Biochimica et Biophysica Acta **861 (1986) 287-301**
Elsevier

BBA 73263

Regulation of transbilayer distribution of a fluorescent sterol in tumor cell plasma membranes

Ann B. Kier [b], William D. Sweet [a], Matthew S. Cowlen [a] and Friedhelm Schroeder [a]

Departments of [a] Pharmacology, School of Medicine and [b] Veterinary Pathology, College of Veterinary Medicine, University of Missouri-Columbia, Columbia, MO 65212 (U.S.A.)

(Received 10 March 1986)

Key words: Plasma membrane; Cholesterol; Metastasis; Cancer; Phosphatidylinositol

The lipid composition and transbilayer distribution of plasma membrane isolated from primary tumor (L-929, LM, A-9 **and C_3H) and nine metastatic cell lines cultured under identical conditions was examined. Cultured primary tumor and metastatic cells differed two-fold in sterol / phospholipid molar ratios. There was a direct correlation between plasma membrane anionic phospholipid (phosphatidylinositol and phosphatidylserine) content and plasma membrane sterol / phospholipid ratio. This finding may bear on the possible link between oncogenes and inositol lipids. The fluorescent sterol, dehydroergosterol, was incorporated into primary tumor and metastatic cell lines. Selective quenching of outer monolayer fluorescence by covalently linked tinitropbenyl groups demonstrated an asymmetric transbilayer distribution of sterol in the plasma membranes. The inner monolayer of the plasma membranes from both cultured primary and metastatic tumor cells was enriched in sterol as compared with the outer monolayer. Consistent with this, the inner monolayer was distinctly more rigid as determined by the limiting anisotropy of 1,6-diphenyl-1,3,5-hexatiene. Dehydroergosterol fluorescence was temperature dependent and sensitive to lateral phase separations in phosphatidylcholine vesicles and in LM cell plasma membranes. Dehydroergosterol detected phase separations near 24°C in the outer monolayer and at 21" C and 37°C in the inner monolayer of LM plasma membranes. Yet, no change in transbilayer sterol distribution was detected in ascending or descending temperature scans between 4 and 45°C. Alterations in plasma membrane phospbolipid polar head group composition by choline analogues (*N*,*N*-dimethylethanolamine, *N*-methylethanolamine, and ethanolamine) also did not perturb transbilayer sterol asymmetry. Treatment with phenobarbital or prilocaine, drugs that selectively fluidize the outer and inner monolayer of LM plasma membranes, respectively, did not change dehydroergosterol transbilayer distribution.**

Introduction

Cholesterol plays a significant role in determining the structure and function of biological membranes and in the development of disease states. For example, cholesterol increases membrane microviscosity and the packing density of phospholipids [1,2], influences lateral lipid phase separations [3], and affects transbilayer fluidity gradients [4,5]. Moreover, cholesterol modulates the expression of surface membrane antigenic determinants [1,2], the activity of membrane-bound enzymes [6], the K_d and B_{max} of drug receptors

Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

Correspondence address: Department of Veterinary Pathology, College of Veterinary Medicine. University of Missouri-Columbia, Columbia, Mb 65212, U.S.A.

0005-2736/86/$03.50 © 1986 Elsevier Science Publishers B.V. (Biomedical Division)

[7–10], membrane contour [11] and membrane permeability [12,13]. In addition, sterol content has been correlated with altered plasma membrane structure and physical properties as well as with metastatic ability in mouse and rat tumor cells [14–17]. Increasing evidence also indicates that there is an asymmetric distribution of sterol across the surface bilayer of many biological membranes including viral membranes [18,19], mycoplasmal [20] and eukaryotic plasma membranes from rat sciatic nerve myelin [21], human, rat, and mouse erythrocytes [4,5,22,23], tumorigenic mouse fibroblasts [4,5], guinea pig sperm tail [24] and insect Kc cells [25]. However, the significance of transbilayer sterol asymmetry with respect to the above structure-function relationships, and the exact mechanisms involved in the maintenance of transbilayer sterol asymemtry remain to be elucidated. Herein, we examined the stability of transbilayer sterol asymmetry in plasma membranes from cultured tumorigenic mouse fibroblasts. In particular, highly metastatic mouse tumor cells had distinctly lower sterol carrier protein content and plasma membrane sterol/phospholipid molar ratios as compared with primary tumor cell lines [16]. Therefore, it was of interest to determine whether or not primary and metastatic cells had different transbilayer sterol distributions. In addition, charged anesthetics such as phenobarbital (anionic) and prilocaine (cationic) are believed to selectively intercalate into and fluidize the outer and inner monolayers of plasma membranes due to the asymmetric enrichment of neutral (zwitterionic) and acidic phospholipids, respectively [26–28]. Moreover, these drugs have been used to demonstrate that some membrane-bound enzymes which are asymmetrically distributed across the bilayer are sensitive to selective modulation of individual monolayer fluidity [27,28]. The alteration of individual monolayer fluidity by charged anesthetics might perturb transbilayer sterol distribution. Similarly, the manipulation of phospholipid polar head-group composition distinctly alters plasma membrane structure and function [29–34] and may thereby also alter the transbilayer sterol distribution.

Herein, the effects of genetic variation resulting in altered plasma membrane sterol/phospholipid molar ratio, phospholipid polar head-group manipulation (choline analogues), lateral phase separation (temperature), and alterations of individual monolayer structure (charged anesthetics) on transbilayer sterol distribution are reported. Fluorescence probe molecules (dehydroergosterol and diphenylhexatriene), trinitrobenzenesulfonic acid quenching, and differential polarized phase fluorometry were used to examine the effect of selective perturbation of the normal biophysical and biochemical integrity of the membranes.

A portion of this work was presented as an abstract at the annual FASEB meeting, Anaheim, CA. Fed. Proc. (1985) 44, 1350 (abstr. 5494).

Materials and Methods

Reagents and fluorescence probe molecules

The fluorescent sterol dehydroergosterol (DHE) ($\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol) was synthesized and purified as described previously [35]. High-performance liquid chromatography of dehydroergosterol and comparison with dehydroergosterol standards purchased from Frann Scientific, Inc. (Columbia, MO) confirmed the purity of DHE. Trinitrobenzenesulfonic acid (picrylsulfonic acid) and phenobarbital were obtained from Sigma Chemical Co. (St. Louis, MO). The fluorescence probe molecule 1,6-diphenyl-1,3,5-hexatriene, choline, *N*, N-dimethylethanolamine, *N*-monomethylethanolamine and ethanolamine were purchased from Eastman Chemical Co. (Rochester, NY). Prilocaine was a generous gift from Astra Pharmaceuticals (Södertälje, Sweden, and Worcester, MA, U.S.A.). Palmitoyloleoylphosphatidylcholine (POPC), dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). 4-Cholesten-3-one and 5-cholesten-3β,7β-diol were purchased from Steraloids (Wilton, NH).

Cell *lines and tissue cultures*

L-929 cells (CCL1) and LM cells (CCL1.2), a subline of the L-929 cell, were tumorigenic mouse fibroblasts obtained from the American Type Culture Collection (Rockville, MD). A-9 cells, another derivative of the L-929 cell, were generously provided by Dr. H.P. Klinger, Albert Einstein College

of Medicine, New York. C_3H primary tumor and five lung metastasis cell lines were derived from LM cells injected into C_3H/Hen (MTV-) mice (Charles River Lab., Wilmington, NJ) as described elsewhere [16,36]. Nude mouse primary tumor and four lung metastasis cell lines were similarly obtained from LM cells injected into Balb/c, *nu/nu* mice (Charles River Labs., Wilmington, NJ) [37]. Cells were cultured in suspension at approximately $1 \cdot 10^6$ cells/ml in serum-free, chemically defined medium at 37°C in 500 ml bottles (250 ml/bottle) as previously described [29]. Dehydroergosterol (5 μg/ml) was dissolved in ethanol and added to cultures 72 h prior to treatment with trinitrobenzenesulfonic acid and isolation of plasma membranes. Ethanol did not exceed 0.5% (v/v) final concentration, and did not adversely effect cell growth.

