol (DHE), differ in monomeric and crystalline forms (26–30). DHE consists of up to 20% membrane sterol in yeast and sponge membranes, is taken up from the culture medium of microorganisms and cultured fibroblasts, codistributes with endogenous sterol among intracellular membranes (replaces nearly 90% of endogenous membrane cholesterol), and does not alter cell growth, membrane structure, or function of sterol-sensitive membrane proteins (reviewed in Refs. 1, 2, 26, and 31). The structural, transfer, and functional properties of DHE closely mimic those of cholesterol in lipoproteins and membranes (1, 2, 22, 26). Second, the development of new fluorescence imaging technologies, i.e. multiphoton laser scanning microscopy (MPLSM), now makes it possible to resolve multiple membrane and cellular forms of cholesterol in living cells (32, 33). Because DHE absorption occurs in the ultraviolet region, single photon excitation (used in conventional, video, and confocal imaging microscopy) results in significant photo-bleaching and phototoxicity (32, 34). In contrast, MPLSM utilizes infrared radiation to overcome these problems (32, 33) and, as shown herein, now provides high resolution images of multiple structural forms of sterol within living cells.

EXPERIMENTAL PROCEDURES

Materials—Sucrose was purchased from Sigma. Cholesterol and ergosterol were obtained from Steraloids (Newport, RI), 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine was from Avanti Polar Lipids (Alabaster, AL). Stock cholesterol (10 mg/ml) solutions were prepared in 95% ethanol with 1 mol % butylated hydroxytoluene and stored at −70 °C. Human recombinant sterol carrier protein-2 was prepared as described earlier (35). Anti-caveolin-1 and anti-flotillin-1 antisera were obtained from Affinity Bioreagents (Golden, CO) and Transduction Laboratories (San Jose, CA), respectively. 1,6-Diphenyl-1,3,5-hexatriene (DPH), LysoTracker Green, BODIPY FL C12-mercapto, and Nile Red were obtained from Molecular Probes (Eugene, OR). Dehydroergosterol (Δ5,7,9(11),22-ergostatetraen-3β-ol) was synthesized from ergosterol (Steraloids, Newport, RI) (36) or purchased from Sigma and further purified by high performance liquid chromatography (HPLC) (36). DHE was prepared in 100% ethanol as a stock solution (5 mg/ml) containing 1 mol % butylated hydroxytoluene and stored at −70 °C. All solvents were HPLC grade or better, and aqueous buffers herein described will be filtered 10 μm PES, pH 7.4.

Cell Culture, Cellular Subfractionation, and Membrane Isolation—L-M1 fibroblasts were obtained and cultured as described earlier (37). Cells were cultured for 2 days at 37 °C in a CO2 incubator with Higuchi medium containing 10% fetal bovine serum supplemented with DHE (20 μg/ml medium) (38). Cells were subfractionated to obtain plasma membranes (38), endoplasmic reticulum (2), lysosomes (39), mitochondria (39), and lipid droplets (41). Lysosomal membranes were resolved from DHE crystalline material and used in the live cell assay in which lytic membrane by lysis of the membrane with a lysis buffer (90 mm NaCl, 1.6 mm MgCl2, pH 5.5) and centrifugation for the lack of polarization change in the absence of acceptor (see Results). The relative enrichment of the lipid raft/caveolar membrane fraction was determined by quantitative Western blotting using antibodies to caveolin-1 and flotillin-1, basically as described for other membrane markers (39).

Measurement of Force-area Isotherms of Pure and Mixed Monolayers—Pure cholesterol or dehydroergosterol monolayers or mixed monolayers containing varying amounts of sterol together with 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine were compressed on 140 mm NaCl substrates at 22 °C (under an argon atmosphere in the dark) with a KSV surface barostat (KSV Instruments Ltd., Helsinki, Finland). The barrier speed during compression did not exceed 3.4 Å/molecule/min. Isotherms at a surface pressure of 35 mN/m were recorded using proprietary KSV software (44).

Model Membrane Vesicle Preparation—Small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) were prepared to contain 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), dehydroergosterol (DHE), and cholesterol in the following indicated proportions (45): 65:35:0 and 65:5:30 (SUV and LUV); 40:60:0, 33:67:0, and 25:75:0 (LUV), the median radii of SUV and LUV curvature were 15 ± 3 and 53 ± 10 nm, respectively, determined by a Coulter N4 Plus Photon Correlation Spectrometer (Beckman Instruments, Miami, FL).

Spectral Properties of DHE in Solvents and Membranes—Absorbance spectra were obtained with a Lambda 2 Dual Wavelength Spectrophotometer (PerkinElmer life sciences). Steady-state fluorescence excitation and emission spectra were obtained with a PC1 Photon Counting Fluorometer (ISS Instruments, Champaign, IL). Spectra were analyzed with Grams22 Thermo Galactic, Salem, NH. Light scatter was avoided by use of narrow monochromator slits, low concentrations, and appropriate cut-off filters. Any Raman scattering was subtracted from all excitation and emission spectral data. Artifacts because of inner filter effects were avoided by keeping the absorbance of sample solutions at the excitation wavelength (324 nm) below 0.15. All absorbance measurements were performed in 1 ml or 3.5 ml quartz cuvettes. Fluorescence measurements of organelles and associated membranes were performed with samples in 2 ml of filtered 10 mm PIPES buffer, pH 7.4, in a quartz cuvette with the temperature regulated at 37 ± 0.3 °C through use of a water heating bath (Fisher). Six integrated intensity measurements (325–600 nm) were performed on concentrations of DHE in 10 mm PIPES, pH 7.4, and 100% ethanol, ranging from 2.5 to 10 μm.

Aqueous Solutions of DHE and Cholesterol for Micelle Determination—Solutions of 2.5 and 10 μM cholesterol or DHE were prepared in 95% EtOH and evaporated onto glassware under dry nitrogen. Each of the deposed sterols was then redissolved in 10 mm PIPES buffer at 70 °C under heavy vacuum. Observation of portions of these solutions was made with a single pass through either an Avanti Mini-Extruder with a 0.1-µm polycarbonate membrane (Whatman) or a 0.5-µm filter (Millipore, Bedford, MA). Similarly, DPH in tetrahydrofuran was evaporated onto glassware followed by addition of the unfiltred and filtered sterol solutions and intense vortexing. The sterol/DPH ratio was maintained at 290:1. Fluorescence emission spectra were obtained as described above.

Determination of DHE Steady-state Polarization during Exchange Assays—Steady-state fluorescence polarization measurements of DHE in plasma membranes, endoplasmic reticulum, mitochondria, lysosomes, and lysosomal membranes at 37 °C were performed as described earlier (39–46). Residual light scatter (from both donor and acceptor membranes) contribution to polarization data was corrected by converting polarization to anisotropy according to the equation r = 2P(3 − P), and subtracting the residual fluorescence anisotropy of both donor and acceptor membranes (i.e. not containing DHE) from all experimental data. Absorbance (324 nm) of sample solutions in 10 mm PIPES buffer, pH 7.4, was kept below 0.1.

Lysosomal Sterol Transfer—Sterol transfer between isolated lysosomes was determined using a fluorescent sterol (DHE) exchange assay as described previously (39, 46). The basis of the assay (release from self-quenching of DHE in the donor lysosome and transfer to acceptor lysosome lacking DHE), validation of DHE as a probe for cholesterol (47), and depth descriptions of the assay, protocol, and justification for the lack of polarization change in the absence of acceptor membranes were provided in the above cited publications. Standard curves for DHE in lysosomal-lysosomal membrane exchanges were determined earlier (39, 46).
Multiphoton Laser Scanning Microscopy (MPLSM) and Image Analysis—MPLSM of DHE, Nile Red, LysoTracker Green, and BODIPY FL C_2-ceramide was performed on intact L-cells (L arpt 'tk') cultured on two-well Lab-Tek chambered cover glasses (VWR, Sugarland, TX). Prior to imaging, cells were supplemented for 2 days with the addition to the medium of 20 mg/ml DHE, either from a 5 mg/ml stock solution of DHE in anhydrous ethanol or with 10 mg/ml hexadecyltrimethylammonium bromide (HHT) and DHE (prepared as described above). For colocalization experiments of DHE with Nile Red, the cells cultured with DHE were washed with Puck's buffer and then incubated with 100–400 μM Nile Red for ~30 min. The concentration was tiered to different levels in this range depending upon the amount of excitation power used in the multiphoton imaging process described below. For imaging with LysoTracker Red L-cells were grown to confluence on a coverslip and washed several times with Puck's buffer. To ascertain crystalline DHE and Golgi colocalization, BODIPY FL C_2-ceramide was purchased as already made complexes with bovine serum albumin. Murine L-cells with DHE (20 μg/ml medium) were grown on chambered cover glasses for 2 days and then washed with a Hanks' buffered saline solution (HBSS) with 10 mg/ml HEPES, pH 7.4, subsequently termed HBSS/HEPES. After washing, the cells were loaded for 30 min with 5 μM BODIPY FL C_2-ceramide-bovine serum albumin at 4 °C in HBSS/HEPES medium. Afterward, the medium was removed, and the cells were washed with HBSS/HEPES at 4 °C. After replacing the medium, the chambered cover glass was then placed back in the incubator for 30 min at 37 °C. The cells were once again washed with HBSS/HEPES medium and immediately imaged under multiphoton excitation.

