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The influence of saturated fatty acid modulation of bilayer physical state on cellular and membrane structure and function

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Cultured chick fibroblasts supplemented with stearic acid in the absence of serum at 37°C degenerate and die in contrast to cells grown at 41°C which appear normal in comparison with controls. These degenerative effects at 37°C are alleviated by addition to stearate-containing media of fatty acids known to fluidize bilayers. These observations suggest that cell degeneration at 37°C may involve alterations in the physical state of the membrane. Fatty acid analysis of plasma membrane obtained from stearate-supplemented cells clearly demonstrates the enrichment of this fatty acid species into bilayer phospholipids. Moreover, the extent of enrichment is similar in cells grown at both 37 and 41°C. Stearate enrichment at either temperature does not appear to alter significantly membrane cholesterol or polar lipid content. Fluorescence anisotropy measurements for perylene and diphenylhexatriene incorporated into stearate-enriched membranes reveals changes suggestive of decreased bilayer fluidity. Moreover, analysis of temperature dependence of probe anisotropy indicates that a similarity in bilayer fluidity exists between stearate-enriched membranes at 41°C and control membranes at 37°C. Calorimetric data from liposomes prepared from polar lipids isolated from these membranes show similar melting profiles, consistent with the above lipid and fluorescence analyses. Arrhenius plot of stearate-enriched membrane glucose transporter function reveals breaks which coincide with the main endotherm of the pure phospholipid phase transition, indicating the sensitivity of the transporter to this transition which is undetectable in these native bilayers. These data suggest the existence of regions of bilayer lipid microheterogeneity which affect integral enzyme function, cell homeostasis and viability.

Introduction

Modification of eukaryotic cell membrane fatty acid composition was first reported by Moskowitz [1]; in this study, saturated fatty acid enrichment at 37°C was reported to result in cellular degeneration and death. Degenerative changes accompanying saturated fatty acid supplementation have more recently been observed by Baker [2], Gordon [3], and Doi et al. [4]. The prokaryote *Acholeplasma laidlawii* exhibits a similar response to saturated fatty acid enrichment [5]. In this latter system there is evidence that altered ($Na^+ + Mg^{2+}$)-ATPase activity [6] potentiates cell death.

In experiments investigating the relationship

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between bilayer fluidity and cell transformation with a temperature-sensitive mutant of Rous sarcoma virus, it was observed that cells supplemented with stearic acid degenerated at 37°C, yet proliferated normally at 41°C [2]. Interestingly, the sensitivity of the system to changes of only 4°C in growth temperature is not without precedent. Maintenance of homeostatic potential and viability of *A. laidlawii* was shown to be sensitive to changes of a single carbon in acyl chain length and exhibits similar growth temperature dependence [7].

Saturated fatty acid-induced cell degeneration was interpreted initially as being due to 'free fatty acid toxicity' [1,3]. Correlated with this apparent toxicity was the reported presence of cleft-like dilatations within the cytoplasm of enriched cells. These clefts were thought to be triacylglycerol crystals which mechanically rupture cellular membranes resulting in cell death. More recent data conflict with this interpretation, and suggest rather that the clefts are artifacts, probably related to routine cell fixation procedures (Chester, D.W. and Tourtellotte, M.E., unpublished observations). Thus, stearate-induced cell death is not due to 'toxicity' and mechanical rupture of the cell. An alternative explanation relates this phenomena to saturated fatty acid enrichment of membrane phospholipids and subsequent effects on bilayer physical state.

There is clear evidence that the physical state of the bilayer affects membrane enzyme function. Sinensky et al. [8] and Engelhard et al. [9] have demonstrated that the basal and fluoride-stimulated activities of adenylate cyclase increase as membranes become more crystalline. Conversely, the activities of the $(Na^+ + K^+)$-ATPase in L cells [10] and $(Na^+ + Mg^{2+})$-ATPase of *A. laidlawii* [6] were reported to be inhibited by increased bilayer crystallinity. Glucose transporter function in the rat adipocyte has also been shown to be sensitive to membrane fluid state changes [11,12].

The purpose of this study is to examine the correlation between the cell growth temperature dependence and saturated fatty acid enrichment of chick embryo fibroblasts, focusing on the physical state and function of the membrane as they relate to stearate-enriched cell growth at 37 and 41°C. Data presented strongly suggest the existence of regions of lipid microheterogeneity within the bilayer which affect integral enzyme function and, ultimately, cell physiology and viability.

Materials and Methods

Monolayer cultures of secondary chicken embryo fibroblasts were used in all experiments (embryonated eggs for cell preparation obtained from Spafas, Inc., Norwich, CT). Cells were grown in Eagle's minimal essential media (Grand Island Biologicals, Co.) supplemented with 5% newborn calf serum (Grant Island Biologicals Co.) and incubated, prior to supplementation, at 37°C under standard growth conditions of 5% CO_2 and 100% humidity. Penicillin and streptomycin were added to the media at a final concentration of 50 U/ml each. At approximately 50–60% confluency, the growth medium was replaced by supplementation medium, described below, and cells reincubated at either 37 or 41°C. Cultures were examined periodically to assess fatty acid enrichment effects on cellular growth characteristics.

Bovine serum albumin-fatty acid conjugation. Sterile 10% lipid-poor fraction V bovine serum albumin (Miles Laboratories, Inc.) was prepared in Eagle's balanced salts minus $CaCl_2$ (pH 7.4). The desired fatty acid (Analabs, Inc.), in ethanol, was added to a rapidly stirring solution of albumin warmed to 56°C yielding a final fatty acid concentration of 0.8 mg/ml. Complete binding of fatty acid under similar conditions has been confirmed by fluorescence polarization studies on parinaric acid binding [13].

Supplementation medium. Supplementation medium was prepared by adding 25 ml of fatty acid-albumin per liter of media to obtain a final fatty acid concentration of 70 μM. This medium was further supplemented with 2 μg/ml desthiobiotin (National Biochem. Corp.), an inhibitor of de novo fatty acid synthesis. In cases of dual lipid supplementation, adjustments were made in the respective fatty acid additions to maintain albumin concentrations constant throughout.

Growth curves. In an effort to count only the live cell population, monolayers were stained 5 min at 37°C with 0.25% trypan blue prepared in phosphate-buffered saline. Monolayers were

washed with saline, lightly trypsinized with 0.1% trypsin, 0.5 mM EDTA, saline, (pH 7.4), and counted with a hemocytometer.