Trinitrobenzenesulfonic acid labeling and plasma membrane isolation

Cells were cultured with dehydroergosterol and split into two aliquots, one which received no treatment and one which was treated with trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C) for 80 min in order to trinitrophenylate outer monolayer amine moieties, or under penetrating conditions (37°C) in order to trinitrophenylate both monolayers [5,15,38]. The reaction was stopped by the addition of ice-cold Tris buffer (0.15 M, pH 7.0). Cells were washed with phosphate-buffered saline (pH 7.4), resuspended in 0.25 M sucrose/1 mM triethanolamine (pH 7.2), homogenized, and plasma membranes, microsomes and mitochondria were isolated by differential and sucrose gradient centrifugation as previously described [29]. Treatment of cells with buffer alone versus no treatment gave identical results. Plasma membranes were also isolated from cells cultured without dehydroergosterol as described above for experiments with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. The percentage of membrane phosphatidylethanolamine trinitrophenylated was determined as described earlier [31].

Determination of sterol/phospholipid ratios

All organic solvents were glass distilled and all glassware was washed with sulfuric acid/dichromate before use. Isolated plasma membranes were suspended in 1.0 ml of phosphate-buffered saline (pH 7.4) and 0.2 ml was removed for the determination of protein by the method of Lowry et al. [39]. Total membrane lipid was extracted from the remaining 0.8 ml by the method of Bligh and Dyer [40] as described by Ames [41]. Sterols and phospholipids were separated by silicic acid column chromatography [29]. Phospholipid was determined as described by Ames [42]. Sterol content and sterol composition were determined by high-performance liquid chromatography (HPLC) basically as described earlier [35] except for the following modifications: neutral lipid fractions were analyzed on a 4.8×150 mm, 3 micron octadecyl reverse-phase column and 3 cm guard column containing the same material (Alltech Assoc. Inc., Applied Science Labs, Deerfield, IL). The solvent system used was HPLC grade (Fisher Scientific, Pittsburgh, PA) 70% acetonitrile/30% methanol at a flow rate of 2 ml/min with a Series 4 HPLC (Perkin-Elmer Inc., Norwalk, CT). The column eluant was monitored for absorbance at 215 nm using a 18 μl flow cell in a LC-95 ultraviolet/visible spectrophotometer detector (Perkin-Elmer, Inc., Norwalk, CT). Retention times of compounds were: dehydroergosterol, 12.5 min; desmosterol, 17.4 min; ergosterol (added as internal standard), 19.5 min. Sterols were quantitated using a 3390A Recording Integrator (Hewlett Packard Inc., Palo Alto, CA). Peak areas were corrected for different response factors of the sterols at 215 nm.

Fluorescence measurements

Absorbance-corrected fluorescence intensity of dehydroergosterol was determined for unlabeled and trinitrophenylated plasma membranes using the computer-centered spectrofluorimeter developed by Holland et al. [43] as described by Schroeder [15]. Samples of membrane (50 μg protein/ml phosphate-buffered saline (pH 7.4)) containing dehydroergosterol were excited at 324 nm and emission was measured at 380 nm. The transbilayer distribution of dehydroergosterol was determined by the selective quenching of outer monolayer dehydroergosterol fluorescence by trinitrophenylated amine moieties in the outer monolayer [5] resulting from trinitrobenzene-

sulfonic acid labeling at 4°C as described above.

Fluorescence lifetime, steady-state polarization, and differential polarized phase fluorometry of diphenylhexatriene in isolated LM cell plasma membranes was determined using a T format SLM 4800 subnanosecond spectrofluorimeter (SLM Instruments, Champaign-Urbana, IL) as described by Schroeder [15]. Narrow band passes in the excitation monochrometer and cut-off filters in the emission monochrometers were used to reduce light scattering during steady-state polarization (P) measurements. Fluorescence lifetimes (τ) were determined by the simultaneous measurement of samples and a reference solution of 1,4-bis(2-(4-methyl-5-phenyloxazolyl))benzene (dimethyl POPOP) dissolved in absolute ethanol [44] with excitation polarized at 0°C and emission polarized at 55°C to eliminate lifetime artifacts due to Brownian motion. Excitation wavelength was 355 nm, while emission was measured above 430 nm using cut-off filters (Schott Optical Co., Duryea, PA).

Rotational relaxation time in ns $(6R)^{-1}$ and limiting anisotropy (r_∞) of 1,6-diphenyl-1,3,5-hexatriene in LM cell plasma membranes were determined by differential polarized phase fluorometry using the methods of Weber [45] and Lakowicz et al. [46] and described by the equation:

$$r_\infty = r + (r - r_0)/6R\tau \quad (1)$$

where r_∞ is the limiting anisotropy, r is the steady-state anisotropy, r_0 is the anisotropy in the absence of rotational motion, R is the rotation rate (radians/s) and τ is the fluorescence lifetime. $(6R)^{-1}$ represents the rotational relaxation time in ns. The value of r_0 for 1,6-diphenyl-1,3,5-hexatriene is 0.3920 [46]. Steady-state anisotropy, r, can be calculated from steady-state polarization (P) by:

$$r = 2P/(3 - P) \quad (2)$$

R, the rotational rate in radians/s, can be determined by differential polarized phase measurements [45,46] according to the equation:

$$(m \tan \Delta)(2R\tau)^2 + (c \tan \Delta - A)(2R\tau) + (D \tan \Delta - B) = 0 \quad (3)$$

where:

$$A = 3B = w\tau(r_0 - r)$$

$$C = (1/3)(2r - 4r^2 + 2)$$

$$D = (1/9)(m + m_0 w^2 \tau^2)$$

$$m = (1 + 2r)(1 - r)$$

and Δ is the phase shift angle and w is the circular modulation frequency.

Calculations of outer monolayer physical properties were based on Weber's law of anisotropy additivity [47] which requires intensity weighting for the addition of fractional anisotropies.

Arrhenius plots of absorbance-corrected fluorescence intensity of dehydroergosterol in LM cell plasma membranes were accomplished with an Exocal 100, Endocal 850, and ETP-3 Temperature Programmer system (Neslab Inst., Portsmouth, NH). The temperature of samples was increased from 4 to 45°C then decreased from 45 to 4°C at 1 Cdeg/min, and was monitored with a WR-700 Digital Thermometer (Kernco Instruments, El Paso, TX) and a thermocouple placed directly above the lightpath in the sample cuvette.

Chemical and drug treatments

The absorbance-corrected fluorescence and dynamic physical properties of dehydroergosterol in LM cell plasma membranes with or without trinitrobenzenesulfonic acid treatment were determined. Samples were exposed to phenobarbital (0.5 mM) or prilocaine (1 mM). These concentrations were chosen because they optimally fluidized the outer versus inner monolayer (phenobarbital) and fluidized the inner versus the outer monolayer (prilocaine) [27]. All samples were incubated at 37°C for various periods of times (described in legends to tables and figures), and fluorescence properties determined at 37°C as described above.

Preparation of liposomes

Small unilamellar vesicles (SUV) were prepared as follows: dehydroergosterol (0.5 mol%) and the indicated phospholipid were dissolved in chloroform or ethanol. Samples were placed in solvent-cleaned glass tubes, evaporated under N_2, and trace amounts of solvents were removed in vacuo

for 24–48 h. Sufficient buffer (10 mM Pipes/0.02% NaN_3 (pH 7.4)) was added at 24°C to provide a final lipid concentration of 4 mM. The samples were vortexed and sonicated for 3 min with a Sonogen bath sonicator (Cole-Parmer Instrument Co., Chicago, IL) to remove the lipid from the sides of the tube and yield a milky suspension of multilamellar vesicles. This suspension was then further sonicated in three 10 min bursts with a titanium microtip and Sonic Dismembrator, Model 300 (Fisher Scientific, Inc., Pittsburgh, PA) set at 33% maximum output. Sonication was always performed under N_2 and above the phase transition temperature of the matrix phospholipid. The resulting SUV were separated from large vesicles and multilamellar liposomes by differential ultracentrifugation for 2 h with a 40 Ti rotor and L6-65 ultracentrifuge (Beckman Instruments, Fullerton, CA) as described earlier [48]. The yield of POPC SUV in the supernatant in the presence of dehydroergosterol was approx. 60%.