MPLSM fluorescence imaging was performed (33) using an MRC1024 Multifluochromat Laser Scanning Microscope controlled by LaserSharp software and equipped with an external descanned 3 detector unit (Bio-Rad) (47). Briefly, the excitation source was a femtosecond Coherent Mira 900 mode-locked Ti:Sapphire laser with broadband optics delivered at 1 kHz to a Coherent F-200 argon ion laser (Coherent, Palo Alto, CA). The excitation light (900–930 nm) was delivered to an Axiovert 135 (Zeiss Inc., New York, NY) microscope stage via a modified epiluminescence light path. A Zeiss 63× Plan-Apochromat (1.4 N.A.) or 100× Fluar (1.3 N.A. with higher transmittance between 350 and 400 nm) oil immersion objective was used for all images. Selection of 900–925 nm as the multiphoton excitation wavelength range allowed simultaneous excitation of crystalline DHE and monomeric DHE (three-photon excitation), with either Nile Red, LysoTracker Green, or BODIPY FL C_2-ceramide (two-photon excitation). The fluorescence emission of the respective probe molecules was collected by the same objective and passed into the Bio-Rad external detector unit where the emission was analyzed separately at different wavelengths and collected by three photomultiplier tubes (PMT) as follows: (i) multi-tialkali PMT1 emitted collection through a D455:30-nm filter; (ii) multi-tialkali PMT2 collected emission through a HG575:150-nm filter; (iii) bi-alkali PMT3 collected emission through a D755:50-nm filter. All filters and dichroics used in detection were from Chroma Technology (Brattleboro, VT). Images (Kalman filtered) were analyzed and presented using a combination of software packages including MetaMorph Image Analysis Software (Advanced Scientific Imaging, Meraux, LA), Adobe Photoshop 5.0 (San Jose, CA), CorelDraw 9 (Ottawa, Ontario, Canada), and National Instruments LabView 6i equipped with IMAQ Vision 5.0 (Austin, TX). MPLSM of autofluorescence in L-cells was observed after each experiment at the respective excitation power and wavelength as used for collecting probe fluorescence. The gain and black levels of each photomultiplier tube were optimized to minimize the autofluorescence signal and to maximize the fluorescence signal in the probe-supplemented cells. Any residual autofluorescence signal at high detection sensitivity in the 375-500-nm channel was subtracted.

To determine structural properties of DHE in membranes of living cells, it was essential that DHE be free of contaminants. A commercially available DHE as well as DHE freshly synthesized and purified herein (see “Experimental Procedures”) were examined. Absorbance spectra of the two preparations differed only slightly in the region where DHE displayed a peak maxima near 311, 324, and 340 nm (Fig. 1A). However, below 275 nm the absorbance of the two preparations deviated significantly, indicating the presence of impurities. HPLCs of commercially available DHE (10 μg/10 μl solvent) revealed four peaks (Fig. 1B). However, only peak 3 (representing 83% of total) exhibited absorption characteristics of DHE. Peaks 1 and 2 were sample impurities, whereas peak 4 was due to a small solvent impurity. In contrast, the DHE synthesized and purified as indicated under “Experimental Procedures” was 98% pure as indicated by HPLC (Fig. 1C). When Peak 3 (pure DHE in Fig. 1C) was pooled from a number of HPLC runs, dried under nitrogen, and reinjected on the HPLC column, the resultant DHE was 99.7% pure (Fig. 1D, peak 3).

Absorbance Spectral Properties of DHE in Ethanol and in Aqueous Buffer—Although DHE is monomeric at low concentrations in ethanol, aggregation of DHE occurs in aqueous buffers due to the low critical micellar concentration (20–30 mM for cholesterol and DHE) of sterols (26–30). Absorbance spectral properties of DHE in aqueous buffers (10 μM in 10 mM PIPES, pH 7.4) differed significantly from those of monomeric DHE (10 μM in ethanol). In ethanol DHE displayed maxima at 311, 324, and 340 nm (Fig. 2A). In contrast, DHE in aqueous buffer (Fig. 2C) exhibited the following differences. (i) The absorption spectrum in aqueous buffer was slightly broader with maxima (314, 329, and 347 nm) that were red-shifted by 3, 5, and 7 nm from those in ethanol. (ii) The absorption peak profile of DHE in aqueous buffer was significantly altered. The ratio of absorption maxima at 329:314 nm was 1.03, signifi-
cantly lower than that for absorption maxima at 324:311 nm in ethanol near 1.14. (iii) The molar extinction coefficient of DHE in 10 mM PIPES, pH 7.4 (ε<sub>324</sub> = 5500 M<sup>-1</sup> cm<sup>-1</sup>) was 2.4-fold lower than for DHE in anhydrous ethanol (ε<sub>324</sub> = 13,000 M<sup>-1</sup> cm<sup>-1</sup>), both measured at 324 nm.

Fluorescence Emission Characteristics of DHE in Ethanol and in 10 mM PIPES Buffer, pH 7.4—The fluorescence emission spectral properties of DHE in ethanol and aqueous buffer differed even more than the absorption spectra. The fluorescence emission of DHE in ethanol (Fig. 2B) was Stokes shifted ~46 nm as compared with the absorption spectrum (Fig. 2A), essentially the same as that obtained for DHE in other alcohols (1-butanol, 2-propanol, and methanol) (28). The fluorescence emission spectrum of DHE in ethanol (Fig. 2B) was a near mirror image of the absorption spectrum (Fig. 2A) and exhibited vibrationally resolved fluorescence emission maxima near 354, 370, and 390 nm (Fig. 2B). In contrast, the aqueous fluorescence emission spectrum of DHE was consistent not only with the presence of monomeric DHE spectral features as observed for DHE in ethanol (Fig. 2B) but also indicated the appearance of a completely new fluorescent entity differing markedly from that in ethanol (Fig. 2B) as follows. (i) DHE emission maxima (356 and 375 nm) were red-shifted as compared with those in ethanol. (ii) New fluorescence emission maxima appeared at 403 and 426 nm (Fig. 2C). (iii) The relative emission intensity of the new emission maxima was severalfold greater than those at 356 and 375 nm (Fig. 2D). This increased intensity was at some expense of the DHE emission at 356 and 375 nm because their intensities were 0.65 that of the emission maxima in ethanol (Fig. 2B). However, the overall integrated intensity of the emission from the DHE in 10 mM PIPES was 2.7 ± 0.2 greater than that in ethanol. (iv) The quantum yield (88) of DHE in 10 mM PIPES, pH 7.4, was increased 6-fold from 0.04 in ethanol (56) to 0.25 ± 0.02, due to the overall increase in integrated emission and decrease in absorption.

A fluorescence emission spectrum of the new DHE fluorescing species, formed in 10 mM PIPES, pH 7.4 (Fig. 2B), was resolved from that of the monomeric DHE by subtraction of the normalized fluorescence emission spectrum of DHE in ethanol from that in aqueous buffer. The new spectral profile of DHE exhibited fluorescence emission maxima at 403 and 426 nm, with a slight shoulder at 480 nm. The appearance of this new fluorescence could be due to an impurity in the DHE preparation. Although this may potentially be the case in studies where partially pure DHE (e.g. Fig. 1, A and C) was used, in the present investigation the DHE was highly purified (99.7% pure, Fig. 1D). In contrast to the commercially available preparation, the highly purified DHE did not contain significant impurities. These data indicated that the DHE species emitting maximally at 426 nm in PIPES, pH 7.4, was not due to a contaminant in the DHE preparation, but rather it was due to the presence of aggregating DHE in the form of microcrystals or micelles. That the DHE was present primarily as microcrystals, rather than micelles, was resolved as follows.

First, aqueous solutions of DHE or cholesterol were examined under polarized light microscopy using the Zeiss Axiovert Microscope with 63× oil objective. Clearly visible under cross-polarization of DHE solutions were small birefringent crystals. For comparison, aqueous solutions of cholesterol were also made and examined using the same technique. Under these conditions, small birefringent crystals of cholesterol monohydrate were detected, confirming earlier results of others (49). Importantly, the DHE microcrystals were similar in size and shape as that those of cholesterol.

Second, aliquots of the aqueous solutions of DHE (2.5 and 10 μM) were filtered through 0.1-μm filters, which would allow passage of micelles (but not microcrystals) with sizes on the order of 4 nm. Emission spectra over the region 350–600 nm of the aqueous solutions of DHE were recorded before and after filtration using an excitation wavelength of 324 nm. Before
filtration, the spectrum of DHE showed the new fluorescing species as shown in Fig. 2D. However, after filtration no spectral emission was observed above the background noise. By increasing the slit sizes on the monochromator, sensitivity was increased but without any detectable DHE emission. In an effort to determine whether there are possible contributions from larger aggregates of micelles, the filtration was repeated using a 0.5-μm filter. Once again no DHE emission was detected in the filtrate.

Third, the fluorescence probe DPH was used to detect the presence of micelles. In order to excite DPH and not DHE, the excitation wavelength was changed to 369 nm, and emission spectra were recorded over the range 385–600 nm. DPH was added to the unfiltrated and filtrated solutions of DHE as described under “Experimental Procedures.” Initially, a spectrum was obtained on DPH in buffer without DHE to determine any background level of DPH emission. No detectable emission was observed. Next, the spectra of DPH added to the DHE solutions before and after filtration were obtained, also showing no DPH emission. As a control, similar concentrations of cholesterol in aqueous buffer were prepared and filtered similarly to that of the DHE. As was the case with DHE, no DPH emission was detected for cholesterol.

These experiments suggest that the spectral emission of the unfiltered solutions of DHE is derived from microcrystals larger than 0.5 μm. This is supported by comparison of spectral characteristics of aqueous dispersions of DHE with those of DHE in the form of crystalline powder (30). Although the purity of the latter preparation of DHE is unknown, the fluorescence emission spectrum and lifetime components of this crystalline powder were remarkably similar to that shown for the new aqueous form (Fig. 2D). In summary, the fluorescence emission spectrum of the new, microcrystalline form of DHE appearing in 10 mM PIPES, pH 7.4, was distinct from that of monomeric DHE appearing exclusively in ethanol.

**Stability of Crystalline DHE in Aqueous Media**—The stabili...
DHE was 10 μM in solvents and model membranes. Where indicated, values represent the mean ± S.E., n = 5–7. LUV refers to large unilamellar vesicle membranes. SUV refers to small unilamellar vesicle membranes. All membrane spectra of DHE emission were obtained in 10 mM PIPES, pH 7.4. Control L-cell fibroblasts were cultured with 10% fetal bovine serum medium containing 20 μg/ml DHE, and subcellular organelles and lysosomes were isolated as described under “Experimental Procedures.” ND, not determined.

