

## Comparative behavior of sterols in phosphatidylcholine-sterol monolayer films

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Received 10 October 2000; received in revised form 18 January 2001; accepted 2 February 2001

### Abstract

The ability of sterols other than cholesterol (CHOL) to support membrane functions in membranes that normally contain CHOL as the primary, if not sole, sterol may be due, in part, to how well such sterols can mimic CHOL's behavior and physical properties in membranes. We compared the mixing properties of CHOL, 7-dehydrocholesterol (7DHC), and desmosterol (DES) in egg phosphatidylcholine-sterol monolayer films containing 10, 20, and 30 mol percent sterol, measuring pressure-area isotherms on a Langmuir–Blodgett trough with the aqueous, buffered subphase maintained at 37°C. Under the conditions employed, the pressure-area isotherms for all three sterols were similar, with 7DHC exhibiting slightly larger molecular areas on the water surface at all compositions. These results are discussed in the context of the ability of sterols such as 7DHC and DES to substitute structurally and functionally for CHOL in biological membranes. © 2001 Published by Elsevier Science B.V.

**Keywords:** Cholesterol; 7-Dehydrocholesterol; Desmosterol; Desmosterolosis; Langmuir film; Phosphatidylcholine; Smith–Lemli–Opitz syndrome

### 1. Introduction

Although sterols are quantitatively and functionally significant constituents of eukaryotic plasma membranes, they differ in their relative abilities to be incorporated into membranes and support membrane functions (reviewed in [1,2]). For example, 7-dehydrocholesterol (cholesta-5,7-dien-3 $\beta$ -ol, 7DHC)

is solvated only about 55% as efficiently as is cholesterol (CHOL) in egg phosphatidylcholine (PC) liposomes, suggesting that 7DHC does not pack spontaneously into membrane bilayers as well as does CHOL [3]. Also, 7DHC and desmosterol (cholesta-5,24-dien-3 $\beta$ -ol; DES) are only 16.5 and 27% as effective, respectively, as CHOL in supporting Na<sup>+</sup>/Ca<sup>2+</sup> exchanger activity derived from sarcoplasmic reticulum membranes, whereas these same sterols are 11.6 and 83.2% as effective, respectively, as CHOL in supporting Na<sup>+</sup>/K<sup>+</sup>-ATPase activity [4]. Indeed, CHOL is required for full activity by both kidney Na<sup>+</sup>/K<sup>+</sup>-ATPase [5] and the erythrocyte Band 3 anion transporter [6]. Cells exhibit different phylogenetic preferences for particular sterol struc-

Abbreviations: CHOL, cholesterol; DES, desmosterol; 7DHC, 7-dehydrocholesterol; PC, phosphatidylcholine; ROS, rod outer segment

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tures; hence, CHOL is the predominant sterol in most mammalian cells, whereas ergosterol (24-methyl-cholesta-5,7,22-trien-3 $\beta$ -ol), which has the same sterol nucleus structure as 7DHC, is the preferred sterol in yeast [1].

Recently, we reported [7–9] that rats exposed to either AY9944 (an inhibitor of 3 $\beta$ -hydroxysterol  $\Delta^7$ -reductase [10,11]) or U18666A (an inhibitor of 3 $\beta$ -hydroxysterol  $\Delta^{24}$ -reductase [12,13]) exhibited normal development of retinal structure and function, despite the fact that the retina and other tissues largely replaced their CHOL (up to 80%) with either 7DHC or DES, respectively. To explain this, we proposed that these ‘alternate’ sterols can substitute both physically and functionally for CHOL in the retina. In the present study, we sought to evaluate the ability of 7DHC and DES to mimic CHOL in a simplified, artificial membrane system, using binary mixtures of egg PC and a given sterol spread as monolayer films on a Langmuir–Blodgett trough. Such monolayers essentially represent one half of a membrane bilayer, and have been used extensively to study the biophysical properties of membrane components and to model biochemical processes that occur in biological membranes (reviewed in [14,15]). Pressure-area isotherms obtained by analysis of Langmuir films can reveal fundamental information about the packing of molecules over a range of molecular areas, and are also useful for determining miscibility of constituents in binary systems. Herein, we demonstrate that DES and, to a somewhat lesser degree, 7DHC exhibit behavior similar to CHOL in binary PC–sterol monolayer films over a range of sterol concentrations that are biologically relevant. These findings are discussed in the context of the ability of DES and 7DHC to replace CHOL structurally and functionally in more complex biological membranes.