Results

Incorporation of dehydroergosterol in LM cell plasma membranes

Cholesterol is the endogenous sterol found in cell surface membranes of most eukaryotic cells. However, LM cells lack the enzymes required to convert desmosterol to cholesterol and therefore their membranes contain desmosterol rather than cholesterol [4]. The fluorescent sterol dehydroergosterol ($\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol) is very similar in structure to the nonfluorescent cholesterol and desmosterol. Dehydroergosterol contains a highly fluorescent conjugated triene system in the B and C rings. Spectral analysis of dehydroergosterol fluorescence in LM cell plasma membranes showed excitation maxima near 315 nm, 324 nm and 340 nm, while emission maxima were observed near 360, 380 and 405 nm.

The mol% sterol and phospholipid in plasma membranes from LM cells cultured with dehydroergosterol (5 μg/ml medium) are presented in Table I. Total sterol (dehydroergosterol + desmosterol) comprised 29 mol% of total lipid in the whole membrane. The mol% sterol in the inner monolayer was 3.3-fold greater than in the outer monolayer as determined by fluorescence quenching (see below). Dehydroergosterol represented 22% of total sterol content in both monolayers (representing 2.7 mol% and 8.9 mol% of total lipid in the outer and inner monolayers, respectively). Phospholipid content was 71 mol% of total lipids in the plasma membrane (representing 88 mol% and 60 mol% in the outer and inner monolayers, respectively). This low content of dehydroergosterol in both monolayers assured that dehydroergosterol-dehydroergosterol interactions resulting in self-quenching did not occur. In addition, dehydroergosterol supplementation did not alter the total sterol content, sterol/phospholipid ratio, phospholipid composition, or phospholipid fatty acid composition of the plasma membranes.

TABLE I

MOL% STEROL AND PHOSPHOLIPID IN LM CELL PLASMA MEMBRANES

Plasma membranes were isolated from LM cells cultured and prepared as described in the legend to Fig. 2. Values represent the average of two determinations. The range in values was less than 5%.

Lipid	mol% of total lipid		
	whole membrane	outer monolayer	inner monolayer
Total sterol	29.1	12.4	40.4
Dehydroergosterol	6.4	2.74	8.9
Desmosterol	22.7	9.70	31.5
Total phospholipid	70.9	87.6	59.6

Trinitrophenylation of fibroblast plasma membranes

In order to accurately determine the transbilayer distribution of fluorescent sterols and the individual monolayer structure of fibroblast plasma membranes, selective quenching techniques utilizing trinitrobenzenesulfonic acid were used. For this purpose it was essential that the extent and nature of the trinitrophenylation reaction was similar in the various cell systems. Indeed, when a variety of cultured primary (LM, A-9, C_3H and L-929) or cultured C_3H mouse lung metastasis cell lines were exposed to trinitrobenzenesulfonic acid under non-penetrating conditions (4°C), the quantity of phosphatidylethanolamine that was trinitrophenylated was essentially similar (Fig. 1). The percentage of tri-

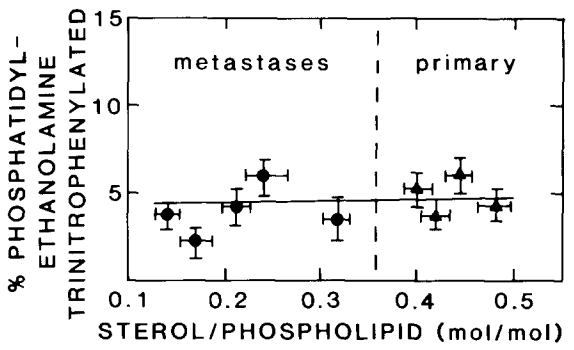

Fig. 1. Transbilayer distribution of phosphatidylethanolamine in plasma membranes from primary and metastatic mouse tumor cells. Cells were cultured in same chemically defined medium and incubated in the absence or presence of trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C). Plasma membranes were isolated and the percentage phosphatidylethanolamine trinitrophenylated was determined as described in Materials and Methods. Primary tumor cell lines (▲) are to the right and metastatic cell lines (●) are to the left of the vertical dashed line. Results are given as the mean ± S.E., $n = 3$–5.

nitrophenylphosphatidylethanolamine ranged between 2 and 6%. These values were maximal, since extending the incubation time did not increase the percentage of trinitrophenylation. In addition, approximately twenty times as many protein amino groups as phospholipid amino groups were trinitrophenylated in the outer monolayer. Most important, if the trinitrobenzenesulfonic acid had penetrated into the cell, extensive trinitrophenylation of intracellular organelles (microsomes or mitochondria) would have occurred. This did not occur. Microsomal and mitochondrial phosphatidylethanolamine were typically trinitrophenylated only to the extent of 0.7 and 2.4%, respectively. In contrast, under penetrating conditions (37°C) plasma membrane, microsomal, and mitochondrial phosphatidylethanolamine were 90, 89 and 91% trinitrophenylated, respectively. Last, since the extent of trinitrophenylation varied neither with cell type nor plasma membrane sterol content, the efficiency of fluorescence probe quenching is expected to be similar in all the cell types investigated.

Homogenization potentially could cause relocation of originally exofacial trinitrophenylphosphatidylethanolamine to the cytofacial leaflet, thereby quenching cytofacial fluorescence. Differences of the sterol asymmetry between plasma membranes in vivo and isolated plasma membranes are also conceivable. Neither possibility can be rigorously excluded by the data presented here. However, the fraction of dehydroergosterol fluorescence quenched in exofacially trinitrophenylated, isolated plasma membrane versus isolated phagosomes (inside-out membranes) trinitrophenylated on the cytofacial leaflet was nearly opposite [5], suggesting no difference in sterol asymmetry and no rearrangement of trinitrophenylated phospholipid, despite the differences in forces acting on the two membranes during homogenization.

Asymmetric transbilayer distribution of dehydroergosterol in tumorigenic mouse fibroblasts

Trinitrophenylation of plasma membrane outer monolayer amine moieties by trinitrobenzenesulfonic acid quenches the fluorescence of dehydroergosterol in that monolayer [4,5]. The degree of quenching reflects the content of dehydroergosterol in the outer monolayer only if trinitrophenylation of both the outer and inner monolayers results in complete quenching. Indeed, when LM fibroblasts and the other cultured cell lines were grown in the presence of dehydroergosterol and then treated with trinitrobenzenesulfonic acid under penetrating conditions (37°C) then dehydroergosterol fluorescence was quenched 96 ± 2%.