Membrane isolation procedures. Cell monolayers were washed with warmed saline (pH 7.4), scraped from the culture dish with a rubber policeman, and pooled at 4°C. All subsequent steps were carried out at 4°C. Cells were pelleted at $600 \times g$ for 6 min and resuspended in 10 mM Tris-HCl (pH 7.4)/250 mM sucrose/50 mM KCl/2 mM $MgCl_2$/1 mM $CaCl_2$ used for nuclear stabilization. Cell disruption was performed by nitrogen cavitation at 300 lb/in^2 and 15 min pre-equilibration in a cell disruption bomb (Parr Instrument Co., Moline, IL). Na_2EDTA (pH 7.1) was added to the lysate to a final concentration of 5 mM. The lysate was differentially centrifuged using the procedure outlined by Bustamonte et al. [14] yielding both crude mitochondrial and microsomal pellets which were subsequently resuspended in a small volume of 10 mM Tris-HCl (pH 7.4)/200 mM mannitol/70 mM sucrose/0.5 mg/ml bovine serum albumin (resuspension buffer). Crude fractions were layered on 10–40% Ficoll 400 (Pharmacia)/5 mM Tris-HCl (pH 7.8)/1 mM $MgCl_2$ gradients containing an additional 10% Ficoll cushion and centrifuged at $53\,000 \times g$ for 2 h. The isolated membrane subfractions were pelleted at $100\,000 \times g$ for 60 min and resuspended in resuspension buffer. Aliquots were quick frozen in solid CO_2/acetone and stored at −70°C prior to use.

Enzyme assays. 5′-Nucleotidase (EC 3.1.3.5) was assayed by a modification of the method of Avruch and Wallach [15] as a plasma membrane marker. The assay system, a final volume of 1 ml, was composed of 50 mM Tris-HCl (pH 8.1)/0.18 μM $MgCl_2$/40 μg membrane protein, and reaction initiated by the addition of 10 μl [^{3}H]adenosine monophosphate (2 μCi/ml; 4 μCi/μM). The reaction mixture was incubated at 41°C for 45 min, terminated with 0.2 ml of 0.25 M $ZnSO_4$ and subsequently iced. Unhydrolyzed [^{3}H]AMP and protein were precipitated with 0.2 ml of 0.25 M $Ba(OH)_2$. The precipitate was spun out and 0.7 ml of the supernatant counted for radioactivity.

NADPH-cytochrome *c* reductase (EC 1.6.2.4) activity was measured as a microsomal marker by the method of Phillips and Langdon [16].

Succinate-cytochrome *c* reductase (EC 1.3.99.1) was measured as a mitochondrial marker by the method of King [17].

Stereospecific D-glucose transport was measured in membranes obtained from control and 18 : 0-enriched cells grown at 41°C by the method of Connell and Romano [18]. For these studies, 60 μg crude microsomal membrane protein were incubated with 2 mM D-[^{14}C]glucose and 2 mM L-[^{3}H]glucose (each 10 μCi/μM) in a final volume of 25 μl for periods of 0 to 60 s at temperatures ranging from 20 to 42°C, to establish initial rates of glucose uptake. Transport was terminated after appropriate incubation periods by adding 4.5 ml ice-cold stop buffer containing 10 mM Tris-HCl (pH 7.5), 0.8 M NaCl, and 0.1 mM phloretin (to prevent efflux). The reaction mixtures were immediately filtered on membrane filters (Millipore HA, 25 mm, 0.45 μm porosity) and washed with an additional 4.5 ml of ice-cold stop buffer. Filters were placed in 10 ml scintillation fluid [19] for double label counting in a Packard Liquid Scintillation Spectrometer. Results were calculated as nmol D-glucose minus nmol L-glucose (correction for adsorption and simple diffusion) per min.

Protein assay. Membrane protein was determined by the method of Lowry et al. [20] using serum albumin as standard.

Lipid analysis. Subcellular membranes and whole cell lipids were extracted with 50–100 volumes of chloroform/methanol (2 : 1, v/v). Phases were split by the addition of 0.58% NaCl according to Folch et al. [21]. Polar and non-polar lipids were separated by single dimension thin-layer chromatography (TLC) against authentic phospholipid standards (Analabs) with chloroform/methanol/water (65 : 25 : 4, v/v) or selectively eluted off silicic acid columns by increased solvent polarity. Phospholipids were transesterified in 6.5% H_2SO_4/dry methanol at 100°C for 60 min. Methyl esters were TLC purified against authentic methyl ester standards (Analabs) with petroleum ester/diethyl ether (90 : 10, v/v) as developing solvent and separated by GLC isothermally at 180°C on polar cyanosilicone SP-2330 columns (Supelco).

Total cholesterol was measured colorimetrically by a modification of the cholesterol oxidase (EC 1.1.3.6) assay described by Heider and Boyett [22]. Briefly, 0.1 ml of 1% Triton X-100 in ethanol was

added to an aliquot of whole lipid extract. Samples were dried under nitrogen, 0.5 ml of 0.2 M NaCl added, the solution vortexed, and 100 μl of the assay system (0.125 U/ml cholesterol oxidase/2.5 U/ml peroxidase/5 mM 4-aminoantipyrene/15 mM phenol/0.5% Triton X-100/5 mM cholate/2 mM NaN_3/50 mM phosphate buffer (pH 7.0)) added. After vortexing lightly, the samples were incubated at 37°C for 30–60 min and absorbance was read at 500 nm.

Total membrane phospholipid content was assayed colorimetrically by the micro-method of Bartlett [23].

Compositional alterations in membrane phosphatidylethanolamine (PE)/phosphatidylcholine (PC) ratios were determined as a function of relative percent ^{32}P in TLC spots as described by Quigley et al. [24]. In these experiments, medium was supplemented on secondary passage of the cells with 1.7 μCi/ml $^{32}PO_4$-carrier free orthophosphate (New England Nuclear) and maintained at this concentration throughout supplementation. 24 h post-supplementation at 37 or 41°C, cells were harvested and plasma membranes prepared and extracted as described above except that a 10% Ficoll cushion replaced the linear gradients. Phospholipid spots were scraped and counted as were regions below, between, and above spots to detect trailing.

All organic solvents were redistilled and glassware was solvent washed prior to use.

Fluorescence spectroscopy. Two hydrophobic fluorophores, perylene and 1,6-diphenyl-1,3,5-hexatriene, were used to examine membrane fluid characteristics. Tandem use of these two probes has been suggested by Shinitzky and Barenholz [25].

Perylene fluorescence anisotropy data were obtained by measuring the emission (474 nm) of plane polarized light both parallel and perpendicular to the excitation beam (437 nm) in a Perkin-Elmer (series MPF-4) Fluorescence Spectrophotometer equipped with a Lauda K2/RD temperature controller. Plasma membranes suspended in buffered saline (pH 7.4) were incubated with perylene at 42°C for 30 min to allow stabilization of fluorescence intensity and re-equilibrated to 25°C prior to isothermal assay. When temperature-dependent studies were carried out, samples were re-equilibrated to 10°C and polarization parameters measured at approximately 2.5°C intervals. Analysis of the temperature dependence of fluorescence polarization gave no indication of hysteresis in up-scale/down-scale scans.

Diphenylhexatriene fluorescence polarization parameters were measured, as with perylene, using 357 and 430 nm for excitation and emission wavelengths, respectively.