Table I

| Solvent/membrane          | DHE, % crystalline | Emission peak ratio |
|---------------------------|--------------------|---------------------|
|                           |                    | 426/355 nm          | 426/373 nm          |
| Solvent                   | Solvent            | 0.42 ± 0.01         | 0.298 ± 0.004      |
| 10 mM PIPES, pH 7.4       | 0                  | 4.8 ± 0.1           | 3.4 ± 0.2          |
| 10% fetal bovine serum (t = 70 h) | 80                | 3.12 ± 0.07         | 2.19 ± 0.05        |
| LUV 65:35:0               | 0.2                | 0.54                | 0.33               |
| LUV 65:5:30               | ND                 | 0.45                | 0.31               |
| LUV 40:60:0               | 7.7                | 0.84                | 0.47               |
| LUV 33:67:0               | 7.1                | 0.78                | 0.45               |
| LUV 25:75:0               | 9.5                | 0.81                | 0.49               |
| SUV 65:35:0               | 6.0                | 0.69                | 0.43               |
| SUV 65:5:30               | ND                 | 0.47                | 0.31               |
| Plasma membranes          | 5.4                | 0.69                | 0.54               |
| Lyosomal membranes        | 6.9                | 0.66                | 0.48               |
| Caveolae (non-detergent)  | 0.3                | 0.47                | 0.33               |
| None                      | 55                 | 2.28                | 1.54               |
| 25% ethanol               | 53                 | 2.28                | 1.49               |
| 50% ethanol               | 31                 | 1.37                | 0.89               |
| >99% ethanol              | ND                 | 0.42                | 0.30               |
| 1.5 μM SCP-2 (t = 0 min)  | 50                 | 1.78                | 1.32               |
| 1.5 μM SCP-2 (t = 270 min)| 3                  | 0.54                | 0.38               |

* Percent crystalline was determined by spectral subtraction as indicated under “Experimental Procedures.”

† DHE donor lysosomes (isolated from cells cultured with 20 μg/ml DHE in the medium as in membranes) in 10 mM PIPES, pH 7.4. At time t = 0 min SCP-2 (1.5 μM) was added, and a DHE emission spectrum was obtained. Another DHE emission spectrum was obtained after 270 min of incubation at 37 °C with 10-fold excess of acceptor lysosomes (containing no DHE).

multiphoton imaging of membrane sterol
energy transfer, the shape of the DHE emission spectrum was unaffected. Consequently, the self-quenching did not account for the presence of the small percentage of crystalline DHE in these membranes.

The fluorescence emission spectrum of DHE in LUV composed of POPC:DHE at 40:60 (i.e. sterol:phospholipid molar ratio of 1.5) exhibited very similar emission maxima near 355, 373, and 395 nm, along with a more distinct shoulder near 426 nm (Fig. 3A) as compared with that at 0.54 DHE:phospholipid ratio. At 1.5 DHE:phospholipid molar ratio, the ratios of intensities of the DHE fluorescence emission peaks at 426:355 and 426:373 nm were 0.84 and 0.47, respectively (Table I). These ratios were significantly higher than those of monomeric DHE in ethanol, higher than for DHE in LUV with 0.54 molar ratio of sterol:phospholipid, and thereby suggested the presence of a small amount of crystalline DHE (Table I). Subtraction of the normalized emission spectrum of DHE in ethanol (normalized to that of 1.5 molar ratio DHE:phospholipid LUV at 373 nm) yielded a difference spectrum, which again exhibited emission maxima (Fig. 3B) characteristic of crystalline DHE in aqueous buffer (Fig. 2E). After correction for the higher quantum yield of crystalline DHE, this indicated that increasing the molar ratio of sterol:phospholipid by 3-fold (from 0.54 to 1.5) increased by 15-fold the amount of crystalline sterol to 7.7% (Table I). Further increment in DHE:phospholipid ratio to 2 and 3 increased the crystalline DHE content to 9.5% (Table I). Thus, emission spectral properties of DHE detected the presence of small amounts (1–10%) of crystalline sterol in LUV membranes composed of a broad range of sterol:phospholipid molar ratios (i.e. 0.54–3.00).

**Force-area Isotherms of Monolayers Formed from DHE or Cholesterol or Mixtures of the Two Sterols with POPC**—In order to examine whether the DHE separation into crystalline form in membrane lipids reflects that of cholesterol, force-area isotherms of DHE and cholesterol in POPC monolayers were compared (Table II) at a surface compression of 35 mN/m, in the range of that typical of lipid bilayers (44, 50). Increasing the mol % of either DHE or cholesterol in the monolayer reduced the mean molecular area (Å²), due to the condensing effect of sterols on phospholipid membranes. At 35 mol % sterol, where no crystalline cholesterol was detected by x-ray crystallography and birefringence (51), the mean molecular area of the monolayer composed of cholesterol and POPC, 43.6 Å², was not statistically different from that composed of cholesterol and POPC, 43.4 Å² (Table II). Because there was no difference in the condensing effects of DHE versus cholesterol in the POPC monolayers, these data are consistent with DHE behaving similarly to cholesterol with regard to formation of crystalline forms in monolayers at high mol % of sterol.

**Fig. 3.** Excitation, emission, and difference emission spectra of DHE in model membrane bilayers. DHE was incorporated into large unilamellar vesicles (LUV, A) or small unilamellar vesicles (SUV, C) as indicated under “Experimental Procedures.” All fluorescence emission difference spectra (B and D) were obtained by subtraction of the emission spectrum of DHE in ethanol, normalized to the emission of DHE at 373 nm in the respective membrane. The values indicated in the figure represent the ratios of POPC:DHE:cholesterol in the respective membrane preparations. Fluorescence (A) spectra of LUV are shown where the proportions of these lipids are as follows: Curve 1, 25:75:0; curve 2, 65:5:35; curve 3, 33:66:0; curve 4, 40:60:0; and curve 5, 65:35:0. Difference (B) spectra of LUV are shown with proportions 65:5:35 (curve 1), 65:35:0 (curve 2), 25:75:0 (curve 3), 33:66:0 (curve 4), and 40:60:0 (curve 5). The proportions of lipids for fluorescence (C) and difference (D) spectra of SUV were 65:5:30 (curve 1) and 65:35:0 (curve 2). Fluorescence excitation spectra (200–360 nm) were obtained with emission detected at 375 nm. Fluorescence emission spectra (340–600 nm) were obtained with excitation at 324 nm.
the one used to sediment debris and multilamellar vesicles.

Likelihood that the spectra (Fig. 2 and after the extrusion were exactly the same, decreasing the for the aqueous solutions of DHE. The spectra obtained before that with decreasing radius of curvature of the membrane the DHE spectrum. Model membrane data from Table I indicated variability in the amount of the crystalline portion of the SUV.

\[ \text{Membrane Curvature} - \] 

The fact that the inner (cytofacial) leaflet of the highest molar ratio of sterol:phospholipid ratio in the cell, – sterol in LUV is nearly equal, packing constraints significantly affect the transbilayer distribution of sterol in small unilamellar model membranes (SUV). In SUV with cholesterol: phospholipid ratios \( \geq 0.3 \) (i.e. \( \geq 23 \text{ mol} \% \text{ cholesterol} \)), the molar ratios of sterol:phospholipid in the innerleaflet and outer leaflet are about 1.3 and 0.9, respectively (52), and sterol in the inner leaflet is much more tightly packed than in the outer leaflet (22). To examine if this increase might induce the formation of crystalline phase sterol, SUVs with limiting radii of curvature were prepared as described under “Experimental Procedures.” The radii of curvature in the SUV and LUV, measured by photon correlations spectroscopy, were 15 \( \pm \) 3 and 53 \( \pm \) 10 nm, respectively. The fluorescence emission spectrum of DHE in SUV (POPC:DHE of 65:35, i.e. sterol:phospholipid molar ratio of 0.54) exhibited emission maxima (Fig. 3C) indistinguishable from those in LUV with the same composition (Fig. 3A). The ratios of intensities of the DHE fluorescence emission peaks in the SUV at 426:355 and 426:373 nm were 0.69 and 0.43, respectively, slightly higher than for monomeric DHE in ethanol, thereby suggesting the presence of low levels of crystalline DHE in SUV (Table I). When the emission spectrum of DHE in ethanol was normalized to that of DHE (at 370 nm) in the SUV and subtracted, the difference spectrum (Fig. 3D) exhibited emission maxima characteristic of crystalline DHE in aqueous buffer (Fig. 2E).

Because any DHE expelled from the membrane would form microcrystals in the buffer and give rise to spectra resembling Fig. 2E, the small unilamellar vesicles were subjected to a single pass extrusion using a 0.1-\( \mu \)m membrane as performed for the aqueous solutions of DHE. The spectra obtained before and after the extrusion were exactly the same, decreasing the likelihood that the spectra (Fig. 2E) originates from extraneous DHE in the buffer. Also, an additional centrifugation run like the one used to sediment debris and multilamellar vesicles (sediments \( >85\% \) of the microcrystals) did not produce any variability in the amount of the crystalline portion of the SUV DHE spectrum. Model membrane data from Table I indicated that with decreasing radius of curvature of the membrane the crystalline sterol increased 30-fold from \(-0.2\% \) in LUV to \(-6\% \) in SUV.

Spectral Properties of DHE in Plasma Membranes Isolated from L-cell Fibroblasts—The plasma membrane exhibits the highest molar ratio of sterol:phospholipid ratio in the cell, 0.5–1.0 (2). The fact that the inner (cytofacial) leaflet of the plasma membrane contains 80–90\% of plasma membrane sterol but only half of the plasma membrane phospholipid (2, 53 54) indicates molar ratios of sterol:phospholipid in the cytofacial leaflet as high as 1.8. However, it is not known if this sterol in the plasma membrane may be phase-separated into crystalline sterol. Therefore, L-cells were cultured in the presence of DHE, and the DHE-enriched plasma membranes were isolated as described under “Experimental Procedures.”