## 2. Materials and methods

L- $\alpha$ -PC, from egg yolk, >99% pure, CHOL, and DES were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were used as received. The fatty acid composition of the egg PC employed throughout this study was reported by the manufacturer to be as follows: 33% C16:0, 13% C18:0, 31% C18:1, and

15% C18:2 (with 8% from other, non-specified fatty acid species). 7DHC (a generous gift from Dr. Seiichi P.T. Matsuda, Rice University, Houston, TX, USA) was recrystallized repeatedly from acetone–water prior to use. All sterols were analyzed by reverse-phase HPLC and found to be >99% pure [7]. Sterols were stored in dry form under argon atmosphere in amber glass vials at  $-20^{\circ}\text{C}$  until ready for use. Spreading solutions containing the desired pure lipid (sterol or PC) or PC–sterol mixtures were prepared in chloroform (HPLC grade; Fisher Scientific, Pittsburgh, PA, USA). A stock PC solution (ca. 1 mg PC/ml  $\text{CHCl}_3$ ) was prepared, to which solid CHOL, DES, or 7DHC was added to achieve final concentrations of 10, 20, and 30 mol percent sterol. Water used for the subphase was purified with a Barnstead Nanopure system (Barnstead/Thermolyne, Dubuque, IA, USA), and had a specific resistivity of 18 M $\Omega$ -cm. Tris base (Fisher Scientific) was added to the subphase at a final concentration of 0.01 M, and the pH was adjusted to 7.4 with hydrochloric acid (Fisher Scientific).

Langmuir films were prepared using a NIMA Model 611D Langmuir–Blodgett trough (Coventry, UK), essentially as described previously [16,17]. In brief, a lipid monolayer was formed by dropping approximately 0.1 ml of a chloroform-solvated lipid spreading solution (containing either pure sterol, pure PC, or sterol–PC mixture) onto the aqueous subphase with a glass syringe. Ten minutes were allowed for solvent evaporation before film compression was initiated. Using the software provided by NIMA, pressure-area isotherms were collected continuously for the compression and expansion cycles at a rate of 12  $\text{\AA}^2/\text{molecule}/\text{min}$ . Trough areas were recorded as a function of surface pressure, and Excel<sup>®</sup> spreadsheets were utilized to calculate the pressure-area isotherms in mean molecular area per molecule. For each composition examined, measurements were made at least three times on three different days ( $N=9$  for each lipid film), and the nine isotherms obtained for each system were averaged. The subphase was maintained at  $37^{\circ}\text{C}$  with a heated circulating water bath, which pumped water through coils in the base of the trough. A NIMA PS4 surface pressure sensor was employed in all studies.

Ideal isotherms for mixed monolayers were calculated using the equations for the ‘additivity rule’ [22]:

$$\underline{A} = x_1 A_1 + x_2 A_2 \quad (1)$$

where  $\underline{A}$  is the average area occupied per molecule in a mixed monolayer at a given pressure,  $x_1$  and  $x_2$  are the mol fractions of the two components in the mixed film, and  $A_1$  and  $A_2$  are the molecular areas occupied at the given pressure for each of the two pure molecular components. The areas  $A_1$  and  $A_2$  were determined at a given pressure from the isotherms obtained for each pure sterol monolayer. A complete ideal isotherm was constructed by calculating  $\underline{A}$  through a range of pressures.