Differential scanning calorimetry. Calorimetric measurements were performed on both native membranes and isolated phospholipids using a Perkin-Elmer DSC II in an effort to discern the position of the order-disorder phase transition relative to fatty acid supplementation and the temperature dependence of stearate-enriched cell growth. Native membranes were prepared as outlined under 'Membrane isolation' above but were maintained as a paste for loading into calorimeter pans. Isolated phospholipids, in chloroform, were thoroughly dried down under vacuum on a clean glass slide, transferred to calorimeter pans, and totally hydrated in 50% ethylene glycol/water. Lipid vesicles were subsequently prepared in the pans by rapid heat-cool cycles. Differential heat flow between sample and reference pans was measured utilizing an equal mass of ethylene glycol/water as a reference standard.

Results

Supplementation-dependent growth characteristics

Growth of chick fibroblasts supplemented with stearate (18 : 0) was compared with serum, equimolar stearate/oleate- (18 : 0-18 : 1*c*, 70 μM total), or oleate- (18 : 1*c*) supplemented controls cultured under identical conditions at 37 or 41°C. Growth curves presented in Fig. 1 highlight the dramatic difference in growth of 18 : 0-supplemented cells between 37 and 41°C in comparison with controls at either temperature. This is further demonstrated photomicroscopically in Figs. 2 and 3.

Cells grown 18–24 h post-supplementation at 37°C in 18 : 0-containing medium begin to exhibit alterations in morphology and growth characteristics (Fig. 2c) which ultimately result in cellular degeneration and sloughing (Figs. 2d and 3b).

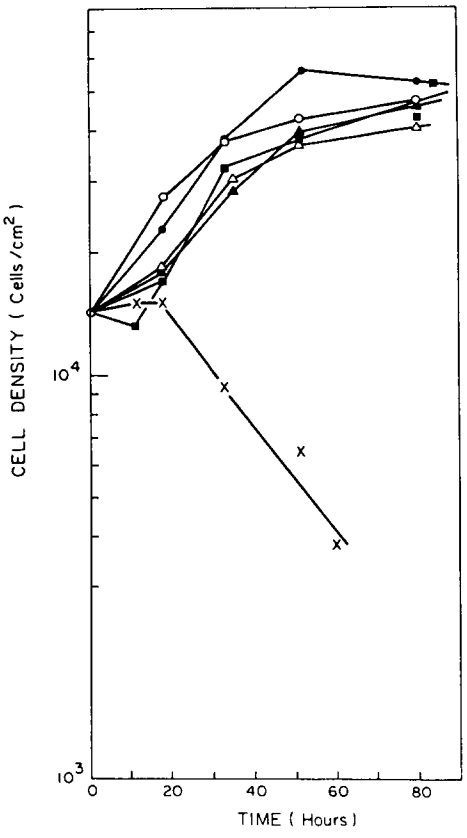


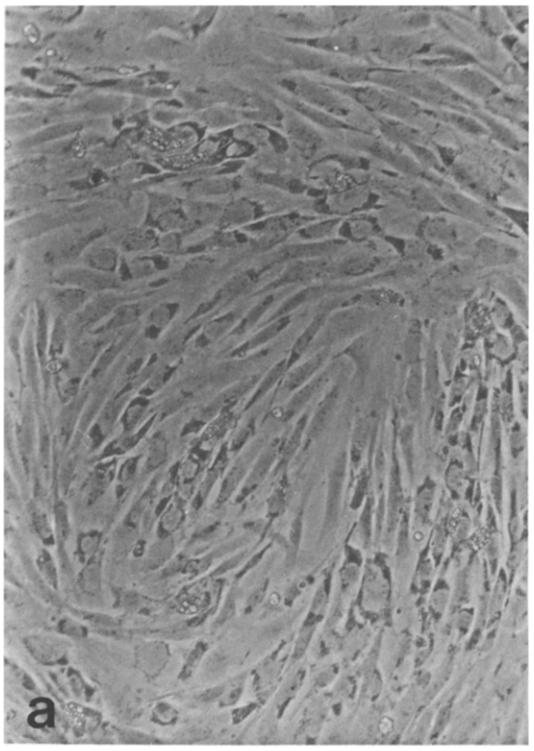
Fig. 1. Growth curves of CEF's supplemented at 37 or 41°C. Cells were supplemented as described in 'Materials and Methods' with: 5% newborn calf serum (○, ●), or 70 μM 18:0 (×, ■), 18:1*c* (△, ▲), and equimolar (70 μM total) 18:0-18:1*c* at 37°C (△).

These changes are characterized by cellular swelling and rounded morphology (Figs. 2c, 2d, and 3b), distension of the endoplasmic reticulum (Fig. 2c, arrow), cell surface blebbing (Fig. 2c, asterisk), cell sloughing, and subsequent decreased state of confluency (Fig. 2d). At higher magnification, Fig. 3 demonstrates the lesions described above for 37°C supplements as well as marked nuclear changes, including loss of nuclear detail and nucleoli, heterochromatin margination, pyknosis, and karryorhexis. In contrast, similar supplements grown at 41°C (Figs. 2b and 3b) proliferated to and maintained a normal state of confluency and were indistinguishable from 18:0-18:1*c*, 18:1*c*, and serum controls at either 37 and 41°C. The slight lag in growth phase of fatty acid-supplemented cells, as compared with serum supplements is also observed in cells grown in lipid-poor serum albumin-containing media and is therefore unrelated to the fatty acid supplement per se. Interpretation of the lag phase is difficult, since cell growth was asynchronous. However, Barnes and Sato [26] have demonstrated in several systems, including chick fibroblasts, that the lag in growth rate under serumless conditions is due to the absence of serum-supplied hormones. It is important to note that the cells in this study overcame the serumless condition, grew to, and maintained similar states of confluency and plate life as observed with serum supplementation.

The growth curves and degenerative changes associated with 18:0 supplementation at 37°C are in good agreement with observations made in other systems [1,3,4,27]. The temperature dependence of 18:0-enriched cell growth, however, has not been previously reported. Moreover, the growth temperature dependence demonstrated herein is not peculiar to these cells, since we have observed it in both MDBK and L-929 cells also (data not shown).

Correlative to 18:0-induced effects on cell growth, cells were supplemented at 37°C with other fatty acids known to either crystallize or fluidize lipid bilayers. Supplementation with laurate (12:0) had no effect on cell growth, consistent with the data of Doi et al. [4], demonstrating the lack of incorporation of 12:0 into membrane phospholipid or effects on LM cell growth. However, supplementation with other saturated fatty acids; palmitate (16:0), heptadecanoate (17:0), and nonadecanoate (19:0), resulted in graded degenerative changes similar to that observed with 18:0 supplementation at 37°C. Palmitate had the least effect on cell growth and morphology, whereas 19:0 resulted in rapid cell death at both 37 and 41°C. The effects of 19:0 enrichment, especially at 41°C, was again indicative of the sensitivity of the system to alterations in bilayer fluid state. Elaidate (18:1*t*) and dihydrosterculate (saturated fatty acid, oleate analog) supplementation at 37°C resulted in normal cell growth indistinguishable from that of 18:1*c* at 37°C.