Excitation and emission spectral intensities of DHE were higher in plasma membranes than any other cellular membrane fractions (Fig. 4A). Whereas emission spectra of DHE in plasma membranes (Fig. 4A) largely resembled those of DHE in model membranes (Fig. 3), after subtraction of the monomeric DHE (Fig. 4C), a small amount of crystalline DHE (about 5.4\% of total) was detectable in the plasma membrane fraction (Table I). This was confirmed by examination of emission spectral peak ratios, all of which were greater than those of monomeric DHE (Table I). Detection of a small amount of crystalline sterol in these membranes was not due to adherence of DHE crystals to the membranes followed by coisolation on sucrose gradients. On the contrary, plasma membrane vesicles appeared at much higher density (27–30\% sucrose) than did DHE crystals (14\% sucrose). Furthermore, mixing of crystalline DHE with membranes not containing DHE, followed by reisolation on sucrose gradients, showed that there was no cross-contamination of the purified membranes with crystalline DHE.

Spectral Properties of DHE in Plasma Membrane Microdomains, Lipid Raft-enriched Subfractions from L-cell Fibroblasts—Plasma membrane sterol is laterally distributed into cholesterol-rich (lipid rafts and caveolae) and cholesterol-poor regions. Lipid rafts/caveolae account for about 10\% of total plasma membrane sterol but consist of only 1\% of plasma membrane surface (reviewed in Ref. 23). Thus, the small amount (i.e. 5.4\%) of crystalline sterol in the plasma membrane may be highly enriched in these microdomains. To examine this possibility, DHE containing lipid raft/caveolar-enriched subfractions were isolated from the plasma membrane fraction by a nondetergent technique (see “Experimental Procedures”). The lipid raft/caveolar subfraction was enriched nearly 3-fold in caveolin-1 and flotillin-1 as compared with the parent plasma membrane fraction (Fig. 4B). The emission spectra of DHE in lipid raft/caveolar membrane subfraction (Fig. 4B) were similar to those of plasma membranes (Fig. 4A). DHE exhibited low intensities of crystalline DHE emission peaks at 403 and 426 nm (Fig. 4B), in the range of those observed for plasma membranes (Fig. 4A) and model membranes containing low molar ratios of DHE:phospholipid (Fig. 3A). Close examination of the DHE emission spectra in lipid raft/caveolar membrane subfraction after subtraction of monomeric DHE (Fig. 4C) showed \(<0.5\% \) crystalline DHE, considerably less than in the plasma membrane. The very small and almost negligible presence of the crystalline DHE in the lipid raft/caveolar membrane subfraction was confirmed by examination of emission spectral peak ratios, all of which were only slightly greater than those of monomeric DHE (Table I).

Spectral Properties of DHE in Lysosomes—In order to examine the structural form of DHE in the intracellular organelle membrane containing the second highest cellular sterol phospholipid ratio, i.e. lysosomal membrane, it was necessary to first isolate the lysosome organelle. Lysosomes contained high quantities of crystalline sterol as follows. (i) The emission spectrum of DHE in isolated lysosomes exhibited large peaks near 416 and 403 nm (Fig. 4, D and E). (ii) The DHE emission spectral peak ratios at 426:355 and 426:373 were 2.02 \( \pm \) 0.15 and 1.42 \( \pm \) 0.06, respectively (\( n = 3 \)) (Table I). These ratios were much higher than for monomeric DHE in ethanol (Table I) but were more similar to those of crystalline DHE in buffer or cell culture medium (Table I). (iii) Quantitative evaluation of the emission spectra showed that 75\% of the fluorescence intensity was representative of the crystalline form. (iv) Lyso-

---

**Table II**

| % POPC\( ^{\text{a}} \) | Cholesterol (Å\(^2 \)) | DHE (Å\(^2 \)) |
|---|---|---|
| 0 | 39.15 | 40.40 |
| 30 | 39.90 | 41.50 |
| 65 | 43.40 | 43.80 |
| 100 | 55.00 | 55.00 |

\( ^{\text{a}} \) Pure cholesterol or dehydroergosterol monolayers, or mixed monolayers containing the indicated amount of sterol together with 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine, were compressed on 140 nm NaCl in water at 22 °C (under an argon atmosphere in the dark) with a KSV surface barostat and proprietary KSV software. The barrier speed during compression did not exceed 3.4 Å/molecule per min.

\( ^{\text{b}} \) Each value is average of 3 isotherms measured at 35 mN/m with an average variation \(<0.5 \text{ Å}^2 \).
**Fig. 4.** Spectral properties of DHE in subcellular membrane fractions and organelles isolated from L-cell fibroblasts. Excitation spectra were obtained at 37 °C while monitoring emission at 375 nm. Emission spectra were obtained at 37 °C upon excitation at 324 nm. All fluorescence emission difference spectra were obtained by subtraction of the emission spectrum of DHE in ethanol, normalized to the emission of DHE at 373 nm in the respective membrane. Protein concentration in all samples was 14 μg/ml in 10 mM PIPES, pH 7.4. 

A. Excitation (left side) and emission (right side) spectra of DHE in isolated plasma membrane vesicles (PM), microsomal membrane vesicles (ER), mitochondrial membrane vesicles (MITO), and lipid droplets (LD). Lipid droplet spectrum shown is the result of subtraction of a spectrum of lipid droplets without DHE from a spectrum of lipid droplets with DHE. 

B. Emission spectra of DHE in lipid raft/caveolar membrane subfractions. The fold enrichment of caveolin-1 and flotillin-1 in these subfractions is shown as a subpanel.

C. Emission difference spectra of DHE in plasma membrane vesicles (PM), lysosomal membrane (LM), and caveolar membrane (CAV)-enriched fractions. 

D. Excitation (left side) and emission (right side) spectra of DHE in isolated lysosomes (LYSO).

E. Effect of increasing ethanol on emission spectra of DHE in isolated lysosomes. Curve 1 shows DHE emission upon addition in lysosomes (14 μg of protein/ml 10 mM PIPES, pH 7.4). Curve 2 shows the same sample as in curve 1 diluted with ethanol to a final concentration 25%. Curve 3 shows the same sample as in curve 1 diluted with ethanol to a final concentration 50%. Curve 4 shows the lysosomes (14 μg of protein) added directly to ethanol (final ethanol concentration >99%).
somess had very high ratios of sterol:phospholipid, consistent with the separation of DHE into a crystalline phase, crystallization of DHE in the lysosomal matrix, and/or incomplete solubilization/efflux of DHE crystals from the lysosomal matrix into the cell interior. The total DHE:phospholipid molar ratios in the lipids extracted from the lysosomes, determined from the extinction coefficient of DHE and by HPLC analysis, were 1.6 and 1.5, respectively.

Spectral Properties of DHE in Lysosomal Membranes—To determine whether the crystalline DHE detected in the lysosome (see above) was largely confined to the lysosomal matrix or actually a part of the lysosomal membrane, lysosomes were subjected to hypotonic lysis followed by an additional sucrose gradient purification to resolve DHE crystals (matrix-derived) from lysosomal membranes (see “Experimental Procedures”). The lysosomal membranes (density >30% sucrose) were clearly resolved from lysosomal matrix-derived crystals of DHE (density >16% sucrose). The molar ratio of total sterol:phospholipid (determined by HPLC of lipids extracted from purified lysosomal membranes) was 0.38, consistent with earlier reports (39). This molar ratio was more than 4-fold lower than that of intact lysosomes, confirming the presence of a large amount of crystalline DHE in the lysosomal matrix.

The emission spectral intensity of DHE in the lysosomal membrane fraction (not shown) was lower than that of DHE in plasma membranes but higher than that of DHE in endoplasmic reticulum and mitochondrial membranes (Fig. 4A). This reflected the relative sterol content of the lysosomal membranes versus plasma membranes, microsomes (endoplasmic reticulum), and mitochondrial membranes. Close examination of the DHE emission spectra in lysosomal membranes showed (after subtraction of monomeric DHE) a clearly resolvable crystalline DHE emission spectrum (Fig. 4C). The crystalline DHE present in the purified lysosomal membrane fraction represented about 6.9% of total sterol. The presence of a small amount of crystalline DHE in the lysosomal membranes was also substantiated by examination of emission spectral peak ratios, both of which were greater than those of monomeric DHE (Table I).

The possibility that the crystalline DHE in the lysosomal membrane was the result of DHE crystals (from the lysosomal matrix) sticking to the lysosomal membrane and cosedimenting at the same density as lysosomal membranes was considered as follows. (i) Crystalline DHE was mixed with isolated lysosomal membranes that did not contain DHE. (ii) The mixture was placed on the same sucrose gradient and the two fractions were clearly separated. Examination of emission spectra of the two fractions revealed that all the crystalline DHE appeared much higher in the density (density <14% sucrose) and was clearly separated from the much denser lysosomal membrane fraction (density >30% sucrose), which did not contain any DHE. Finally, it is important to note that the small amount of lysosomal membrane DHE found in the crystalline form was in the same range as that observed for DHE in model membranes wherein no crystalline DHE was present during the membrane preparation (Table I).

Spectral Properties of DHE in Microsomal Membrane and Mitochondrial Membrane Fractions Isolated from L-cell Fibroblasts—The ratio of sterol:phospholipid in microsomes (endoplasmic reticulum) and mitochondrial membranes, near 0.2 and <0.1, respectively, was much lower than that in plasma membranes or lysosomal membranes (reviewed in Refs. 32 and 39). The excitation and emission spectral intensities of DHE in the microsomal and mitochondrial membranes were both lower than in plasma membranes (Fig. 4A) or lysosomal membranes (not shown). The low intensities of DHE emission spectra in these membrane fractions (Fig. 4A) produced, upon subtraction of monomeric DHE, spectra with large signal to noise but indicated only small amounts of crystalline DHE, <1% of the total.