### 3. Results

Representative averaged pressure-area isotherms obtained for pure sterol, pure PC, and mixed PC–sterol monolayers at 37°C are shown in Fig. 1, with the data for films containing CHOL, DES, and 7DHC given in Fig. 1A–C, respectively. The pure CHOL isotherm (Fig. 1A, curve *a*) exhibits condensed phase behavior (i.e., a rigid film), characterized by a steep rise in the isotherm at molecular areas below 50 Å<sup>2</sup>/molecule. The monolayer collapsed at a pressure of 43.5 mN/m at a molecular area of 35.0 Å<sup>2</sup>/molecule. For comparison, Demel et al. [22] reported a collapse pressure of 37.2 mN/m (at 22°C) at a molecular area of 39.0 Å<sup>2</sup>/molecule for pure CHOL (see Section 4). In contrast, the isotherm for pure egg PC (Fig. 1A, curve *e*) demonstrates phase behavior characteristic of a liquid phase film. The first increase in surface pressure upon film compression occurs at 138 Å<sup>2</sup>/molecule, and the pressure rises steadily as the molecular area is decreased. The PC monolayer could be compressed to nearly 40 mN/m before collapse; however, the isotherm is only shown for compression to ca. 33 mN/m for comparison with the mixed sterol–PC isotherms. The presence of CHOL in the PC monolayer film alters its behavior, such that the experimentally determined isotherms (Fig. 1A, curves *b*, *c*, and *d*) are shifted to smaller mean molecular areas compared to the calculated isotherms obtained for ideal mixing (Fig. 1A, curves *b'*, *c'*, and *d'*) using the additivity rule [22]. This ‘condensing effect’ is well known for CHOL, and results from favorable hydrophobic interactions between the CHOL and lipid hydrocarbon chains (re-

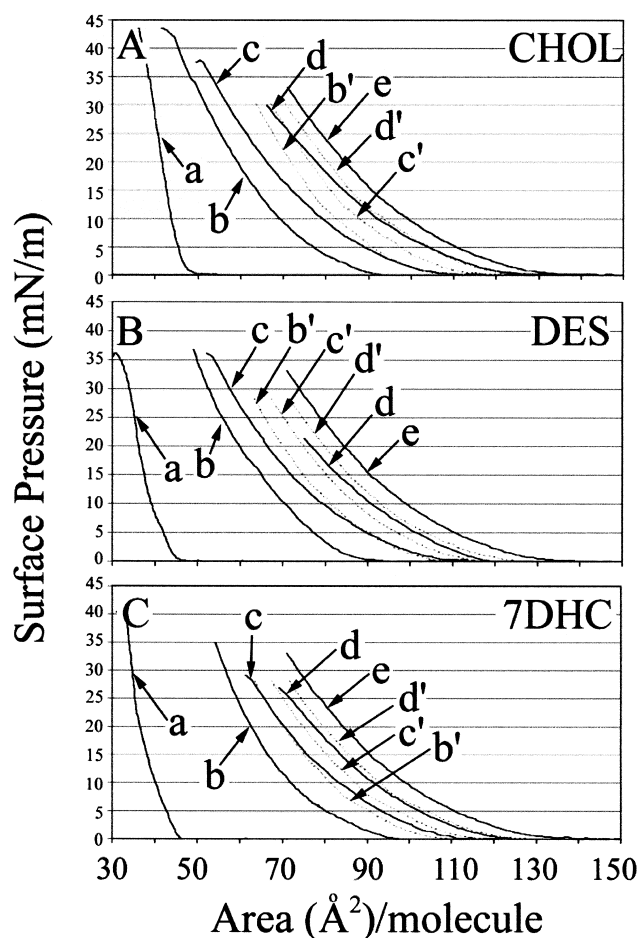


Fig. 1. Pressure-area isotherms obtained at 37°C for mixed PC–sterol monolayers containing (A) CHOL, (B) DES, and (C) 7DHC. Empirically determined isotherms (solid lines); calculated isotherms for ideal mixing (dotted lines). Isotherms: *a*, pure sterol; *b* and *b'*, 30 mol percent sterol; *c* and *c'*, 20 mol percent sterol; *d* and *d'*, 10 mol percent sterol; *e*, pure egg PC.

viewed in [21]). Monolayer collapse was not observed for many of the mixed monolayers; rather, the condensed films were pushed off the water surface above 20 mN/m. This effect is due to the film stiffness caused by the tight packing of surface molecules [14,15].