The distribution of dehydroergosterol across the bilayer of plasma membrenas isolated from cultured primary (L-929, LM, A-9, and C_3H) and cultured nude mouse or cultured C_3H mouse lung metastasis tumor cell lines was constant, despite large variation in plasma membrane sterol/phospholipid molar ratio (Fig. 2). The mean values of primary tumor cell line ($n = 4$) sterol/phospholipid molar ratio and of the percentage of dehydroergosterol fluorescence quenched by outer monolayer trinitrophenylation were 0.43 ± 0.05 mol/mol and 19.1 ± 3.0%, respectively. The mean sterol/phospholipid molar ratio of the metastatic cell lines ($n = 5$) was 52.8% lower ($P < 0.005$) than that of primary tumor cell lines cultured under identical conditions. Since sterols are known to move rapidly and spontaneously between the inner and outer monolayers of membranes, the

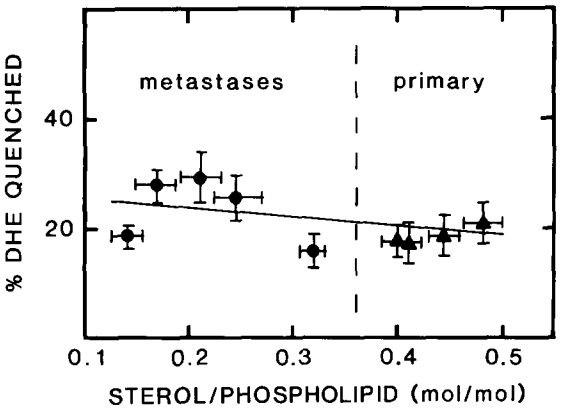

Fig. 2. Transbilayer distribution of sterol in plasma membranes from primary and metastatic mouse tumor cells as a function of sterol/phospholipid molar ratio. Cells were cultured in the presence of 5.0 μg/ml $\Delta^{5,7,9(11),22}$-ergostatetraen-β-ol (DHE) at 37°C for 3 days, incubated in the absence or presence of trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C), homogenized, plasma membranes isolated, and the molar ratio of total sterol/phospholipid and absorbance-corrected fluorescence intensity of dehydroergosterol determined as described in Materials and Methods. Quenched dehydroergosterol fluorescence represents dehydroergosterol in the outer membrane monolayer. Primary tumor cell lines (▲) are to the right and metastatic cell lines (●) are to the left of the vertical dashed line. Results are given as mean ± S.E., $n = 3$–5.

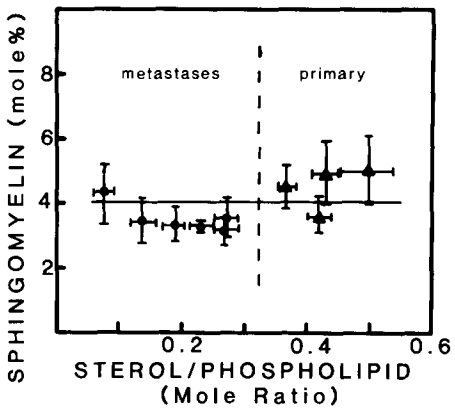

Fig. 3. Correlation between plasma membrane sterol/phospholipid ratio and sphingomyelin content. All conditions were as described in legend to Fig. 2 except that phospholipid composition was determined. ▲, primary tumor cell lines to the right of the dashed line; ●, lung metastasis cell lines to the left of the dashed line.

negative results may appear surprising. The present results would be more secure if positive controls were provided. Therefore, plasma membranes isolated from LM fibroblasts cultured with dehydroergosterol and treated with or without trinitrobenzenesulfonic acid as above were exposed to 10^{-5} M 4-cholesten-3-one or 5-cholesten-3β,7β-diol for 30 min at 37°C. The percentages of dehydroergosterol fluorescence quenched after treatment with 4-cholesten-3-one or 5-cholesten-3β,7β-diol were 6 and 40%, respectively. Thus, the transbilayer sterol distribution can be altered by the oxidized sterols but not by changes in plasma membrane sterol/phospholipid ratio.

The plasma membrane sterol/phospholipid ratio did not correlate with the content of neutral zwitterionic phospholipids (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine) as shown in Figs. 3 and 4. In contrast, the plasma membrane sterol/phospholipid ratio did correlate with anionic phospholipid content (phosphatidylinositol and phosphatidylserine). Since all cell lines were cultured in chemically defined, serum-free, lipid-free medium, the native sterol of all cell lines was desmosterol, not cholesterol, which they are unable to synthesize. In summary, the percentage of dehydroergosterol quenched was not significantly different between primary and meta-

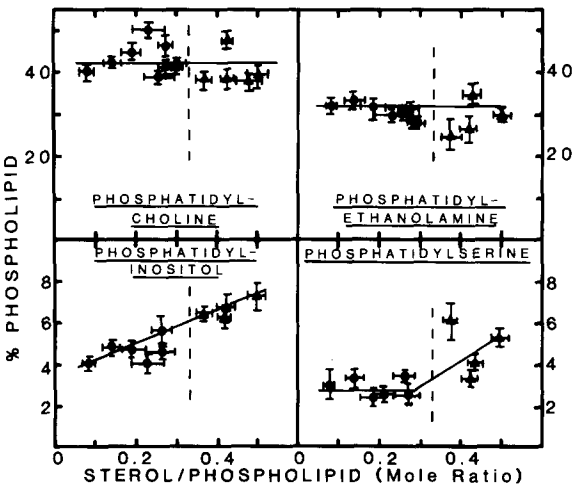

Fig. 4. Correlation between plasma membrane sterol/phospholipid ratio and anionic phospholipid content. All conditions were as described in legend to Fig. 3. Primary tumor cell lines (▲) are to the right and metastatic lines (●) are to the left of the vertical dashed line.

static cell lines. Thus, transbilayer sterol asymmetry did not appear to be dependent on sterol/phospholipid molar ratios in these cells.

Effect of phospholipid polar head-group manipulation on transbilayer sterol distribution

The effect of phospholipid composition on transbilayer sterol distribution in LM cell plasma membranes was examined for two reasons. First, a number of reports indicate that sterols may preferentially interact with phosphatidylcholine and only poorly with phosphatidylethanolamine (reviewed in Ref. 3). Second, enrichment of LM plasma membranes with phosphatidylethanolamine dramatically altered the transbilayer [49] but not bulk [30] membrane fluidity. In order to assess the effect of altered plasma membrane phospholipid polar head-group composition on the transbilayer distribution of sterol, LM cells were cultured in the presence of choline or the choline analogues *N,N*-dimethylethanolamine, *N*-methylethanolamine, or ethanolamine (Table II). The plasma membranes of these cells contained 68% less phosphatidylcholine, which was replaced by analogue-containing phospholipids. The presence of choline analogue-containing phospholipids in the plasma membranes did not perturb either the sterol/phospholipid molar ratio or the transbilayer distribution of dehydroergosterol in LM cells as determined by trinitrophenyl quenching of outer monolayer fluorescence. Thus, the asymmetric transbilayer distribution of sterol in plasma membranes from cultured mouse fibroblasts was stable to large alterations of phospholipid polar head-group composition.

Effects of charged anesthetics on transbilayer sterol distribution and dynamic properties of diphenylhexatriene in LM plasma membranes

The effects of phenobarbital and prilocaine on the absorbance-corrected fluorescence and trinitrophenyl quenching of dehydroergosterol as a function of time are presented in Fig. 5. None of the treatments altered the absorbance-corrected fluorescence intensity of dehydroergosterol (Fig. 5) or lifetime (data not shown) in unlabeled or trinitrophenylated plasma membranes at any time up to 20 min following drug addition. Therefore, the extent of dehydroergosterol quenching by trinitrophenyl groups remained constant at approx. 22% during all treatments.

The effects of the charged amphipathic anesthetics phenobarbital (anionic) and prilocaine (cationic) on the limiting anisotropy of the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene are presented in Table III. Consistent with an asymmetric transbilayer sterol distribution, untreated plasma membranes exhibited a distinct transmembrane fluidity gradient as determined by selective outer monolayer quenching of 1,6-diphenyl-1,3,5-

TABLE II

TRANSBILAYER DISTRIBUTION OF STEROL IN PLASMA MEMBRANES FROM LM CELLS WITH ALTERED PHOSPHOLIPID POLAR HEAD-GROUP COMPOSITION

Cells were cultured in the presence of 5 μg/ml dehydroergosterol and 40 μg/ml choline or the choline analogues *N,N*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine at 37°C for 3 days, incubated in the absence or presence of trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C), homogenized, plasma membranes isolated, and the molar ratio of total sterol/phospholipid and the absorbance-corrected fluorescence intensity of dehydroergosterol determined as described under Materials and Methods. Quenched dehydroergosterol fluorescence represents dehydroergosterol in the outer membrane monolayer, while unquenched fluorescence represents dehydroergosterol in the inner monolayer. Results are given as mean ± S.E., $n = 3$ or 4.