Disruption of Crystalline DHE in Lysosomes, Potential Role of Sterol Carrier Protein-2—Whereas crystalline DHE clearly accumulated in lysosomes of L-cells supplemented with crystalline DHE in the medium (Fig. 4D), crystalline DHE can be converted to monomeric form both in vitro and in intact cells. Addition of ethanol converted the emission spectrum of DHE in the lysosomes from primarily crystalline to essentially monomeric (Fig. 4E). This was confirmed upon examination of the DHE emission peak ratios at 426:355 and 426:373 nm (Table I). The spontaneous transfer of DHE from lysosomal donors (enriched in crystalline DHE) to lysosomal acceptors (containing no DHE) was determined as the release from self-quenching and detected as increased DHE polarization (see “Experimental Procedures”). Spontaneous DHE transfer from lysosomes was relatively slow (Fig. 5A, solid circles) with an initial rate of molecular steroid transfer of 0.048 ± 0.020 pmol/min. However, upon addition of 1.5 μM sterol carrier protein-2, the DHE fluorescence polarization and anisotropy increased rapidly, consistent with transfer of DHE from donor lysosomes to acceptor lysosomes (Fig. 5A, open circles). The initial rate of DHE transfer was enhanced nearly 13-fold, from 0.048 ± 0.020 to 0.601 ± 0.096 pmol/min. To determine whether SCP-2-mediated DHE transfer from the lysosomes disrupted the crystalline DHE or simply transferred DHE from the lysosomal membranes, emission spectra of DHE were obtained for lysosomes (isolated from L-cells supplemented with DHE) at the beginning (t = 0 min, spectrum 1, Fig. 5B) and at the end (t = 270 min, spectrum 2, Fig. 5B) of SCP-2-mediated DHE transfer from lysosomal donors (enriched in crystalline DHE) to lysosomal acceptors (containing no DHE). DHE emission spectral peak ratios (426:355 and 426:373 nm) decreased 3–4-fold to 0.54 and 0.36, respectively, after 270 min of SCP-2-mediated sterol exchange (Table I). These ratios resembled those observed for DHE in purified lysosomal membranes, 0.66 and 0.48, respectively (Table I). It should be noted that simply adding SCP-2 to DHE crystals in the intact lysosomes did not alter the DHE emission spectral properties. These data suggest that SCP-2 converted the crystalline DHE to the monomeric spectral form by quickly transferring the DHE from the crystalline DHE in the donor lysosomes to acceptor lysosomes.

In order to confirm further that sterol exchange from DHE crystals was facilitated by SCP-2, the anisotropy of the transfer of sterol between DHE crystals and lysosomal membrane acceptors was monitored. The experiment consisted of three steps involving donors of DHE crystals created by adding 2.5 μg of DHE (similar to that seen in lysosomes) to 2 ml of 10 mM PIPES buffer. (i) Anisotropy of donor crystals was monitored for 3 h (the flat curve in Fig. 5C). (ii) A spontaneous transfer was performed between the donor crystals and lysosomal membrane acceptors (middle curve of Fig. 5C). (iii) 1.5 μM SCP-2 was included and once again the anisotropy of donor crystal and lysosomal membrane acceptors was monitored for 3 h (the top curve of Fig. 5C). Initial rates were calculated by using a standard curve based upon previously published lysosomal membranes exchange data. Exchanges between crystals and lysosomal membrane acceptors showed that 1.5 μM SCP-2 increased the initial rate of molecular sterol transfer nearly 7-fold, from 4.67 ± 0.65 (spontaneous) to 30.73 ± 5.2 pmol/min (with 1.5 μM SCP-2). Clearly, anisotropy increased for spontaneous sterol transfer from both lysosomal and pure crystalline DHE donors to acceptor lysosomal membranes, an effect 13- and 7-fold larger, respectively, in the presence of SCP-2.

Experimental Procedures

Spectral Properties of DHE in Lysosomal Membranes

Multiphoton Imaging of Membrane Sterol
makes visualization by conventional and confocal fluorescence microscopy difficult because this requires the use of quartz optics, results in significant photobleaching and photodestruction, and yields images with limited resolution and excessive photobleaching over time (32, 34). These difficulties are largely avoided by the use of multiphoton excitation with infrared light at 900–925 nm resulting in simultaneous absorption of three infrared photons equivalent to single photon excitation in the ultraviolet range (i.e. 300–305 nm). Furthermore, in multiphoton excitation only those DHE molecules within the objective focal volume (about 0.01 cu. μ) are excited, thereby allowing emission to be measured only from the DHE molecules excited in the focal volume. Through use of scanning optics and external detectors, images similar to those obtained by confocal microscopy are obtained but lacking in the drawbacks of the latter and exhibiting dramatically reduced photobleaching. These features of multiphoton excitation were therefore used to determine whether crystalline and monomeric forms of DHE could be discriminated by multiphoton laser scanning microscopy.

DHE was crystallized on coverslips by evaporation of carrier ethanol solvent under a stream of nitrogen followed by three-photon excitation at 900 nm (Fig. 6). To resolve crystalline and monomeric DHE, fluorescence emission was monitored simultaneously through separate dichroic filters as described under “Experimental Procedures”: crystalline DHE, distinctly visible as needle-like structures (up to 50 μm long and usually 1–10 μm wide), was preferentially detected by monitoring emission with a 455:30-nm dichroic filter (Fig. 6A, green channel); monomeric DHE was preferentially detected with a 375:50-nm dichroic filter (Fig. 6B, blue channel). For the sake of imaging the crystals without saturation, the gain was decreased in the 455:30-nm channel to prevent saturation, whereas the gain in the 375:50-nm channel was maximal. The red channel (Fig. 6C, 575:150-nm dichroic filter) detected neither monomeric nor crystalline DHE and was used to establish whether DHE fluorescence emission spilled over into this wavelength range. Analysis of the merged image (comprised of A–C) revealed primarily green DHE crystals (Fig. 6D). Thus, multiphoton excitation at 900 nm was useful for determining the presence of crystalline DHE by MPLSM utilizing basically the same spectroscopic techniques as for the in vitro studies except that emission was collected simultaneously in the 455:30- and 375:50-nm channels.

Multiphoton Laser Scanning Microscopy of Crystalline DHE in Lysosomes of Living L-cell Fibroblasts—As indicated above, DHE crystals were stable over many days in 10% fetal bovine cell culture medium incubated at 37 °C. Because crystalline DHE was detected in lysosomes isolated by subfractionation of L-cells cultured with DHE crystals, consistent with the observation that L-cells actively phagocytose particles as large as several microns (55), the possibility that these crystals could be visualized in lysosomes by MPLSM was examined. L-cell lysosomes, ranging from 0.4 to 3.5 μm in diameter, were readily visualized by staining intact cells with LysoTracker and monitoring LysoTracker emission with a 575:150-nm dichroic filter (Fig. 7, red pixels). Microcrystalline DHE was simultaneously visualized by monitoring emission through a 455:30-nm dichroic filter (blue pixels). Finally, some of the crystalline DHE taken up by the L-cells was solubilized/metabolized as indicated by the presence of monomeric DHE detected by monitoring emission with a 375:50-nm dichroic filter (Fig. 7, green pixels). The merged image of all three photomultiplier tubes (Fig. 7) revealed the following.

First, distinct white pixilated areas (indicating colocalized crystalline DHE (blue), monomeric DHE (green), and Lyso-
Tracker (red)) were present in some lysosomes (Fig. 7, arrow 1). Second, distinct magenta pixilated areas (indicating colocalized crystalline DHE (blue), but not monomeric DHE, and LysoTracker (red)) were present in some lysosomes (Fig. 7, arrow 2). Third, distinct yellow/orange pixilated areas (indicated colocalization of monomeric DHE (green), but little crystalline...
DHE, and LysoTracker (red) were present in some lysosomes (Fig. 7, arrow 3). Fourth, distinct red pixilated areas (indicating LysoTracker staining regions without either monomeric or crystalline cholesterol) showed that L-cell fibroblasts had a substantial population of lysosomes that did not appear to contain significant amounts of either monomeric or crystalline DHE (Fig. 7, arrow 4). Fifth, distinct green (Fig. 7, arrow 5) and cyan (Fig. 7, arrow 6) pixels showed that some monomeric and crystalline/monomeric DHEs, respectively, were not present in the lysosomes.

**Resolution of the Two Forms of DHE in Living L-cell Fibroblasts**—Due to the complexity of visually distinguishing the different color tones necessary for deciphering the spatial colocalization of crystalline and monomeric forms, a procedure for separating the two forms utilizing the differences in spectral ratios was developed (see “Experimental Procedures”).

First, the 455:30-nm channel and the 375:50-nm channel were pseudo-colored red and green, respectively, and merged (Fig. 8A). This was imported into the software program that plotted the fluorogram (Fig. 8B) as described under “Experimental Procedures.” This fluorogram showed that there was a strong correlation in the more red (crystalline) pixels, which clearly have a higher ratio over the more green (monomeric) pixels and appeared in somewhat linear fashion along the ordinate axis. The more green pixels appeared in a more complex pattern due to the saturation of monomeric regions of localization, with a population aligned linearly along the horizontal axis and another population saturating along the right vertical axis. Some saturation was allowed in order to detect lower concentrations. As will be shown later, at least some of those saturated monomeric DHE regions in the pixelgram represented monomeric DHE in lipid droplets.

Second, the more red pixels (pixels with $I_{red}/I_{green} \geq 1$) were selected by drawing a line along the diagonal and then enclosing a triangle around the upper left scattering of more red pixels. The resulting gray scale image was created (Fig. 8C) and then merged with the third or 575:150-nm channel (LysoTracker emission, not shown). This merged image (Fig. 8E) showed the crystalline (green) colocalizing with LysoTracker Green (red) displayed as a fluorogram (Fig. 8F) and colocalization coefficients: $C_{green} = 0.97$ (crystalline DHE) exhibited a strong correlation with the LysoTracker Green, whereas $C_{red} = 0.32$ (LysoTracker Green) did not. This was corroborated by the image (Fig. 7) that showed many lysosomes colored in bright red and thereby evidencing the fact that they did not have any crystalline DHE.