The ideal isotherm for 10 mol percent CHOL (Fig. 1A, curve *d'*) was calculated using the additivity rule and the isotherm data collected for the pure CHOL (curve *a*) and PC (curve *e*) monolayers. The experimental isotherm for 10 mol percent CHOL (Fig. 1A, curve *d*) shows a slight shift to smaller areas, which is most notable above 25 mN/m. Similarly, for CHOL compositions of 20 mol percent (Fig. 1A, curve *c*)

Table 1  
Deviation from ideal behavior<sup>a</sup>

Mol percent sterol:	10			20			30		
Surface pressure <sup>b</sup>	10	15	20	10	15	20	10	15	20
Sterol									
CHOL	2.3	2.9	3.9	7.8	8.1	8.2	13.1	13.3	13.0
DES	1.9	2.3	3.1	5.5	6.6	7.2	10.5	10.6	10.3
7DHC	1.7	1.2	1.1	4.4	4.5	4.6	8.9	8.5	7.6

All measurements taken at 37°C (*N* = 9).

<sup>a</sup>Values (in Å<sup>2</sup>) represent the difference between the empirically determined isotherm and that calculated for an ideal mixture of pure egg PC and pure sterol at the given mol percent sterol and film pressure.

<sup>b</sup>Values in mN/m.

and 30 mol percent (Fig. 1A, curve *b*) in mixed PC–sterol films, the experimental isotherms are shifted to smaller molecular areas compared to the ideal curves (Fig. 1A, curves *c'* and *b'*, respectively), with the largest condensing effect observed for 30 mol percent CHOL. For the sake of direct quantitative comparison with the results obtained previously by Demel et al. [22], we determined the mean molecular areas and calculated shift values at a pressure of 12 mN/m, as follows: 86.8 Å<sup>2</sup>/molecule with a shift of 2.4 mN/m from ideal (10 mol percent CHOL), 76.7 Å<sup>2</sup>/molecule with a shift of 8.1 mN/m (20 mol percent CHOL), and 66.4 Å<sup>2</sup>/molecule with a shift of 13.2 mN/m (30 mol percent CHOL). Demel et al. [22] measured isotherms for films containing a 1:1 molar ratio of CHOL with 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (18:1/18:0-PC), and reported a mean molecular area of 82.1 Å<sup>2</sup>/molecule at a pressure of 12 mN/m (37°C), with a deviation from ideal behavior of 11.5 Å<sup>2</sup>/molecule. Hence, despite systematic differences between the two studies (see Section 4), our results are in reasonable agreement with those of Demel et al. [22].

Further quantitative analysis of the isotherm data was pursued by calculating the shifts from ideal behavior for all three PC–sterol compositions at pressures of 10, 15, and 20 mN/m (see Table 1). The corresponding mean molecular areas are summarized in Table 2. The deviation from ideal behavior increases from about 2.3 mN/m at 10 mol percent CHOL to 13.1 mN/m at 30 mol percent CHOL incorporation. The increased deviation at higher CHOL concentrations indicates significant condensation of the monolayer.

The isotherms for DES and PC–DES mixtures are shown in Fig. 1B. As was shown for CHOL (see above), the pure DES film (Fig. 1B, curve *a*) behaves as a condensed phase film, with collapse at 36.1 mN/m at an area of 33.6 Å<sup>2</sup>/molecule. To our knowledge, isotherms of pure DES have not been reported previously. The collapse pressure is lower compared to that for CHOL, likely due to the Δ<sup>24</sup> double bond located on the side-chain of DES, which apparently alters the intermolecular interactions above the water surface. Although the collapse area for the pure DES film is apparently smaller than for CHOL, the differ-

Table 2  
Mean molecular area<sup>a</sup>

Mol percent sterol:	10			20			30		
Surface pressure <sup>b</sup> :	10	15	20	10	15	20	10	15	20
CHOL	90.6	82.9	76.2	79.6	72.2	66.5	68.9	63.5	58.5
DES	91.8	83.4	77.0	81.1	73.2	67.5	70.1	64.9	59.7
7DHC	94.4	86.4	80.7	82.5	75.6	70.2	72.0	66.3	62.4

All measurements taken at 37°C (*N* = 9).