Supplement	Sterol/phospholipid (mol/mol)	% total dehydroergosterol fluorescence	
		quenched	unquenched
Choline	0.415 ± 0.049	20.0 ± 3.0	80.0 ± 3.0
N,N-Dimethylethanolamine	0.484 ± 0.066	18.0 ± 2.8	82.0 ± 2.8
N-Methylethanolamine	0.441 ± 0.078	22.8 ± 3.2	77.2 ± 3.2
Ethanolamine	0.366 ± 0.051	18.4 ± 2.2	81.6 ± 2.2

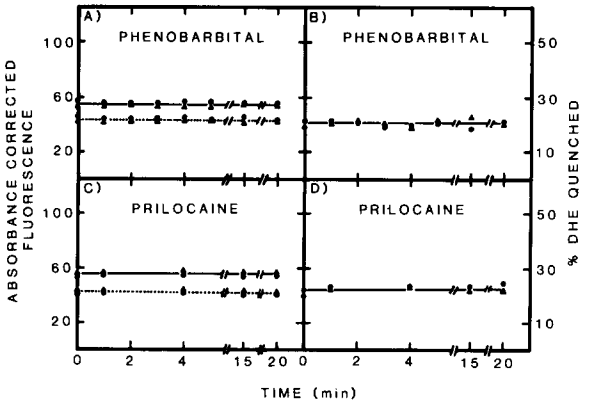

Fig. 5. Transbilayer distribution of sterol in LM cell plasma membranes treated with charged amphipathic anesthetics. LM cells were cultured and plasma membranes isolated as described in the legend to Fig. 3. Isolated membranes were incubated in the presence of 0.5 or 10.0 mM phenobarbital, or 1.0 mM or 10.0 mM prilocaine and dehydroergosterol fluorescence determined as a function of time. A, absorbance-corrected fluorescence of DHE in unlabeled (———) and trinitrophenylated (- - - - -) membranes treated with 0.5 mM (●) or 10.0 mM (▲) phenobarbital; B, % dehydroergosterol quenched by trinitrobenzenesulfonic acid in membranes treated with 0.5 mM (●) or 10.0 mM (▲) phenobarbital; C, absorbance-corrected fluorescence of dehydroergosterol in unlabeled (———) and trinitrobenzenesulfonic acid labeled (- - - - - -) membranes treated with 1.0 mM (●) or 10.0 mM (▲) prilocaine; D, % DHE quenched by trinitrobenzenesulfonic acid in the presence of 1.0 mM (●) or 10.0 mM prilocaine (▲).

hextriene fluorescence by trinitrophenyl groups. The limiting anisotropy (r_∞) of 1,6-diphenyl-1,3,5-hexatriene in the inner monolayer was 30.2% greater than in the outer monolayer. Treatment of plasma membranes with 0.5 mM phenobarbital significantly reduced r_∞ of 1,6-diphenyl-1,3,5-hexatriene in the outer monolayer by 8.0% but did not alter inner monolayer r_∞. Thus, 0.5 mM phenobarbital increased the transbilayer fluidity difference. At a 20-fold higher phenobarbital concentration (10.0 mM), both the inner and outer monolayers were significantly more fluid, based on the reduced limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene in both monolayers, as compared with untreated controls. In direct contrast to phenobarbital, prilocaine (1 mM) selectively lowered the limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene in the inner monolayer with respect to controls. Prilocaine at a concentration of 10.0 mM decreased r_∞ of 1,6-diphenyl-1,3,5-hexatriene in both monolayers as compared with controls and decreased the r_∞ in the inner monolayer by 16% as comapred to no drug treatment.

Thus, the treatment of plasma membranes with phenobarbital and prilocaine can be used to selectively modulate individual monolayer fluidity in lipid domains detected by 1,6-diphenyl-1,3,5-hexatriene, and to determine whether or not transbilayer dehydroergosterol distribution is affected by these perturbations.

Thermal properties of dehydroergosterol in LM cell plasma membranes

In order to assess if dehydroergosterol underwent lateral phase separation in LM plasma membranes, if was first necessary to demonstrate whether dehydroergosterol was sensitive to lateral phase alterations in a model membrane system. Therefore, 0.5 mol% fluorescent dehydroergosterol

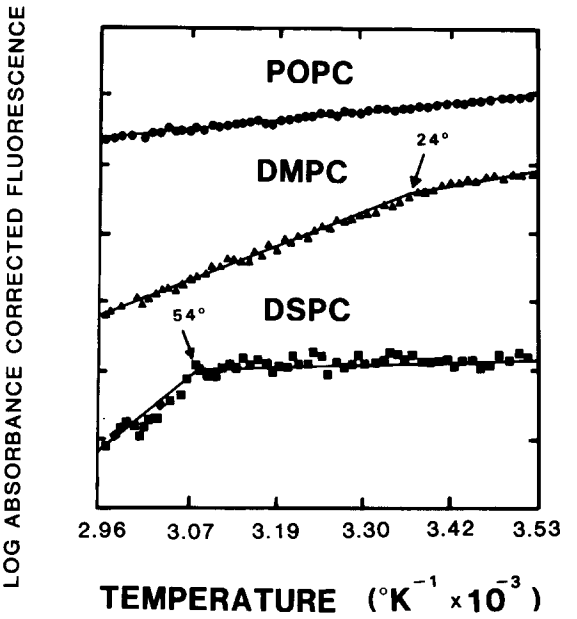

Fig. 6. Arrhenius plots of dehydroergosterol fluorescence in phospholipid membranes. Dehydroergosterol (0.5 mol%) was incorporated into small unilamellar vesicles composed of POPC (●); DMPC (▲); or DSPC (■). Absorbance-corrected fluorescence was measured as a function of temperature. only ascending temperature scans are shown. Similar data are obtained with descending temperature scans.

TABLE III

LIMITING ANISOTROPY OF DIPHENYLHEXATRIENE IN LM CELL PLASMA MEMBRANES TREATED WITH CHARGED AMPHIPATHIC ANESTHETICS

LM cells were cultured without dehydroergosterol for 3 days at 37°C, labeled with trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C), homogenized, plasma membranes isolated, and limiting anisotropy of the exogenous fluorescent probe 1,6-diphenyl-1,3,5-hexatriene determined as described in Materials and Methods. Membranes were incubated with or without the indicated concentrations of phenobarbital or prilocaine for 10 min at 37°C prior to the determination of limiting anisotropy. Limiting anisotropy of trinitrobenzenesulfonic acid-labeled membranes represents inner monolayer anisotropy, and outer monolayer anisotropy was calculated from trinitrobenzenesulfonic acid-labeled and unlabeled membranes according to Weber's law of additivity of anisotropy weighting by fractional intensities. Values represent mean ± S.E., $n = 3$.

Treatment	Limiting anisotropy		
	whole membrane (untreated)	inner monolayer (trinitrophenylated)	calculated outer monolayer
None	0.137 ± 0.002	0.155 ± 0.003	0.119 ± 0.003 [b]
0.5 mM phenobarbital	0.132 ± 0.004	0.154 ± 0.003	0.110 ± 0.002 [ab]
10.0 mM phenobarbital	0.128 ± 0.001 [a]	0.145 ± 0.002 [a]	0.111 ± 0.001 [ab]
1.0 mM prilocaine	0.128 ± 0.002 [a]	0.143 ± 0.002 [a]	0.113 ± 0.003 [b]
10.0 mM prilocaine	0.113 ± 0.003 [a]	0.130 ± 0.002 [ac]	0.096 ± 0.002 [ab]

[a] Significantly less than untreated controls, $P < 0.05$.
[b] Significantly less than inner monolayer, $P < 0.05$.
[c] Significantly less than inner monolayer, +10 mM phenobarbital, $P < 0.05$.

was incorporated into phospholipid liposomes composed of pure POPC, DMPC or DSPC. Absorbance-corrected fluorescence of the sterol was measured as a function of temperature (Fig. 6). over the temperature range 10–65°C, dehydroergosterol was sensitive to the lateral phase separation of DMPC and DSPC occurring near 24°C and 54°C, respectively, but no phase alteration was detected in POPC vesicles. These results were in excellent agreement with those obtained by other investigators using differential scanning calorimetry or fluorescence probe molecules such as 1,6-diphenyl-1,3,5-hexatriene or pyrene-labeled phospholipids [50–52]. Thus, dehydroergosterol was sensitive to lateral phase separations in phospholipid membrane vesicles.