Third, the more green pixels (pixels with $I_{red}/I_{green} < 1$) were selected by drawing a triangle around the lower right scattering of more green pixels. This resulting image was created (Fig. 8D) and then merged with the 575:150-nm channel (LysoTracker emission, not shown). The resulting new merged image showed monomeric DHE pseudo-colored green and LysoTracker pseudo-colored red (Fig. 8G). The fluorogram of the merged image (Fig. 8H) indicated that the LysoTracker, $C_{red} = 0.54$, exhibited a slightly larger amount of colocalization with the monomeric DHE, $C_{green} = 0.46$. Both coefficients indicated additional separate localization of each probe in other cellular sites.

In summary, MPLSM allowed simultaneous three-photon excitation (crystalline and monomeric DHE) and two-photon excitation (LysoTracker Green) and separate, simultaneous detection of each in living cells in real time. This noninvasive technology took advantage of the enhanced emission properties of crystalline DHE to image multiple structural forms of sterol in living cells both within lysosomes and outside of the lysosomal compartment. It should be noted that the intensities of the crystalline DHE in L-cells were so bright that, to avoid saturating the photomultiplier tubes, it was not possible in these images to visualize simultaneously the DHE enriched in the plasma membrane. However, the latter was clearly imaged under other conditions (see below).

**Multiphoton Laser Scanning Microscopy of DHE and BODIPY FL C12-ceramide, a Golgi Marker, in L-cell Fibroblasts**—To determine whether crystalline DHE entered the Golgi compartment of L-cell fibroblasts, cells were incubated with BODIPY FL C12-ceramide (see “Experimental Procedures”), MPLSM at 900 nm excitation resulted in simultaneous three-photon excitation of DHE (monomeric and crystalline) and emission detected as above. Concomitantly, MPLSM at 900 nm resulted in simultaneous two-photon excitation of BODIPY FL C12-ceramide whose emission was detected through a dichroic filter (575:150 nm). When the images were treated as described above (not shown), the fluorogram of the Golgi marker versus crystalline DHE (not shown) indicated that only 9% of DHE in Golgi was crystalline. Thus, once the DHE left the lysosomal compartment it did not significantly accumulate in the Golgi in a crystalline form.

**Multiphoton Laser Scanning Microscopy of DHE and Nile Red, a Lipid Droplet Marker, in L-cell Fibroblasts**—To determine whether DHE was translocated for storage in intracellular lipid droplets of L-cell fibroblasts, the cells, as described under “Experimental Procedures,” were cultured on chambered cover glass and incubated with Nile Red, a probe that accumulates primarily in neutral lipid droplets. Tuning the Ti:Sapphire laser to 925 nm resulted in simultaneous three-photon excitation of DHE (monomeric and crystalline) and two-photon excitation of Nile Red. The fluorescence emission of all fluorophores was simultaneously detected as follows: Nile Red with a 575:150-nm dichroic filter; crystalline and monomeric with a 455:35- and a 375:50-nm dichroic filter, respectively. Two levels of sensitivity and objectives were used to collect images as follows: (i) no attenuation of excitation power and a Zeiss 100× Fluar oil immersion lens (high transmission characteristics for 350–400 nm but decreased flatness of field); (ii) 80% attenuation of excitation power and a Zeiss 63× Plan Apochromat oil immersion lens. To determine the degree of colocalization of the monomeric DHE with Nile Red in L-cell lipid droplets, a region of cells was selected showing little crystalline DHE; sensitivity was increased, and the images were merged (Fig. 9A). The blue/cyan regions showed the presence of very low crystalline DHE (Fig. 9A, arrow 1). Importantly, monomeric DHE was clearly visualized in plasma membranes and other membranes (Fig. 9A, arrow 2). The bright saturated yellow regions (Fig. 9A, arrow 3; upper right corner of fluorogram, Fig. 9B) were lipid droplets wherein monomeric DHE colocalized with the lipid droplet probe, Nile Red. The colocalization coefficient, $C_{red} = 0.98$ showed that almost all of the Nile Red colocalized with monomeric DHE, whereas $C_{green} = 0.42$ showed that just under half of the monomeric DHE intensity was colocalized with Nile Red in the lipid droplets (Fig. 9B). This implied that >50% of monomeric DHE intensity was in membranes, vesicles, and in diffuse distributions in the cytoplasm.

Some of the bright pixilated areas in the merged image were cyan (Fig. 9A, arrow 1), representing crystalline DHE colocalized with monomeric DHE. To obtain a more quantitative estimation of the distinction between crystalline and monomeric DHE with Nile Red, another image was taken at lower laser excitation power and with the 63× objective centered upon cells containing more phagocytosed DHE crystals. Once again the images were merged under the same pseudo-colors as previously (Fig. 9C), where the blue regions represented crystalline...
DHE (Fig. 9C, arrow 1), green regions showed monomeric DHE (Fig. 9C, arrow 2), and the yellow regions represented monomeric DHE colocalized with Nile Red in lipid droplets (Fig. 9C, arrow 3). Once again, an image was formed (not shown) composed of DHE alone: crystalline (red) and monomeric (green). Two distinct and well separated populations of pixels were
Fig. 9. Multiphoton laser scanning microscopy of DHE and Nile Red, a lipid droplet marker, in L-cell fibroblasts. L-cell fibroblasts were cultured on cover glass for 2 days in medium containing 10% fetal bovine serum and DHE (20 μg/ml) as described under “Experimental Procedures.” The cells were washed 3–4 times with Puck’s buffer, followed by incubation with Nile Red as indicated under “Experimental Procedures.” Excitation at 925 nm elicited simultaneous three-photon excitation of DHE (monomeric and crystalline) and Nile Red. Nile Red emission (red pixels in A and C) was detected through a 575:150-nm dichroic filter; crystalline DHE (blue pixels in A and C) was detected through a 455:30-nm dichroic filter; monomeric DHE (green pixels in A and C) was detected through a 375:50-nm dichroic filter. A shows the merged image obtained from high power excitation on a region of cells with small amounts crystals (arrow 1), plasma membrane (arrow 2) as well as other intracellular membranes, and lipid droplets (arrow 3). B is the fluorogram resulting from colocalization of the monomeric DHE (green) that was obtained using our software as described under “Experimental Procedures” and Nile Red (red). C is a merged image obtained from slightly lower power excitation of a region of cells showing larger amounts of crystals (arrow 1), organelles with DHE but with no Nile Red (arrow 2), and lipid droplets (arrow 3). D is the fluorogram of the merged images detected through the dichroic filters 455:30 (red) and 375:50 nm (green). The crystalline (upper reddish correlated pixels) and the monomeric forms (lower greenish correlated pixels) are clearly separated across the diagonal. As described under “Experimental Procedures,” each clustering of pixels was selected to produce individual gray scale images representative of crystalline and monomeric DHE. E is the fluorogram determined by merging the crystalline portion with Nile Red, and F is the fluorogram obtained from merging the monomeric portion with Nile Red. Clearly, the correlation coefficients confirm that the majority of Nile Red emission from lipid droplets colocalizes with monomeric DHE.

obtained in this pixel fluorogram (Fig. 9D). The high slope, upper pixel population was composed of crystalline DHE, whereas the lower pixel population was composed of monomeric DHE. Each population was encircled, and a corresponding gray scale image was created as described under “Experimental Procedures.” Each of these images was merged with the 575:150-nm channel (Nile Red in lipid droplets, not shown). The fluorograms resulting from the merged images of crystalline DHE (green) with Nile Red (red) and monomeric DHE (green) with Nile Red (red) were shown in Fig. 9, E and F, respectively. The coefficients of colocalization with respect to the crystalline form were quite small for both DHE and Nile Red: $C_{red} = 0.01$, $C_{green} = 0.03$ (Fig. 9E). However, the monomeric form colocalized quite well with Nile Red in lipid droplets. With this concentration of Nile Red, most of the Nile Red was observed in the bright lipid droplets, confirmed in the image (yellow round regions and no visible red areas), by the fluorogram, and the coefficient, $C_{red} = 0.99$ (Fig. 9F). The monomeric DHE, on the other hand, clearly resided in other areas illustrated by regions of green. At this excitation level, 74% of the 375:50 nm intensity integrated over the whole image colocalized to some extent with the Nile Red in lipid droplets, as seen by a coefficient of $C_{green} = 0.74$, whereas the other 25% was in other cellular structures (Fig. 9F). Thus, monomeric DHE was significantly colocalized with lipid droplets as well as other intracellular structures and the plasma membrane. Crystalline DHE was primarily associated with lysosomes.

Multiphoton Laser Scanning Microscopy of L-cell Fibroblasts Cultured with Large Unilamellar Membrane Vesicles Containing DHE—To determine whether L-cells cultured with non-crystalline DHE might exhibit the absence of crystalline DHE and/or a different intracellular DHE distribution, L-cell fibroblasts were cultured for 2 days with medium supplemented with DHE (20 μg/ml) in the form of LUV composed of POPC: DHE (65:35) as described under “Experimental Procedures.” As
shown in Fig. 3, DHE in these LUVs was primarily in monomeric form with <0.2% in crystalline form. After the cells were washed 3–4 times with Puck’s buffer, Filters were as described in the legend to Fig. 9. A shows the merged image. Arrow 1 points to the plasma membrane region showing only monomeric DHE; arrow 2 points to a representative white pixelated area wherein mostly crystalline DHE was localized with Nile Red; arrow 3 points to a representative yellow/orange area where monomeric DHE colocalized with Nile Red. B is a pixel fluorogram of only the 455:30-nm emission (red) and 375:50-nm emission (green). The crystalline and monomeric portions were separated out as discussed. C shows the monomeric portion merged with Nile Red emission. D is a pixel fluorogram corresponding to C.

of these images were merged with the Nile Red gray scale image. The merged image of monomeric DHE with Nile Red was shown in Fig. 10C with its corresponding fluorogram (Fig. 10D). With a $C_{\text{red}} = 0.96$, most of the Nile Red colocalized with monomeric DHE even though it was not all in lipid droplets in this figure (Fig. 10D). A higher concentration of the Nile Red probe was used in order to delineate other nonpolar lipid regions within the cell. Again, the DHE in monomeric form was distributed among cellular components, but >50% of the total intensity of monomeric DHE colocalized to some degree with Nile Red staining regions (including lipid droplets) as indicated by $C_{\text{green}} = 0.64$ (Fig. 10D). Whereas the brightest regions were the lipid droplets, the perinuclear regions also appeared to accumulate significant colocalized Nile Red and monomeric cholesterol.