<sup>a</sup>Values in Å<sup>2</sup>/molecule.

<sup>b</sup>Values in mN/m.

ence is within experimental error ( $\pm 1.2 \text{ \AA}^2/\text{molecule}$ ). The incorporation of DES into the PC monolayer leads to large shifts in molecular area compared to the calculated ideal mixing isotherms (cf. Fig. 1B, curves *d* and *d'*, *c* and *c'*, and *b* and *b'*, respectively). As the amount of DES incorporated in the PC monolayer increases, larger condensing effects are observed (see Tables 1 and 2). At 10 mol percent DES incorporation, shifts of ca.  $2 \text{ \AA}^2/\text{molecule}$  are noted, while shifts of greater than  $10 \text{ \AA}^2/\text{molecule}$  were observed at 30 mol percent DES incorporation. The condensing effect for DES is not quite as large as that observed for CHOL incorporation into PC monolayers, since smaller deviations from ideal behavior are observed at all compositions (e.g., compare curves *b* and *b'* in Fig. 1A and B, and the data in Table 1).

Isotherms for pure 7DHC and mixed PC–7DHC films are reported in Fig. 1C. The isotherm for pure 7DHC (Fig. 1C, curve *a*) shows collapse at  $40.8 \text{ mN/m}$  at a molecular area of  $34.5 \text{ \AA}^2/\text{molecule}$ . The packing appears to be similar to that of a pure CHOL monolayer, with similar areas observed at collapse (cf. curve *a* in Fig. 1A vs. Fig. 1C). The 7DHC monolayer does collapse at a lower pressure, however, due to the presence of a double bond in the nuclear ring system, which may lead to out-of-plane puckering during film compression. An isotherm was previously reported by Demel et al. [22] for 7DHC at  $22^\circ\text{C}$ , and a collapse pressure of  $36.1 \text{ mN/m}$  was noted. Again, despite systematic differences between the two studies, both show that pure 7DHC films collapse at lower pressure than do films of pure CHOL.

When 7DHC is incorporated into mixed PC–sterol monolayers, shifts to smaller mean molecular areas are observed, and deviations from ideal mixing behavior are found to increase with increased 7DHC incorporation (see Fig. 1C, curves *b*, *c*, and *d*, and Tables 1 and 2). The overall shifts are smaller compared to those observed for both DES and CHOL, indicating that 7DHC does not pack as tightly in mixed PC–sterol monolayers as do DES and CHOL. The shifts from ideal behavior range from  $1 \text{ \AA}^2/\text{molecule}$  at 10 mol percent 7DHC to around  $9 \text{ \AA}^2/\text{molecule}$  at 30 mol percent 7DHC incorporation (Table 1). The mean molecular areas for 7DHC-

containing films are also larger than those for DES and CHOL in all cases (Table 2).

The fact that 7DHC exhibits packing somewhat different from DES and CHOL can be seen more clearly in Fig. 2. In this figure, the isotherms have been plotted for all three PC–sterol mixed films at similar compositions. While the CHOL and DES isotherms are within  $2 \text{ \AA}^2/\text{molecule}$  throughout the compression range, the 7DHC isotherm is consistently located at larger molecular areas. The 7DHC isotherm does not overlap well with those of DES and CHOL, and deviates significantly at a composition of 30 mol percent. Taken together, these data indicate that CHOL and DES exhibit similar packing at all compositions studied, while 7DHC exhibits packing which is slightly different from these two sterols.