The absorbance-corrected fluorescence of dehydroergosterol in LM cell plasma membranes was determined as a function of temperature in order to determine whether or not this sterol detected temperature-dependent lateral phase separations and to elucidate the effect of temperature and phase transitions on the asymmetric transbilayer distribution of sterols (Fig. 7). The fluorescence intensity of dehydroergosterol was inversely pro-

TABLE IV

CHARACTERISTIC TEMPERATURES IN ARRHENIUS PLOTS OF DEHYDROERGOSTEROL FLUORESCENCE IN LM CELL PLASMA MEMBRANES

Characteristic temperatures were determined from breakpoints in ascending Arrhenius plots of absorbance-corrected fluorescence intensity of LM cell plasma membranes isolated from cells cultured with dehydroergosterol (5 μg/ml) for 3 days and labeled with trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C) as described under Materials and Methods. The concentration of phenobarbital and prilocaine was 0.5 mM and 1.0 mM, respectively. Samples were incubated at 37°C for 10 min with the respective drug, then cooled to 5°C prior to starting ascending temperature scans from 5° to 45°C.

Treatment	Characteristic temperature (°C)		
	whole membrane	inner monolayer	outer monolayer (calculated)
None	21 ± 1 37 ± 1	21 ± 2 36 ± 2	24 ± 2
Phenobarbital	37 ± 1	27 ± 2	24 ± 3 38 ± 1
Prilocaine	27 ± 2	27 ± 2	none

portional to temperature from 4 to 45°C in both unlabeled and trinitrophenylated membranes. Heating scans revealed characteristic temperatures near 21 ± 1°C and 37 ± 1°C for dehydroergosterol in both the whole membrane and the inner monolayer (Fig. 7 and Table IV). The average slopes of Arrhenius plots of dehydroergosterol in the inner monolayer were 0.590 (below 21°C), 0.870 (between 21 and 37°C), and 0.700 (above 37°C) fluorescence units/(10^3/K), respectively. The thermotropic behavior of dehydroergosterol fluorescence in the outer monolayer (calculated) during heating scans demonstrated a characteristic temperature at 24 ± 2°C which, unlike the 21°C transition detected in the inner monolayer, was not evident in cooling scans. The slope in Arrhenius plots below and above the 24°C transition was 0.320 and 0.900, respectively.

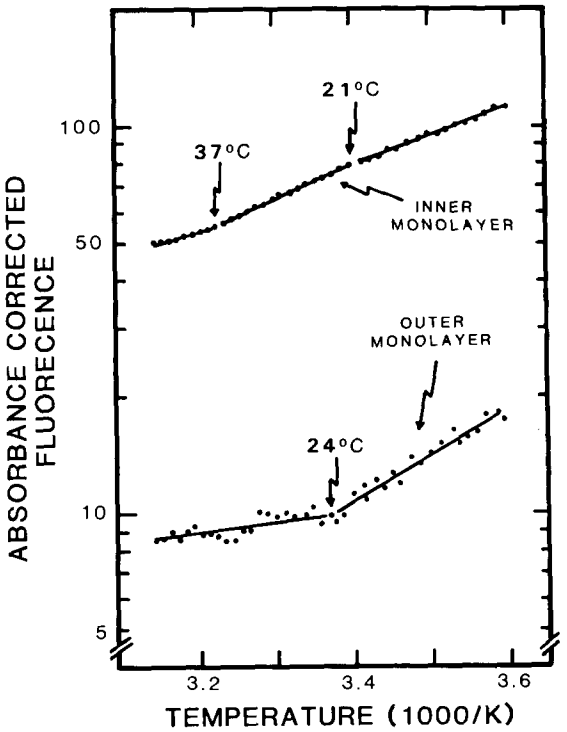

Fig. 7. Arrhenius plots of dehydroergosterol fluorescence in isolated LM cell plasma membranes. Cells were cultured with 5 μg/ml dehydroergosterol, trinitrobenzenesulfonic acid labeled at 4°C, and plasma membranes isolated as described in Materials and Methods. Absorbance-corrected fluorescence was measured as a function of increasing temperature from 5 to 45°C.

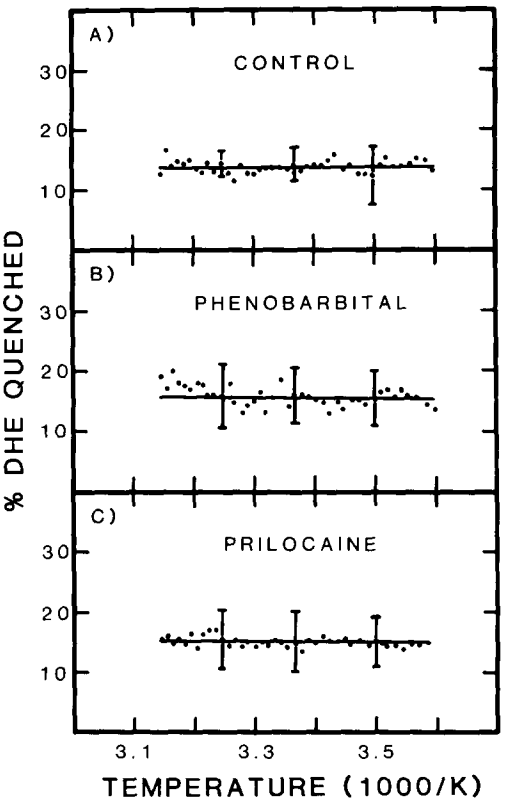

Fig. 8. Transbilayer distribution of sterol in LM cell plasma membranes as a function of temperature. A, untreated membranes; B, membranes treated with 0.5 mM phenobarbital for 10 min at 37°C prior to cooling to 5°C; C, membranes treated with 1.0 mM prilocaine for 10 min prior to cooling to 5°C and beginning scan. Data were obtained from ascending temperature scans. For clarity, error bars have been eliminated on all but three points which illustrate the range of standard error values ($n = 3$).

The asymmetric transbilayer distribution of dehydroergosterol was unaltered at temperatures above or below phase transitions in untreated membranes (Fig. 8A) or in membranes treated with 0.5 mM phenobarbital (Fig. 8B) or 1.0 mM prilocaine (Fig. 8C). Both of the drugs depressed the breakpoint detected by dehydroergosterol in the inner monolayer from 37°C to 27°C (Table IV). In addition, in the outer monolayer both drugs eliminated the breakpoint at 24°C and caused a new breakpoint near 37–40°C. A slight breakpoint at 24°C was also detected in the outer monolayer of phenobarbital-treated samples. Thus, transbilayer sterol asymmetry appeared to be sta-

ble to large alterations in whole membrane structure due to temperature changes and lateral phase separations of sterols in each monolayer.