The above fatty acids were assessed as to their ability to prevent 18:0-induced cell death at 37°C. As expected, addition of 14 μM 16:0 or 17:0 to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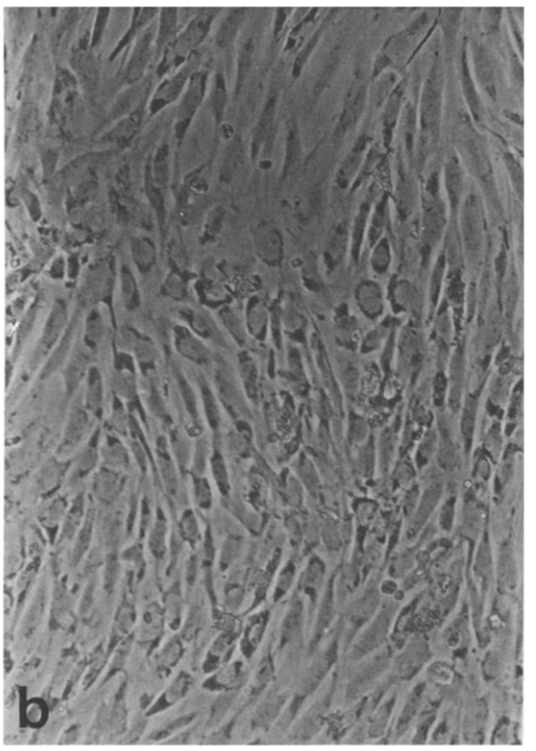
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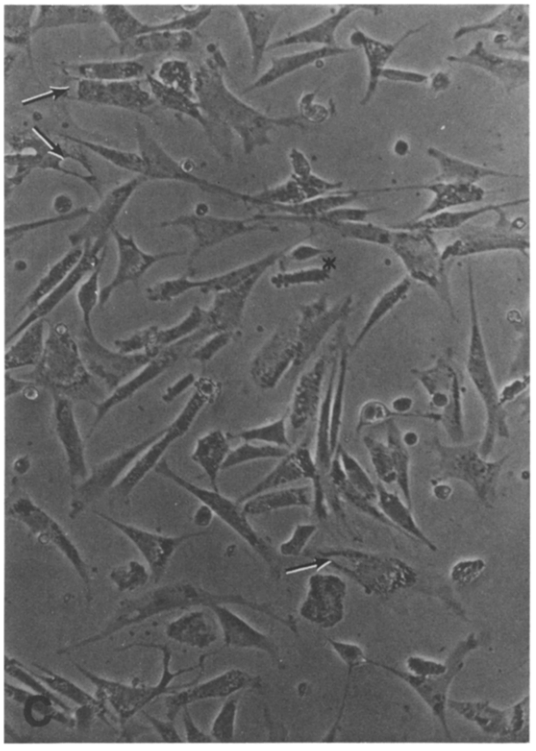
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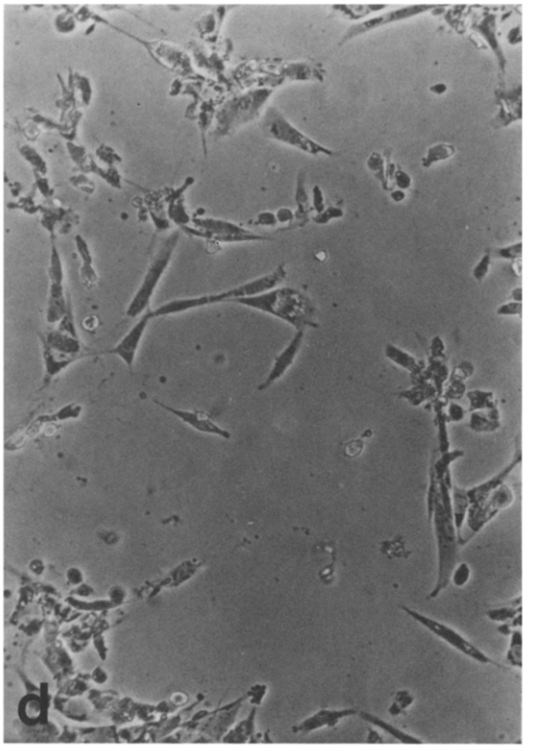
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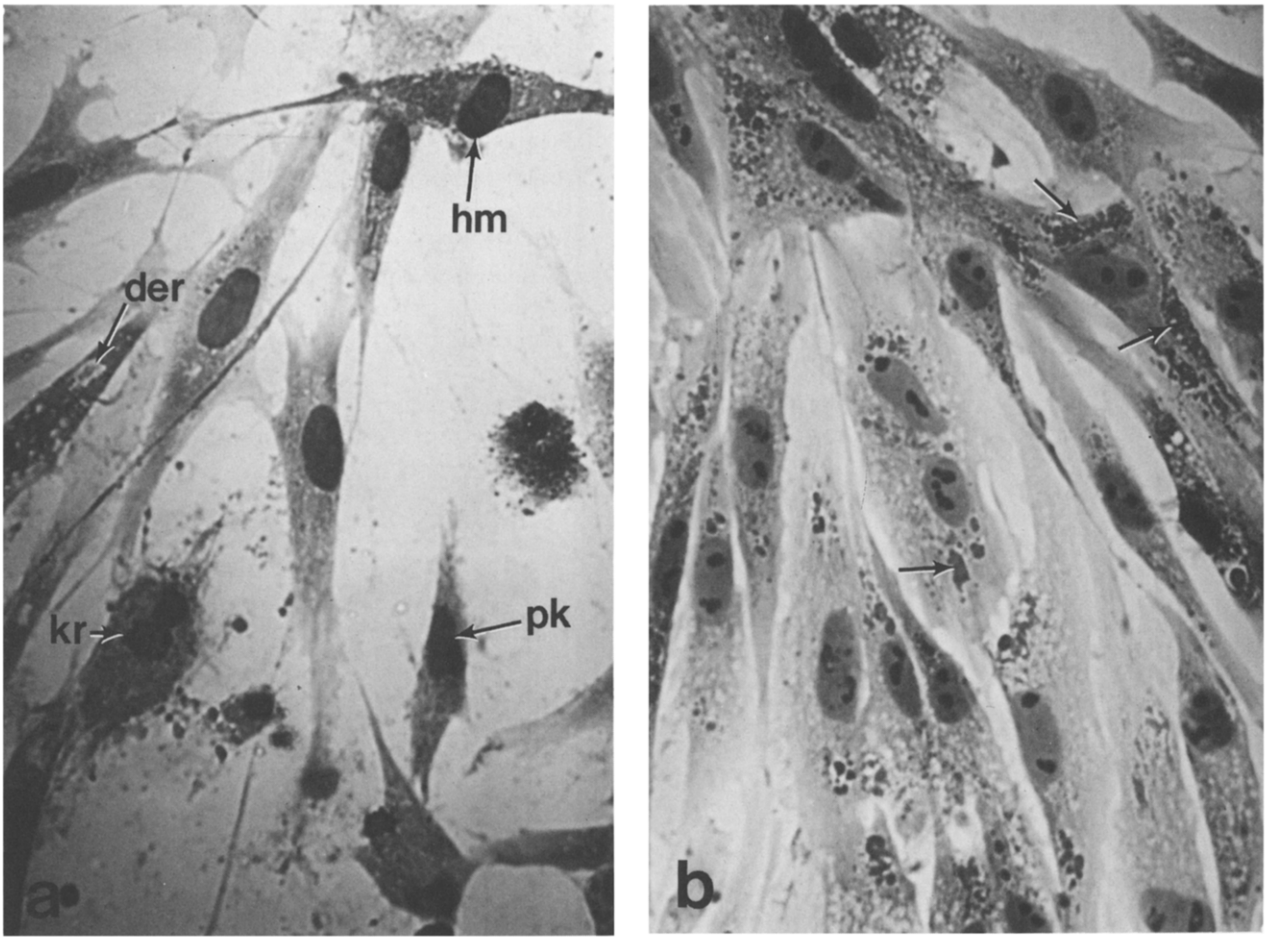