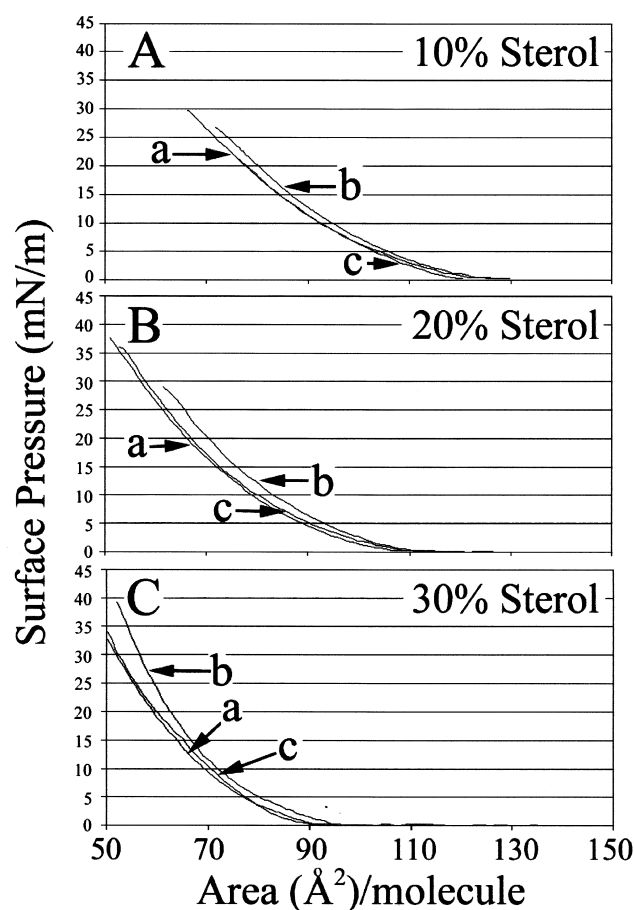


Fig. 2. Pressure-area isotherms obtained at  $37^\circ\text{C}$  for mixed PC–sterol monolayers containing (A) 10, (B) 20, and (C) 30 mol percent sterol. Sterol: a, CHOL; b, 7DHC; c, DES.

#### 4. Discussion

Our results are in reasonably good agreement with those of others (reviewed by Demel and deKruyff [21]), but must be considered within the context of the present technology for preparing and measuring Langmuir films and the specific conditions employed, compared with those used in prior studies (especially those reported in the 1970s). For example, the variance between the findings of Demel et al. [22] and those in the present study likely are due to systematic differences in the Langmuir trough design, water temperature (e.g., 22°C, vs. 37°C), subphase composition (pure water, vs. Tris-buffered water), PC composition (e.g., a pure, single molecular species with defined chain lengths and composition, vs. the multiple molecular species found in egg PC), and the method of data collection (e.g., individual measurements at specific points during film compression, vs. continuous collection during film compression). Based upon the pressure-area data shown in Figs. 1 and 2, the packing of CHOL and DES in mixed PC–sterol monolayer films is very similar at all compositions studied, with the closest packing behavior observed at compositions with 10 mol percent sterol incorporation. The data at 10 mol percent suggest that substitution of DES for CHOL may represent a minimal perturbation that likely would be well tolerated in membranes that normally contain only ca. 10 mol percent CHOL (e.g., retinal rod outer segment (ROS) membranes). This is consistent with our finding that pharmacological alterations of sterol metabolism leading to replacement of the majority of the CHOL with either 7DHC or DES do not cause structural or functional defects in the rat retina [7–9]. Although the mean molecular area of PC–7DHC monolayers was found to deviate from that of PC–CHOL and PC–DES films by about 2 Å<sup>2</sup>/molecule (Table 2), this small difference in molecular area may not be significant in considering alternate sterol substitution. The presence of additional double bonds in DES ( $\Delta^{24}$  double bond in the side-chain) apparently affects sterol packing minimally in the mixed monolayers, but the presence of the side-chain double bond leads to some restricted chain rotation above the water surface. As a result, intermolecular interactions above the water surface during film compression

lead to looser molecular packing compared to films containing CHOL. Good overlap of isotherms for the three PC–sterol compositions examined is observed with DES and CHOL, while the mean molecular areas at the three pressures chosen for quantitative analysis (10, 15, and 20 mN/m) show minimal variation. The tabulated surface areas for DES indicate differences greater than for CHOL of about 1.0–1.5 Å<sup>2</sup>/molecule, which is within experimental error. 7DHC contains a  $\Delta^7$  double bond in ring B of the sterol nucleus, and consistently alters the packing density compared to CHOL, which is likely due to ‘puckering’ (loss of planarity of the ring system). This increases steric hindrance between adjacent molecules, thereby preventing the sterol from packing as tightly in the phospholipid matrix. Thus, the area requirements per molecule are larger for 7DHC than for the other two sterols, as indicated by the fact that the mean molecular areas of 7DHC are about 3–4 Å<sup>2</sup>/molecule larger than CHOL at all compositions examined.