Discussion

The regulation of transbilayer sterol asymmetry in eukaryotic membranes in response to genetic variations among cell sublines, large alterations in sterol/phospholipid ratio and membrane phospholipid polar head-group composition, perturbations of bilayer and individual monolayer fluidity and lateral phase separations has not been previously reported. The results presented herein describe for the first time the stability and maintenance of transbilayer sterol asymmetry using a fluorescent sterol in plasma membranes isolated from transformed mouse fibroblasts and subjected to the above perturbations. The fluorescence probe molecule, dehydroergosterol, is a close structural analogue of native membrane sterols and is functionally similar as determined by a variety of biochemical and biophysical techniques [3–5]. Moreover, dehydroergosterol was taken up by cultured mouse fibroblasts entirely without deleterious effects, and quantitatively replaced native desmosterol up to 90% of total membrane sterol [3–5]. Thus, dehydroergosterol is an excellent, nonperturbing probe of membrane sterol motional properties and asymmetric distribution, especially when used in concert with selective quenching techniques such as trinitrophenylation of the outer monolayer by trinitrobenzenesulfonic acid under nonpenetrating conditions [3]. These techniques have now been used to demonstrate the response of transbilayer sterol asymmetry to a variety of membrane perturbations.

The majority of plasma membrane sterol was localized in the inner monolayer of primary tumor cells (L-929 and the L-929 sublines LM, A-9 and C_3H primary), and metastatic derivative cell lines (C_3H and nude mouse lung metastasis cells) grown in chemically defined, serum-free medium. The genetic heterogeneity of tumor cell populations has been well recognized [53,54] and is reflected in lung metastasis cells obtained by injecting LM cells into athymic (nude) mice and subsequently cultured without serum, and in highly metastatic B16-F10 melanoma cells, which demonstrated markedly lower sterol carrier protein content and plasma membrane sterol/phospholipid molar ratios as compared with primary or low metastatic cell lines [16]. Thus, although cultured highly metastatic cells were deficient in both sterol carrier protein and plasma membrane sterol, they exhibited nearly identical transbilayer sterol distribution as compared with primary tumor cells.

It is interesting to note that some data obtained with model membranes (reviewed in Ref. 3) indicate that cholesterol may interact preferentially with specific phospholipids in the order sphingomyelin > phosphatidylcholine > anionic phospholipids. The data obtained herein with biological membranes from a variety of L cell derivative cell lines is not consistent with model membrane data. Likewise, despite large alterations in phospholipid composition (choline analogues) in a single cell line, the plasma membrane sterol/phospholipid ratio and transbilayer sterol distribution were unaltered.

The plasma membrane sterol/phospholipid ratio correlated with the anionic, not neutral zwitterionic, phospholipid content (Fig. 3 and 4). The most prominent observation was a direct correlation between plasma membrane phosphatidylinositol content and both sterol/phospholipid ratio and malignancy. Tumor-promoting phorbol esters have been reported to directly affect inositol lipid kinases and/or phosphatase, thereby regulating the mass of polyphosphoinositides and phosphatidylinositol in human platelets [55]. Similar observations have been made with diacylglycerol [56]. More important, the $pp60^{V\text{-}5RC}$ and UR2 $p68^{V\text{-}ROS}$, oncogene products in transformed cells, are involved in catalyzing polyphosphoinositide formation [57,58]. The data presented herein thus may bear on the possible link between oncogenes and inositol lipids. The metastatic cells may be considered to be more malignant cells than primary tumor cells. One may therefore predict that the lower phosphatidylinositol content of the metastatic cells may correlate with increased mass and/or turnover of polyphosphoinositide-containing lipids.

LM cells cultured with choline analogues and having large alterations in plasma membrane phospholipid polar head-group composition [29] had transbilayer sterol distributions that were the

same as control cells cultured with choline. Phospholipid polar head-group manipulation of LM cell plasma membranes induced a compensatory redistribution of fatty acyl moieties among the various phospholipids [30], changed membrane surface charge and altered transmembrane acyl chain and aminophospholipid asymmetry [31], abolished transbilayer fluidity gradients [49], and modulated adenylate cyclase activity [32], phagocytosis and pinocytosis [33], and concanavalin A receptor-mediated homotypic and heterotypic agglutination [34]. Despite these influences, the regulation of transbilayer sterol distribution appears to be independent of the structural alterations which occur as the result of phospholipid polar head-group manipulation by choline analogues.

The manipulation of individual monolayer fluidity by low concentrations of charged anesthetics, or both monolayers by either high anesthetic concentrations or temperature, did not perturb transbilayer sterol distribution of LM plasma membranes. The results reported here confirm the selective fluidization of individual monolayers by charged anesthetics as determined by the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene. Limiting anisotropy of diphenylhexatriene indicated a distinctly more rigid inner monolayer as compared to the outer monolayer in the absence of drugs. This observation was consistent with that obtained with steady-state anisotropy and the Perrin equation [49]. However, unlike the present work, the earlier data did not discriminate between dynamic and static aspects of probe motion. Limiting anisotropy indicates that the resistance to probe motion ro order, not just rate of motion, differs significantly between outer and inner monolayer lipids of the LM plasma membrane. In contrast to the above observations with diphenylhexatriene and anesthetics, in the presence of either phenobarbital or prilocaine the outer monolayer anisotropy of dehydroergosterol increased. In addition, prilocaine abolished the characteristic temperature assigned to the inner monolayer of LM cell plasma membranes detected by 1,6-diphenyl-1,3,5-hexatriene ,while phenobarbital had no effect (unpublished observation). In contrast, both drugs induced a new characteristic temperature of dehydroergosterol in the outer monolayer and depressed the characteristic temperature of dehydroergosterol in the inner monolayer. Other investigations have shown the induction of new characteristic temperatures by these anesthetics [59]. These results indicated quite clearly that the fluorescent sterol detected membrane environments which were distinctly different from those detected by 1,6-diphenyl-1,3,5-hexatriene. On the other hand, dehydroergosterol detected asymmetric characteristic temperatures across the bilayer which were very similar to the characteristic temperatures in each monolayer of LM cell plasma membrane phagosomes as determined by Wisnieski and Iwata [60] using a non-flipping glucosamine derivative of the ESR probe glucosamine-12-nitroxystearic acid. Both dehydroergosterol and glucosamine-12-nitroxystearic acid detected characteristic temperatures in the inner monolayer near 22°C and 37°C as well as breakpoints in the outer monolayer near 24–28°C. Dehydroergosterol did not detect the characteristic temperature near 13°C that was detected by the ESR probe in the outer monolayer of LM cell phagosomes, possibly because of small differences between phagosomal membranes and the total plasma membrane, or between the lateral partitioning of dehydroergosterol and glucosamine-12-nitroxystearic acid in the outer monolayer. Dehydroergosterol was also sensitive to phase transitions in DMPC and DSPC model membranes (small unilamellar vesicles), indicating that the breakpoints in Arrhenius plots of dehydroergosterol fluorescence were likely due to lateral phase separations in plasma membrane sterols. However, the possibility that temperature and charged anesthetics altered dehydroergosterol fluorescence parameters through changes in phospholipid-sterol interactions including microdomains, clusters, hexagonal phases and/or annuli should be considered.

Thus, none of the perturbations examined in this investigation were capable of altering transbilayer sterol distribution in LM cell plasma membranes. In *Mycoplasma gallisepticum,* cholesterol exists in two kinetic pools representing inner and outer monolayer sterol, as determined by exchange experiments in which sterol flip-flop was determined to be rate limiting [61]. When sterol/phospholipid molar ratios were decreased from 0.36 to 0.25, there was a concomitant increase in sterol exchange rate between the mycoplasmal and

small unilamellar phospholipid vesicle membranes. Since the translocation of sterol from the inner to the outer monolayer was the rate-limiting step, these experiments represent a change in the rate of flip-flop, yet no change in the size of the inner and outer pools was noted. The half-time of flip-flop of cholesterol in the human erythrocyte was determined to be extremely rapid – on the order of 3 s [62]. In view of the effect of sterol on membrane contour [11] and the short half-time of transmembrane movement of sterol, it was suggested that a rapid redistribution of sterol across the bilayer might occur and thus modulate a change in membrane contour [11]. Yet, our results obtained from LM cell plasma membranes in a variety of highly perturbed physicochemical states indicated no net transbilayer reorientation of sterol. In a related study, other investigators have shown that major alterations in sterol/phospholipid ratio (0.6 to 1.4) did not alter phospholipid transbilayer distribution across monkey red blood cell membranes [63]. The above observations on the stability of transbilayer sterol distribution are also consistent with results obtained with cholesterol oxidase and vesicular stomatitis virus membranes [18]. Cholesterol depletion had no effect on the transbilayer distribution of cholesterol in the virus membranes.