Fig. 3. Light microscopy of 18:0-supplemented cells grown 24 h at 37 (a) and 41°C (b) stained with oil-red-O. Cells were fixed at 37°C with 2% glutaraldehyde/1% acrolein/1 mM $CaCl_2$/50 mM cacodylate-HCl (pH 7.2). In recent studies (Chester, D.W. and Tourtellotte, unpublished observation) it was determined calorimetrically that all neutral lipids were melted above 45°C. As a result, neutral lipids were stained with oil-red-O in propylene glycol at 50°C to facilitate adequate dye incorporation. Stained neutral lipid in both photomicrographs is highlighted with arrows. (b) is typical of control cells (18:0-18:1*c*, serum) grown at either 37 or 41°C. Lesions identified in (a) are characteristic of degenerative changes observed upon 18:0 supplementation at 37°C: der, distended endoplasmic reticulum; hm, heterochromatin margination; kr, karyorrhexis (nuclear fragmentation); pk, pyknosis (nuclear condensation). Magnification, 1350×.

56 μM 18:0 did not effect a 'saving', while 18:1*c*, 18:1*t*, and dihydrosterculate prevented 18:0-induced degenerative changes (data not shown). The predictability of the above observations, as well as the correlations with the *A. laidlawii* system suggest that degeneration at 37°C is a function of altered membrane physical state. Moreover, the fact that small concentrations of unsaturated fatty

Fig. 2. Phase contrast photomicroscopy of supplemented cell growth characteristics at 37 and 41°C. (a) is representative of cells supplemented at 37°C with equimolar (70 μM total) 18:0-18:1*c* for 48 h and characteristic of other controls used in this study. (b) represents 70 μM 18:0-supplemented cells grown 48 h at 41°C in comparison with growth at 37 for 24 h (c) and 48 h (d). Arrows and asterisks in (c) highlight distension of endoplasmic reticulum and cell-surface blebbing, respectively, resulting from 18:0 supplementation at 37°C. Magnification, 300×.

acids are sufficient to prevent 18 : 0-induced cell death is consistent with the effects of raising growth temperature only 4°C allowing normal growth.

Since 18 : 0-enriched cells grow normally at 41°C, it was of interest to determine whether lowering incubation temperature of these cells, after grown to confluency at 41°C (96 h post-supplementation), would result in alterations in cell morphology and function. In an effort to increase the rate at which degenerative changes could be observed, 18 : 0- and control-supplemented cells were reincubated at 25°C and these observations demonstrated in Fig. 4. Reincubation at 25°C was associated with a rapid onset of degenerative changes in 18 : 0-supplemented cells as early as 1.5 h after temperature down-shift. These changes are highlighted by progression toward a more rounded morphology, decreased cell/cell contact and substratum adhesions, and sloughing, culminating in a decreased state of confluency. The severity and rapid onset of these changes in 18 : 0-enriched cells relative to controls is indicative of alterations in membrane permeability characteristics and loss of homeostasis leading to cell lysis.