Considering the results obtained in this study, it is possible that in some membrane systems the substitution of DES for CHOL may be reasonably well tolerated at sterol levels between 10 and 30 mol percent. In the high pressure region of the pressure-area isotherm, which is comparable to the lipid packing in natural membranes, there is approach and overlap between the isotherms obtained for PC–CHOL and those of the PC–DES films (see Figs. 1 and 2). The isotherm for the 7DHC-containing film, although shifted to the right of that for PC–CHOL, shows a similar curvature. Hence, despite quantifiable differences, 7DHC exhibits behavior similar to that of CHOL and DES in this type of membrane system. This provides the necessary, but not sufficient, condition for 7DHC to support biological membrane functions comparable to CHOL at the given sterol mol percent values. Clearly, this would depend upon both the type of membrane, its composition, and the particular function being examined.

It should be noted that Demel et al. [22] have shown previously that 7DHC and CHOL exhibit similar behavior in Langmuir films, both as pure sterols and also in 1:1 molar ratio with 18:1/18:0-PC, at both 22 and 37°C. Our results complement and extend that study, using somewhat different con-

ditions (see above), and also include comparative data for DES, which was not included in the prior study.

Marked, pharmacologically induced increases in 7DHC and DES and compensatory decreases in CHOL appear to be well tolerated in the neonatal and developing rat retina [7–9], where 75–80 mol percent of the CHOL normally present is replaced by these alternate sterols without alteration of the total sterol content. The present results are consistent with these findings. However, it is well known that genetic defects that lead to comparable perturbations of CHOL metabolism in humans result in devastating diseases, such as the Smith–Lemli–Opitz syndrome [23,24] and desmosterolosis [25,26]. Hence, physical properties alone are not sufficient predictors of biological functionality. In the case of pharmacologically induced changes in sterol metabolism, either the retina evades such insults by virtue of its relatively low sterol content (and the ability of these alternate sterols to effectively mimic CHOL at low concentrations), or else other factors may help to preserve normal structure and function. For example, compensatory, adaptive changes in the fatty acid composition might occur in response to altered sterol metabolism, as has been noted in cultured LM fibroblast sterol mutants [27,28]. Indeed, we recently have obtained evidence for such alterations in AY9944-treated rats [29]. Alternatively, ‘sterol synergism’ may be responsible for this phenomenon [30–32]. Currently, we are performing similar studies with binary sterol mixtures in PC monolayers, varying the mol ratio of 7DHC and DES to CHOL and the mol ratio of total sterol to PC in an effort to examine this problem further. In addition, it has been shown [33] that CHOL can interact directly with rhodopsin with some degree of specificity (e.g., ergosterol does not), in addition to exerting effects on the bulk phase lipids of ROS membranes. Whether or not 7DHC or DES can do the same remains to be determined.

Finally, it should be appreciated that the findings obtained with simple binary lipid systems such as used here may not reflect accurately the effects obtained with a given sterol in a particular biological membrane system. Indeed, our choice of egg PC to represent the phospholipid component of biological membranes was done for simplicity, while still main-

taining some degree of biological relevance to the findings. The complexity of naturally occurring membrane lipid classes and their constituent molecular species also adds another significant determinant to the behavior of sterols in biological membranes, particularly as may affect phase separations, localized domain structure, and the activity of various membrane-bound enzymes, receptors, and ion channels [1,2,19,20]. This is certainly true for retinal membrane lipids, which contain hundreds of phospholipid molecular species, in addition to sphingolipids and sterols [18]. Hence, the direct extrapolation of these results to a given biological system should be done with due caution.

### Acknowledgements

Supported by U.S.P.H.S. (NEI/NIH) Grant EY07361 (to S.J.F.). A.B.S. is the recipient of a Summer Research Award from Saint Louis University. We thank Michael J. Richards for technical assistance with preparation of the manuscript and figures for publication.

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