As indicated above, supplementation of L-cells with DHE in liposomal form resulted in the near absence of crystalline DHE, with only a few crystalline pixels detected colocalizing with Nile Red in the perinuclear region (not shown). With a $C_{\text{red}} = 0.04$, almost none of the Nile Red colocalized with crystalline DHE.

**DISCUSSION**

Because of its influence on membrane fluidity, permeability, cell-cell recognition, transport, receptor-effector coupling, and microdomain (e.g. caveolae and rafts) function, the cholesterol content of cellular membranes must be tightly regulated (reviewed in Refs. 2 and 53). This is especially important in view of the fact that abnormal regulation of membrane cholesterol content and/or distribution impairs membrane function (reviewed in Refs. 2, 32, 53, and 56–60) and thereby cell survival as indicated by cytotoxicity, sickle cell acanthocytosis, Niemann-Pick C disease, Alzheimer’s disease, and atherosclerosis (reviewed in Refs. 12–16). Despite these findings relatively
little is known regarding the organization of membrane cholesterol, especially when cholesterol is present in molar excess over membrane phospholipid. Likewise, almost nothing is known regarding the real time, direct visualization of different structural forms of cholesterol in membranes or cells (reviewed in Refs. 18 and 25). The data presented here address these issues and provide the following new insights.

First, crystalline and monomeric forms of sterol were differentiated spectroscopically. Monomeric DHE exhibited emission maxima near 354, 370, and 394 nm in anhydrous ethanol, whereas those of crystalline DHE were significantly red-shifted to 356, 403, and 426 nm. These emission characteristics of crystalline DHE were not due to the presence of impurities. The onset of the red-shift was concomitant with the formation of DHE microcrystals in aqueous buffers (27, 30) at concentrations similar to that where cholesterol has been shown to form microcrystals in aqueous buffers (49, 61–63). Several techniques were applied to determine whether the change in spectral characteristics from ethanol to water was due to formation of microcrystals or of micelles. Observation by polarizing light microscopy revealed birefringent structures resembling those reported to be cholesterol monohydrate microcrystals (60–63) and are therefore presumed to be dehydroergosterol monohydrate crystals. Filtration up to 0.5 μm eliminated all detectable emission, indicating that the fluorescence is derived from an aggregate with a lower size limit of >0.5 μm, most likely the observed microcrystals. In corrobororation, the addition of DPH to μM concentrations of DHE in aqueous buffer did not reveal any incorporation into micelles of DHE, regardless of the possible occurrence of micellar agglomeration. These results compared favorably with the same experiments applied to cholesterol at similar concentrations and conditions.

Although determination of the exact photophysical nature of crystalline DHE involves a complex discussion of exciton theory (64), one can consider a simple interaction mechanism with a nearest neighbor in the form of a physical sandwich dimer, because steroid fused rings form α-face-to-α-face dimers as well as β-face-to-β-face dimers in crystals (65). For example, the crystalline absorption spectrum exhibited characteristics such as a bimodal absorption peak accompanied by spectral broadening, some increase in intensity of higher order vibrational bands, and overall hypochromism. The crystalline spectral emission exhibited a red shift in the emission maxima (354 to 356 nm and 370 to 375 nm) whose intensities were decreased from those in the monomer, whereas the lower energy transitions (exhibiting maxima at 403 and 426 nm) were increased dramatically in intensity. This was indicative of enhanced Franck-Condon factors and fast relaxation times into the lowest energy level where there must be less nonradiative processes and stronger wave function overlap with higher vibrational levels in the ground state. A larger lifetime component consistent with a smaller nonradiative transfer rate was observed in the crystalline form (30). The possibility that the enhanced emission could also be due to the formation of excited state dimers (excimers) was considered based on the fact that the conjugated triene double bonds in DHE are part of a planar, ring structure. Such structures, when stacked face-to-face, allow the overlap of the electron clouds of the two molecules and thereby facilitate excimeric interaction. Typically, excimer formation is concentration-dependent and fluoresces at longer wavelengths than monomers as well as exhibits higher quantum yield than the monomer. However, excimeric emission is usually broad and structureless because the excimer dissociates due to the strong repulsive force between the molecules in the ground state. As can be seen in Fig. 1D, vibrational structural features were present in the subtracted spectrum. Regardless of whether the DHE formed physical dimers and/or excimers, these differences in the spectral emission characteristics of the monomeric and crystalline DHE allowed the determination of their relative proportions in solvents, model membranes, biological membranes, and organelles (e.g. lysosomes).

Second, these differences in the spectral emission characteristics of the monomeric and crystalline DHE permitted determination of their relative proportions in model membranes. The structural form of DHE in model membranes was primarily monomeric even at molar ratios of sterol:phospholipid substantially >1. This observation was surprising in view of previous studies on sterol packing in model membranes. While at low mol % the sterol is uniformly dispersed/solubilized in the membrane, and increasing the sterol:phospholipid molar ratio from 0.25 to 1 results in formation of interdigitated, transbilayer sterol dimers (66). Over the same concentration, DHE self-quenches in model membranes by Forster energy transfer (29, 30). The Forster energy transfer distance for this homotransfer between face-to-face oriented DHE molecules is 13.3 Å (30). Because sterol and phosphatidylcholine are 6 (13) and 8 Å (30) thick, respectively, Forster homotransfer of energy is efficient even if the DHE molecules are separated by one phospholipid molecule. In contrast, at sterol:phospholipid molar ratios >1 an immiscible sterol phase forms in model membranes (reviewed in Refs. 13, 67, and 68). If massive formation of pure crystalline DHE had occurred in membranes with DHE:phospholipid molar ratios >1, the concomitant formation of face-to-face DHE dimers would have resulted in preponderant emission at longer wavelength. On the contrary, the data presented here with DHE clearly showed that even at sterol:phospholipid molar ratios >1 there was only a small amount of phase-separated DHE with properties identical to that in pure DHE crystals. The molecular basis for this apparent contradiction between results obtained by the various techniques (x-ray and NMR versus fluorescence) may be explained as follows.

One possibility is that the different techniques detecting phase-separated immiscible sterol may not necessarily report on the same aspects of sterol structural packing in membranes. Although the x-ray and NMR techniques largely report on transbilayer thickness/distance between sterol molecules, it is unclear whether these techniques differentiate the multiple potential forms of lateral phase-separated sterol in membranes: pure cholesterol patches, ribbons of sterol, and/or sterol-sterol with different orientations (edge-to-edge, α-face-to-α-face, β-face-to-β-face, and α-face-to-β-face), etc. (reviewed in Refs. 65, 69, and 70). In contrast, DHE emission characteristics detect the formation of face-to-face lateral interactions between sterols, which emit with greater quantum yield and at higher wavelength. As shown here over the DHE:phospholipid molar ratio ranging from 0.5 to 3, DHE detected only small amounts (1–10%) of immiscible DHE whose structure (identified by longer wavelength emission maxima) appeared identical to pure crystalline DHE or other face-to-face sterol packings.

An alternative explanation of the different results obtained by the x-ray and NMR of cholesterol versus fluorescence of DHE is that the phase behavior of DHE may differ markedly from that of cholesterol in model membranes. Although it is important to recognize that DHE and cholesterol are not identical, this latter possibility was considered unlikely based on the very similar behavior of these sterols in a variety of model membrane systems (reviewed in Refs. 26, 30, 71, and 72) summarized briefly as follows. (i) In pure and mixed monolayers at pressures similar to bilayer systems, cholesterol and DHE
showed similar packing arrangements even at mol % where cholesterol has been shown by x-ray crystallography and birefringence to separate into crystalline structures (51). (ii) The dissociation constants of spontaneous desorption of cholesterol and DHE from membranes were 0.044 and 0.031 mol/mol, respectively (30). In contrast, due to the apparent higher affinity of cyclodextrin for DHE, the cyclodextrin-catalyzed extraction of cholesterol from model membranes was severalfold slower than that of cyclodextrin-catalyzed extraction of DHE (73). (iii) The rate constants of spontaneous transfer of cholesterol and DHE between POPC containing model membranes did not significantly differ (71). (iv) The fractional distribution of cholesterol into kinetically resolvable sterol domains was not significantly different from that of DHE in POPC containing model membranes (71). (v) Cholesterol and DHE similarly shifted the midpoint of the phase transition temperature of POPC similarly to higher temperature (reviewed in Ref. 26). (vi) Cholesterol and DHE similarly abolished the phase transition of POPC at high mol % sterol, although slightly higher DHE was required (reviewed in Ref. 26). (vii) DHE (fluorescence, lifetime, and polarization) detected the same phase transition of POPC at high mol % sterol, although slightly higher DHE was required (reviewed in Ref. 26). (viii) DHE codistributed with cholesterol (i.e. did not phase separate from cholesterol) in membranes and formed superlattices similarly to those of cholesterol in model membranes (reviewed in Refs. 30 and 72). These observations were in dramatic contrast to those obtained with nitroxide-labeled sterols or other types of fluorescent-labeled cholesterol whose properties in model membranes differed much more markedly (4-10-fold) from those of cholesterol (reviewed in Refs. 26 and 34). Thus, the phase properties of DHE in model membrane bilayers reflect those containing cholesterol.