Membrane structure

Since it appears that bilayer physical state may

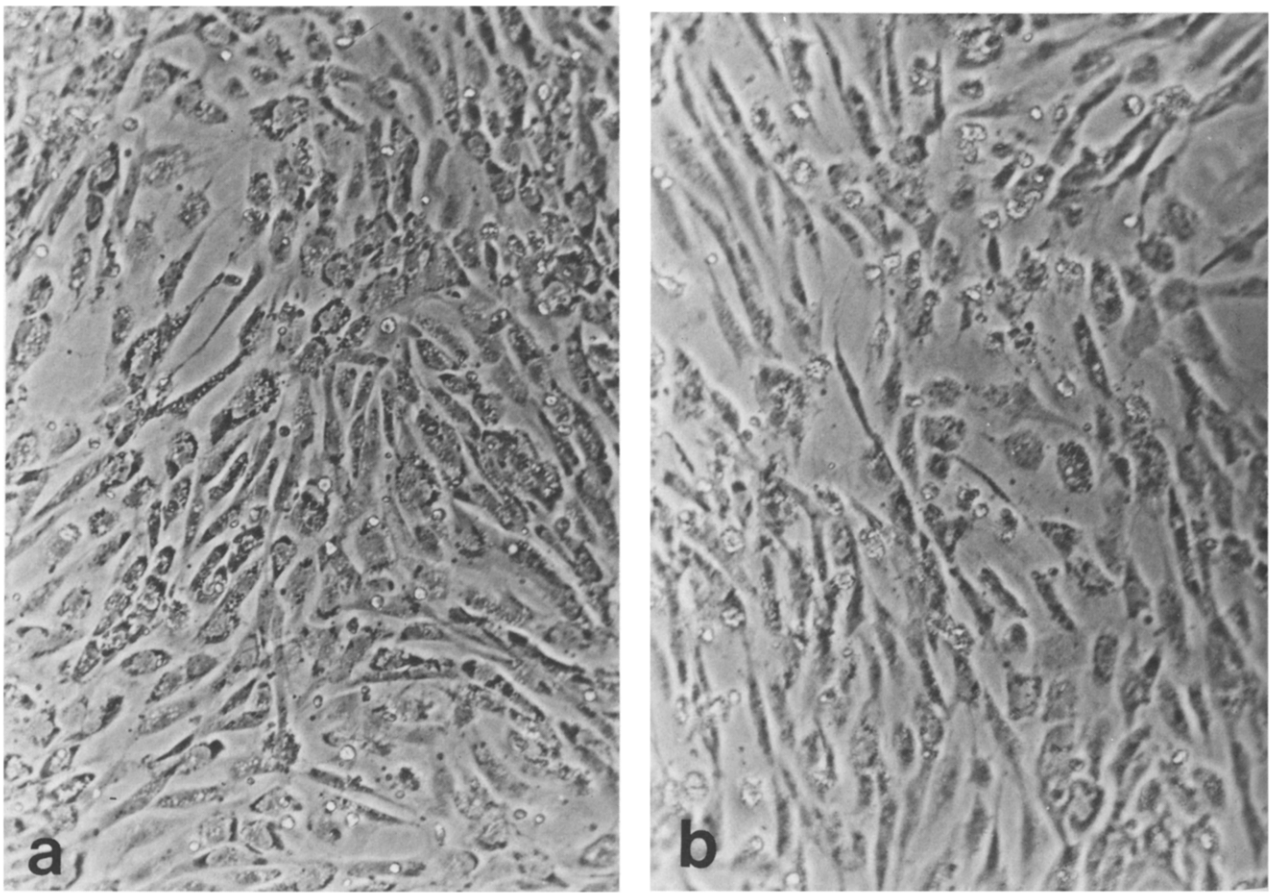

Fig. 4. Phase contrast photomicroscopy of temperature down-shift experiments. Cells were supplemented with 18 : 0 or 18 : 0-18 : 1*c* at 41°C for 96 h and subsequently shifted to 25°C. Represented are 18 : 0-18 : 1*c*- (a) and 18 : 0- (b) supplemented cells 5 h post temperature down-shift. Note the rounded morphology of 18 : 0-enriched cells as compared with stellate appearance of controls. Magnification, 357×.

be mediating the 18 : 0-induced degenerative changes, correlation was sought between membrane structure and function in relation to phospholipid fatty acid modification and the temperature dependence of 18 : 0-enriched cell growth. To this end, plasma membranes were isolated from cells grown at both temperatures and subjected to biochemical and biophysical analysis. Results using plasma membranes are reported in this study, since essentially similar data were obtained from other membrane subfractions. Moreover, these data are most relevant to understanding subsequent glucose transporter data.

The plasma membrane enriched fraction layers on top of the 10% Ficoll cushion in the gradient system used and is therefore easily separated from other subfractions. Marker enzyme data indicate that the 5′-nucleotidase activity was distributed mainly in the plasma membrane subfraction with smaller amounts partitioned throughout the others. Approximately one third of the microsomal marker activity, NADPH-cytochrome *c* reductase, was associated with this fraction. In addition, only 9% of the succinate-cytochrome *c* reductase activity was found. As such, the membrane preparation used in this study, while to a small extent contaminated with microsomal membrane, is enriched in plasma membrane.

Membrane lipid analysis

Membrane fatty acid data were obtained to ascertain the extent of exogenous fatty acid enrichment into TLC-purified phospholipid. Plasma membrane fatty acid composition is presented in Table I for cells supplemented with 70 μM 18 : 0, 18 : 0-18 : 1*c*, and 18 : 1*c* at 37 and 41°C. Plasma membrane fatty acid data from 37°C serum-supplemented cells is included in this table as a reference. Stearate supplementation resulted in increased membrane 18 : 0 content from 26 to 41%. Most importantly, there was no significant difference in 18 : 0 enrichment as a result of the shift in growth temperature from 37 to 41°C. Oleate content decreased upon 18 : 0 enrichment, while the levels of other fatty acid species remained essentially unchanged. The overall changes in phospholipid fatty acid composition are reflected in the saturate/unsaturated fatty acid ratios presented. Oleate supplementation results in levels of enrichment to 67% with subsequent decreases in

TABLE I

PLASMA MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION

Plasma membranes were isolated as described in Materials and Methods from cells supplemented with 70 μM 18 : 0, 18 : 0-18 : 1*c*, 18 : 1*c*, or 5% newborn calf serum at 37 or 41°C. As detailed, lipids were extracted, phospholipids TLC purified, and transesterified generating fatty acid methyl esters. Methyl esters were further TLC purified and separated by GLC. The data presented are representative of that from several experiments yielding similar changes and levels of enrichment relative to the media supplement. Values represent percent of total fatty acid composition from isolated phospholipid.

Fatty acid	Growth temperature					
	37°C			41°C		
Media suppl.:	18 : 0	18 : 0-18 : 1	18 : 1	Calf serum	18 : 0	18 : 0-18 : 1
14 : 0	4.6	2.2	0.5	1.8	3.9	2.9
14 : 1	1.2	0.7	0.9	2.6	0.9	0.9
15 : 0	3.3	2.4	0.7	3.5	2.9	2.3
16 : 0	29.7	22.7	15.1	33.2	38.8	23.3
16 : 1	4.3	6.3	1.8	3.7	2.3	3.8
17 : 0	5.5	5.2	0.8	1.7	4.2	6.9
18 : 0	40.9	26.6	12.8	26.1	38.5	34.5
18 : 1	6.9	24.0	66.8	20.0	6.9	22.1
18 : 2	0.5	2.9	–	2.4	–	0.5
20 : 0	1.0	1.2	–	–	0.8	1.0
Sat/Unsat.	6.6	1.8	0.4	2.3	8.4	2.6

18 : 0 and 16 : 0 content. Dual supplementation with equimolar 18 : 0-18 : 1*c* yields the expected result of equal incorporation into membrane phospholipid at 37°C. At 41°C, however, there appears to be an increased amount of 18 : 0 incorporation into these membranes which, again, is reflected in the saturate/unsaturate ratio. These data on fatty acid enrichment are consistent with those reported in other systems [28,29].

An adaptive response to shifts in phospholipid fatty acid composition might involve alterations in bilayer cholesterol content. Membrane cholesterol content was therefore determined and these results are presented in Table II. From these data, no significant changes are apparent in the phospholipid/cholesterol ratios as a result of fatty acid enrichment or cell growth temperature.

As an alternate adaptive response, phospholipid class composition could be modulated to accommodate saturated fatty acid enrichment. This potential is substantiated in studies conducted by McGee [27] in which unsaturated fatty acid enrichment resulted in increased PE/PC ratios (decreased head group size). Conversely, Weislander et al. [30], in the *A. laidlawii* system, demonstrated that unsaturated fatty acids increased polar head group size (mono- to diglucosyldiacylglycerol). Therefore, PE/PC ratios were determined to detect growth temperature- and/or supplementation-dependent phospholipid class changes and these results are shown in Table II.

In general, the temperature shift from 37 to 41°C was accompanied by increased PC and decreased bilayer PE content. Stearate enrichment increased PE, while decreasing PC content in comparison with 18 : 0-18 : 1*c* controls. Most important, however, no growth temperature dependence was exhibited in 18 : 0-enriched membranes, since PE/PC ratios were the same at both growth temperatures. Refeeding 18 : 0-enriched cells 24 h post-supplementation further increased the PE/PC ratio (data not shown), increasing the significance of the 18 : 0-induced changes in phospholipid head group composition. Oleate enrichment, on the other hand, decreased PE and increased PC, directionally consistent with temperature-shift changes.