Thus, the results presented herein demonstrate for the first time that although fluorescent sterols are sensitive to the induced changes in individual monolayer and bilayer structure, the transbilayer sterol asymmetry is remarkably stable to those perturbations of the membrane lipid structure. The mechanism(s) by which transbilayer sterol asymmetry is/are regulated and maintained, and the functional significance of sterol asymmetry remain unclear, and certainly require further investigation in order to obtain a more precise understanding of sterol structure and function in membranes.

Acknowledgements

We gratefully acknowledge the following individuals for their support in contributing to this research effort: Mr. Robert Fischer, Mr. John Waters, Mr. Jack Gardiner, Mr. Eugene Hubert and Mrs. Larissa Schuster for technicial assistance; Mrs. E. Eckenfels and Mrs. D. Eagle for secretarial work. This investigation was supported, in part, by the USPHS (GM 31651) and the National Cancer Institute (CA 24339).

References

1 Shinitzky, M., Skornick, Y. and Haran-Ghera, N. (1979) Proc. Natl. Acad. Sci. USA 76, 5313–5316
2 Muller, C.P., Stephany, D.A., Shinitzky, M. and Wunderlich, J.R. (1983) J. Immunol. 131, 1356–1362
3 Schroeder, F. (1984) Prog. Lipid Res. 23, 97–113
4 Schroeder, F. (1981) FEBS Lett. 135, 127–130
5 Hale, J.E. and Schroeder, F. (1982) Eur. J. Biochem. 122, 649–661
6 Incerpi, S., Baldini, P. and Luly, P. (1983) Cell. Mol. Biol. 29, 285–289
7 Heron, D.S., Shinitzky, M., Hershkowitz, M. and Samuel, D. (1980) Proc. Natl. Acad. Sci. USA 77, 7463–7467
8 Heron, D., Israeli, M., Hershkowitz, M., Samuel, D. and Shinitzky, M. (1981) Eur. J. Pharmacol. 72, 361–364
9 Shih, J.C. and Ohsawa, R. (1983) Neurochem. Res. 8, 701–710
10 Tandon, N., Harmon, J.T., Rodbard, D. and Jamieson, G.A. (1983) J. Biol. Chem. 258, 11840–11845
11 Lange, Y. (1984) in Erythrocyte Membranes 3: Recent Clinical and Experimental Advances pp. 137–151, Alan R. Liss, Inc., New York
12 De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 666–675
13 Demel, R.A., Bruckdorfer, K.R. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 321–330
14 Schroeder, F. and Gardiner, J. (1984) Cancer Res. 44, 3262–3269
15 Schroeder, F. (1984) Biochim. Biophys. Acta 776, 299–312
16 Schroeder, F., Kier, A.B., Olson, C.D. and Dempsey, M.E. (1984) Biochem. Biophys. Res. Commun. 124, 283–289
17 Mountford, C.E., Wright, L.C., Holmes, K.T., Mackinnon, W.B., Gregory, P. and Fox, R.M. (1984) Science 226, 1415–1418
18 Pal, R., Barenholz, Y. and Wagner, R.R. (1981) Biochemistry 20, 530–539
19 Sefton, B.M. and Gaffney, B.J. (1979) Biochemistry 18, 436–442
20 Clejan, S., Bittman, R. and Rottem, S. (1978) Biochemistry 17, 4579–4583
21 Casper, D.L.D. and Kirschner, D.A. (1971) Nature New Biol. 231, 46–52
22 Fisher, K.A. (1976) Proc. Natl. Acad. Sci. USA 73, 173–177
23 Lange, Y. and Slayton, J.M. (1982) J. Lipid Res. 23, 1121–1127
24 Elias, P.M., Friend, D.S. and Goerke, J. (1979) J. Histochem. Cytochem. 27, 1247–1260
25 Silberkang, M., Havel, C.M., Friend, D.S., McCarthy, B.J. and Watson, J.A. (1983) J. Biol. Chem. 258, 8503–8511
26 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4457–4461

27 Sweet, W.D. and Schroeder, F. (1986) Biochem. J., in the press
28 Houslay, M.D., Dipple, I. and Gordon, L.M. (1981) Biochem. J. 197, 675–681
29 Schroeder, F., Perlmutter, J.F., Glaser, M. and Vagelos, P.R. (1976) J. Biol. Chem. 251, 5015–5026
30 Schroeder, F. (1978) Biochim. Biophys. Acta 511, 356–376
31 Schroeder, F. (1980) Biochim. Biophys. Acta 599, 254–270
32 Engelhard, V.H., Esko, J.D., Storm, D.R. and Glaser, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4482–4486
33 Schroeder, F. (1981) Biochim. Biophys. Acta 649, 162–174
34 Schroeder, F. (1982) Biochemistry 21, 6782–6790
35 Fischer, R.T., Stephenson, F.A., Shafiee, A. and Schroeder, F. (1985) J. Biol. Phys. 13, 13–24
36 Feller, D.J., Schroeder, F. and Bylund, D.B. (1983) Biochem. Pharmacol. 32, 2217–2223
37 Kier, A.B. and Schroeder, F. (1982) Transplantation 33, 274–279
38 Fontaine, R.N. and Schroeder, F. (1979) Biochim. Biophys. Acta 558, 1–12
39 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
40 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
41 Ames, G.F. (1968) J. Bacteriol. 95, 833–843
42 Ames, B.N. (1966) Methods Enzymol. 8, 115–118
43 Holland, J.F., Teets, R.E. and Timnick, A. (1973) Anal. Chem. 45, 145–153
44 Lakowicz, J.R., Cherek, H. and Balter, A. (1981) J. Biochem. Biophys. Methods 5, 131–146
45 Weber, G. (1978) Acta Phys. Pol. A54, 859–865
46 Lakowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 508–519
47 Weber, G. (1952) Biochem. J. 51, 145–167
48 Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E. and Carlson, F.D. (1977) Biochemistry 16, 2806–2810
49 Schroeder, F. (1980) Eur. J. Biochem. 112, 293–307
50 Lavialle, F. and Levin, I.W. (1980) Biochemistry 19, 6044–6050
51 Silvius, J.R., Read, B.D. and McElhaney, R.N. (1979) Biochim. Biophys. Acta 555, 175–178
52 Janiak, M.J., Small, D.M. and Shipley, G.G. (1976) Biochemistry 15, 4575–4580
53 Nowell, P.C. (1976) Science 194, 23–28
54 Fidler, I.J., Gersten, D.M. and Hart, I.R. (1978) Adv. Cancer Res. 28, 149–250
55 Halenda, S.P. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 124, 507–513
56 De Chaffoy de Courcelles, D., Roevens, P. and Van Belle, H. (1984) Biochem. Biophys. Res. Commun. 123, 589–595
57 Sugimoto, Y., Whitman, M., Cantley, L.C. and Erikson, R.L. (1984) Proc. Natl. Acad. Sci. USA 81, 2117–2121
58 Macara, I.G., Marinetti, G.V. and Balduzzi, P.C. (1984) Proc. Natl. Acad. Sci. USA 81, 2728–2732
59 Dipple, I., Gordon, L.M. and Houslay, M.D. (1982) J. Biol. Chem. 257, 1811–1815
60 Wisnieski, B.J. and Iwata, K.K. (1977) Biochemistry 16, 1321–1326
61 Clejan, S. and Bittman, R. (1984) J. Biol. Chem. 259, 441–448
62 Lange, Y., Dolde, J. and Steck, T.L. (1981) J. Biol. Chem. 256, 5321–5323
63 Alsam, A. (1985) Ind. J. Biochem. Biophys. 22, 38–42