Third, it was demonstrated that crystalline and monomeric membrane sterol could be differentiated spectroscopically in vitro in subcellular membrane fractions isolated from L-cell fibroblasts. Plasma membranes, lysosomal membranes, microsomes (endoplasmic reticulum), and mitochondrial membranes differ markedly in sterol:phospholipid ratio ranging from near 1.0 in plasma membranes to as low as 0.04 in mitochondria (reviewed in Refs. 2 and 53). When these membrane fractions were isolated from L-cell fibroblasts supplemented with DHE, only a small proportion (1-7%) of crystalline DHE was detected. This was confirmed by MPLSM of L-cells supplemented with DHE in monomeric form (i.e. LUV) and represents the first real time imaging of a fluorescent sterol in the plasma membrane of a living cell as well as the first real time resolution of multiple structural forms of DHE in the plasma membrane of a living cell. Consistent with the findings of only a small amount of crystalline sterol in the plasma membrane, other investigators (18) using x-ray crystallography also showed that plasma membranes isolated from control smooth muscle cells did not contain significant levels of crystalline sterol.

The lack of substantial quantities of crystalline sterol in plasma membranes, the most highly sterol-enriched membrane in the cell, was especially interesting because the transbilayer distribution of sterol in the plasma membrane is asymmetric, i.e. 4-fold higher in the cytofacial leaflet (reviewed in Refs. 22 and 58). Because the L-cell plasma membranes in this study exhibited a sterol:phospholipid molar ratio of 1.2 (38), this would imply that the sterol:phospholipid molar ratio in the cytofacial leaflet was almost 2.0. Nevertheless, neither DHE in isolated plasma membranes in vitro, x-ray crystallography in vitro, nor DHE in real time multiphoton images of living cells detected very much crystalline sterol in plasma membranes. It should be noted that these observations for the structure of DHE in biological membranes were not due to DHE perturbing biological membrane structure or function because of the following: DHE is a natural product found in high percentage in the membranes of eukaryotes such as sponge and yeast; DHE can be supplemented to microorganisms and cultured L-cells to replace nearly 90% of endogenous sterol; DHE has no adverse effect on sterol:phospholipid ratio or phospholipid composition, fatty acid composition, cell growth, or cholesterol-sensitive enzymes; DHE codistributes with endogenous sterol among intracellular membranes; and DHE does not alter the structure of the plasma membrane or the function of sterol-sensitive membrane proteins (reviewed in Refs. 26 and 31).

Fourth, the detection of crystalline DHE in the isolated plasma membrane fraction suggested that this small amount of crystalline sterol might represent microdomains present in small amounts in the plasma membrane. It is known that the lateral distribution of sterol in the plane of the bilayer is not uniform because cholesterol-rich lipid rafts/caveolae have been detected in the cell surface and isolated by several methods (reviewed in Ref. 23). Caveolae represent only 1-2% of the cell surface membrane area, but lipid raft/caveolar membrane-enriched fractions exhibit severalfold higher sterol content than the plasma membrane (reviewed in Ref. 23). If the small amount of crystalline DHE detected in L-cell plasma membranes was due to its presence in lipid raft/caveolar membrane domains, then an enrichment of severalfold might be predicted therein with DHE:phospholipid ratios near 2-3. However, model membranes with such DHE:phospholipid ratios contained 7.1-9.5% crystalline DHE. Surprisingly, the level of crystalline DHE in isolated lipid raft/caveolar membranes was <0.3%, 18- and up to 32-fold lower than in the bulk plasma membrane and model membranes, respectively (Table I). These observations, showing that lipid rafts/caveolar membranes contain very little crystalline sterol despite their high sterol content, may explain for the first time why so many processes (signaling, transport, etc.) important to cell viability readily function in these domains. The future challenge will be to determine what features of lipid raft/caveolar proteins and/or lipids prevent crystalline sterol formation.

Fifth, MPLSM for the first time resolved the uptake and intracellular distribution of crystalline sterol in real time in living cells. L-cell fibroblasts ingested large amounts of crystalline DHE that accumulated in lysosomes as evidenced by colocalization with LysoTracker Green, a lysosomal stain, in living cells. The lysosomal localization of substantial amounts of crystalline DHE was confirmed by examination of the spectral properties of DHE in lysosomes isolated from L-cells cultured with crystalline DHE. In addition, some crystalline DHE was also detected outside the lysosomal compartment. However, very little extralysosomal crystalline DHE colocalized with BODIPY FL C5-ceramide (Golgi marker) or Nile Red (lipid droplet marker) in living cells. Consistent with this observation, crystalline cholesterol has been detected in macrophage foam cells, both within lysosomes and outside (as crystals surrounded by a membrane) the lysosomal compartment (13, 14, 19). The phagocytosis of DHE crystals was consistent with the known ability of L-cells to phagocytose particles as large as several microns and by the fact that L-cells endocytose the equivalent of their entire cell surface membrane within about 2 h (55, 74), similar to the activity of macrophages (14, 19).

Sixth, MPLSM for the first time resolved the uptake and intracellular distribution of monomeric sterol in real time in living cells. The pattern of monomeric sterol distribution depended on the mode of DHE supplementation to the cells. (i)
Although most cells incubated with crystalline DHE exhibited high amounts of crystalline form in lysosomes, those cells, which had digested/solubilized the ingested crystals, distributed monomeric DHE throughout the cell. The highest concentrations of monomers were in the plasma membrane and lipid droplets, followed by lower levels in the perinuclear regions with much less monomeric DHE was present in a punctate vesicular pattern throughout the cytoplasm. Thus, the use of multiphoton laser scanning microscopy together with the spectral differences in monomeric versus crystalline DHE allowed for the first time the noninvasive, real time imaging of multiple forms of sterol in living cells.

In summary, the data presented herein demonstrate that the spectral properties of DHE, together with multiphoton laser scanning microscopy, form a powerful tool to noninvasively resolve and visualize the individual dynamics of multiple structural forms of sterol in real time in living cells. Crystalline DHE was enriched within lysosomes but not plasma membranes or lipid raft/caveolar membrane-enriched subfractions. Interestingly, the presence of sterol carrier protein-2 in vitro significantly enhanced the sterol transfer of crystalline DHE (in donor lysosomes) to acceptor lysosomes (containing no DHE) as evidenced by the change in spectral characteristics of the DHE emission from crystalline to monomeric. These data for the first time showed a potential protective role for sterol carrier protein-2 in mitigating the deleterious effects of crystalline DHE in the cell. Consistent with this possibility, the cellular level of sterol carrier protein-2 was up-regulated as much as 3-fold in macrophage foam cells (7, 75) wherein crystalline DHE was enriched within lysosomes but not plasma membranes, which had digested/solubilized the ingested crystals, distributed monomeric DHE were found in the plasma membrane and lipid droplets, followed by perinuclear regions with much less monomeric DHE was present in a punctate vesicular pattern throughout the cytoplasm. Thus, the use of multiphoton laser scanning microscopy together with the spectral differences in monomeric versus crystalline DHE allowed for the first time the noninvasive, real time imaging of multiple forms of sterol in living cells.
58. Igbavboa, U., Avdulov, N. A., Schroeder, F., and Wood, W. G. (1996) J. Neurochem. **66**, 1717–1725
59. Wood, W. G., Chochina, S. V., Igbavboa, U., O’Hare, E. O., Schroeder, F., Cleary, J. P., and Avdulov, N. A. (1997) J. Neurochem. **68**, 2086–2091
60. Wood, W. G., Schroeder, F., Avdulov, N. A., Chochina, S. V., and Igbavboa, U. (1999) **Lipids** **34**, 225–234
61. Haberland, M. E., and Reynolds, J. A. (1973) Proc. Natl. Acad. Sci. U. S. A. **70**, 2313–2318
62. Renshaw, P. F., Janoff, A. S., and Miller, K. W. (1983) **J. Lipid Res.** **24**, 47–51
63. Small, D. M., and Shipley, G. G. (1974) *Science* **185**, 222–229
64. Pope, M. (1999) in *Electronic Processes in Organic Crystals and Polymers* (Pope, M., and Swenberg, C. E., eds) pp.1–189, Oxford University Press, Oxford
65. Martin, R. B., and Yeagle, P. L. (1978) *Lipids* **13**, 594–597
66. Sankaram, M. B., Marsh, D., Giersch, L. M., and Thompson, T. E. (1994) *Biophys. J.* **66**, 1959–1968
67. Rice, P. A., and McConnell, H. M. (1989) Proc. Natl. Acad. Sci. U. S. A. **86**, 6445–6448
68. Mantripragada, B., Sankaram, B., and Thompson, T. E. (1991) Proc. Natl. Acad. Sci. U. S. A. **88**, 8689–8690
69. Castanhe, M. A., Brown, W., and Prieto, M. (1992) *Biophys. J.* **63**, 1455–1461
70. Chung, D. S., Benedek, G. B., Kunitz, F. M., and Donovan, J. M. (1993) Proc. Natl. Acad. Sci. U. S. A. **90**, 11341–11345
71. Nemecz, G., Fontaine, R. N., and Schroeder, F. (1988) *Biochim. Biophys. Acta* **943**, 511–521
72. Chong, P. L., Liu, F., Wang, M. M., Truong, K., Sugar, I. P., and Brown, R. E. (1996) *J. Fluorescence* **6**, 221–224
73. Ohvo-Rekila, H., Akerlund, B., and Slotte, J. P. (2000) *Chem. Phys. Lipids* **105**, 167–178
74. Schroeder, F., and Kier, A. B. (1983) *J. Immunol. Methods* **57**, 363–371
75. Hirai, A., Kino, T., Tokinaga, K., Tahara, K., Tamura, Y., and Yoshida, S. (1994) *J. Clin. Invest.* **94**, 2215–2223
Fluorescence and Multiphoton Imaging Resolve Unique Structural Forms of Sterol in Membranes of Living Cells

Avery L. McIntosh, Adalberto M. Gallegos, Barbara P. Atshaves, Stephen M. Storey, Deepak Kannoju and Friedhelm Schroeder

J. Biol. Chem. 2003, 278:6384-6403.
doi: 10.1074/jbc.M205472200 originally published online November 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205472200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 67 references, 21 of which can be accessed free at http://www.jbc.org/content/278/8/6384.full.html#ref-list-1