Therefore, upon close examination, there are small, consistent, directional fatty acid supplementation and temperature-dependent changes in PE and PC content. The direction of change is interesting, since increases in membrane PE content upon 18 : 0 enrichment would tend to further crystallize the bilayer due to tighter packing of PE. As such, the shift toward PE would tend to augment rather than compensate for the effects of 18 : 0 enrichment. However, while interesting, these changes do not explain the differences in growth potential of 18 : 0-supplemented cells at 37 and 41°C.

Membrane physical state

Isothermal (25°C) fluorescence anisotropy studies were carried out on isolated plasma membranes using two structurally divergent hydrophobic probes, perylene and diphenylhexatriene. These data, shown in Table III, indicate that the 37 to 41°C growth temperature shift does not

TABLE II

PHOSPHOLIPID/CHOLESTEROL (PL/CHOL) AND PHOSPHOLIPID CLASS CHANGE RATIO

Isolated plasma membranes of cells supplemented in culture with 70 μM 18 : 0, 18 : 0-18 : 1*c*, or 18 : 1*c* at 37 or 41°C were assayed for phospholipid phosphorus, class composition and cholesterol content as described in Materials and Methods. In class change experiments, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are reported since, as cited in the text, these lipid species are of greatest significance and in other studies have been shown to change as a result of membrane fatty acid modification.

Temp. (°C)	PL/CHOL				PE/PC		
	Media suppl.:	18 : 0	18 : 0-18 : 1	18 : 1	18 : 0	18 : 0-18 : 1	18 : 1
37		2.37	2.64	2.70	0.67	0.57	0.44
41		2.65	2.58	2.32	0.67	0.49	0.39

TABLE III

ISOTHERMAL FLUORESCENCE ANISOTROPY

Plasma membranes were isolated from cells supplemented with 70 μM 18:0, 18:0-18:1, 18:1, or 5% newborn calf serum at 37 to 41°C as described in Materials and Methods. These membrane preparations were then assayed for both perylene (A) and diphenylhexatriene (B) fluorescence anisotropy as described in Materials and Methods. Membranes were equilibrated to 25°C prior to obtained anisotropy values.

Temp. (°C)	Media supplement			
	18:0	18:0-18:1	18:1	Calf serum
A. Perylene				
37	0.081	0.067	0.057	0.068
41	0.079	–	0.058	0.068
B. Diphenylhexatriene				
37	0.189	0.168	0.133	0.142
41	0.175	–	0.132	0.172

result in different fluorescence anisotropy values obtained with perylene or diphenylhexatriene. This is an important finding, since the 4°C difference in growth temperature, while having dramatic effects on cell growth, has no apparent effect on the extent of 18:0 enrichment or subsequent isothermal fluorescence anisotropy values. There are, however, supplementation-dependent changes in fluorescence anisotropy indicated by comparison of 18:0, 18:1*c* and 18:0-18:1*c* controls. Moreover, the anisotropy values are directionally consistent with anticipated values resulting from the fatty acid enrichment scheme.

The temperature dependence of fluorescence anisotropy (Fig. 5) reveals a similar probe anisotropy relationship between 18:0 and other supplements through the temperature range of 10–45°C. The smooth curves are indicative of the lack of major transitions in anisotropy values within this temperature range. In addition, there is little difference between serum, 18:0-18:1*c*, and serum albumin (not shown) supplements.

Since fluorescence anisotropy is a measure of average probe mobility within its environment in the bilayer, extrapolation to fluid state can be made. Thus, 18:0 enrichment results in more crystalline membranes, whereas 18:1*c* enrichment results in more fluid bilayers as compared with

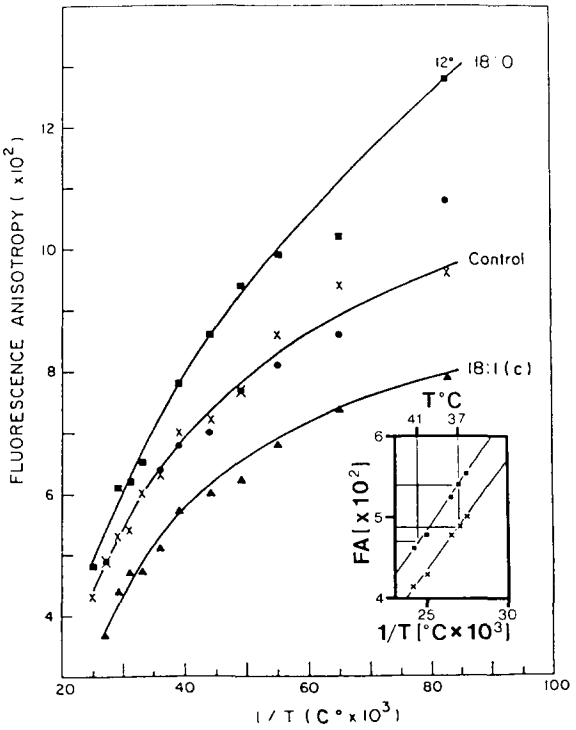

Fig. 5. Temperature dependence of perylene fluorescence anisotropy. Plasma membranes isolated from 18:0 (■), control (18:0-18:1*c*, ×; serum, ●), and 18:1*c*- (▲) supplemented cells, suspended in phosphate-buffered saline (pH 7.4), were equilibrated to 10°C after perylene incorporation at 42°C as detailed in Materials and Methods. Fluorescence anisotropy (FA) data points were subsequently obtained on temperature up-scan. No evidence of hysteresis was observed during temperature down-scan. Inset highlights the FA curves for 18:0- (■) and 18:0-18:1*c*- (×) enriched membranes as related to cell-growth temperature at 37 and 41°C.

controls. Moreover, these fluorescence data are consistent with the structural data presented above. It is interesting to note from data presented in Fig. 5 that the fluorescence anisotropy values from 18:0-enriched membranes at 41°C are the same or slightly lower than that of 18:0-18:1*c* controls at 37°C (see inset, Fig. 5). This suggests that that bulk fluidity of 18:0 membranes at 41°C, as measured by perylene, is similar to that of 18:0-18:1*c* controls at 37°C.

Differential scanning calorimetry was then performed on native 18:0-enriched membranes and isolated phospholipids to correlate changes in bilayer fatty acid composition and fluidity with thermal phase transitions. These data are pre-

sented in Fig. 6. Differential scanning calorimetry performed on native membranes isolated from 18 : 0-enriched cells grown for 96 h at 41°C showed no thermal phase transition other than the irreversible protein denaturation endotherm. The magnitude of the protein transition suggests that sufficient material was present to observe a bulk lipid endotherm if indeed one occurred. The absence of a lipid transition indicates that no cooperative unit of phospholipid exists large enough to produce a detectable transition.

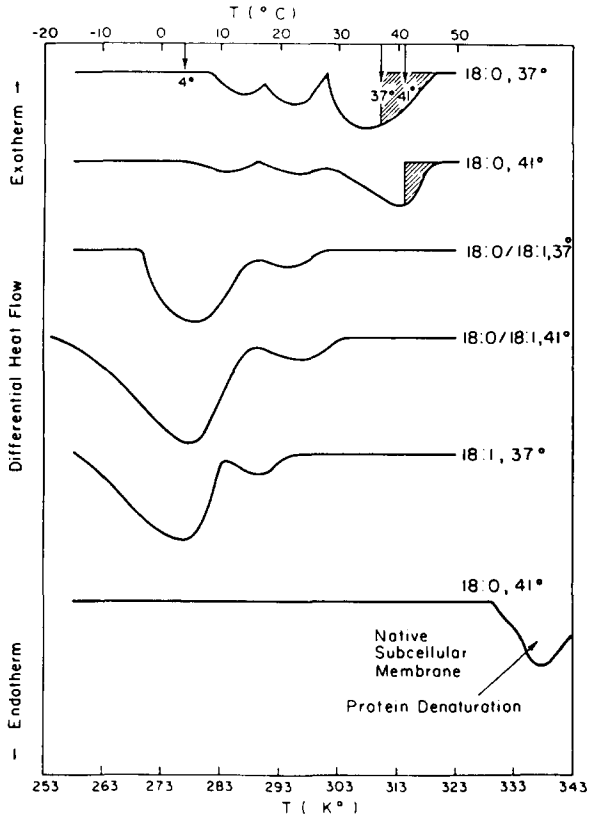

Fig. 6. Differential scanning calorimetry on native membranes and isolated phospholipids. Samples were prepared as described in 'Materials and Methods' and differential heat flow assessed relative to 50% ethylene glycol/water reference standard. Native membrane scan is representative of data obtained from scans of the membrane subfractions obtained from cells highly enriched in 18:0 at 41°C for 96 h. Hatch marks in the 37 and 41°C 18:0 scans highlight the position of the pure phospholipid transition at their respective growth temperatures. Calorimetric baselines were normalized for the purpose of clarity.

Isolated membrane phospholipids from these cells, however, do elicit thermal phase transitions which reflect the enriched fatty acid species. Stearate-enriched phospholipids (cells grown at either 37 or 41°C) yield transitions which terminate near 47°C, well above growth temperature, while 18 : 1*c* and 18 : 0-18 : 1*c* controls terminate much lower (20 and 27°C, respectively). Further, the main endotherm of the 18 : 0-enriched phospholipids which occurs between 28 and 47°C is shifted markedly in comparison with 18 : 1*c* and controls. Extrapolation of growth temperatures through these transitions reveals a 21% difference in amount of pure lipid yet to be melted as a result of the 4°C temperature shift. Therefore, while no transition is observed in native membranes, there are significant differences in the physical state of phospholipids which correlate well with the fatty acid and fluorescence data presented above.

Membrane function: glucose transporter

Having ascertained the structural and physical state characteristics of these fatty acid-modified membranes, a functional parameter was assessed to determine whether enrichment altered activity. Initial rates of glucose transporter function were determined in membranes isolated from 18 : 0- and 18 : 0-18 : 1*c*-enriched cells, since the transporter has been shown to be sensitive to fluid state changes [11,12]. In these initial studies, 2 mM glucose, the K_m for the transporter in chick fibroblasts [31], was used, since maximal sensitivity to perturbations in the transport kinetic parameters (e.g. V_{max}, K_m, etc.) would be attained at this substrate concentration. Arrhenius plots of this data are presented in Fig. 7. For illustrative purposes, the respective calorimetric data shown in Fig. 6 (inverted for clarity) are included in these plots.

Arrhenius plot of control membrane data (Fig. 7a) yields a straight line, suggesting there are no major changes in transporter function over the temperature range of 20–42°C. In contrast, 18 : 0-enriched membranes (Fig. 7b) elicited a triphasic plot of transporter function, suggesting that 18 : 0 enrichment of membrane phospholipid does effect glucose transporter kinetics. Moreover, the slope changes clearly correlate with the onset and midpoint of the main endotherm of the pure phos-

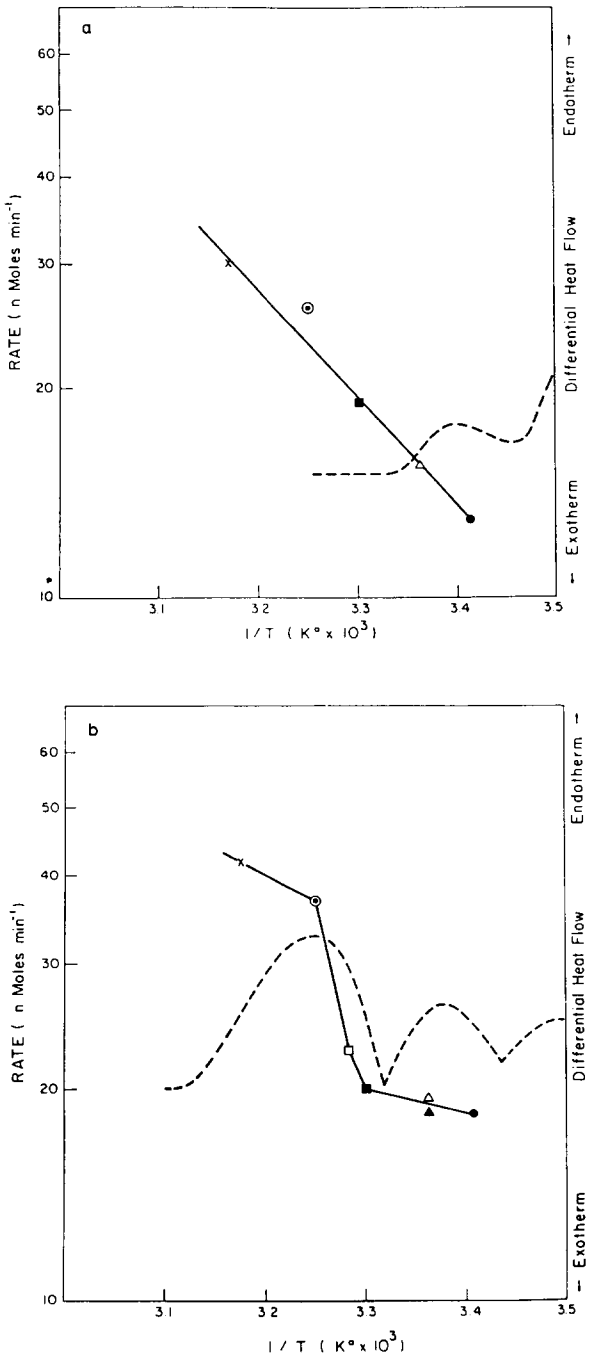

Fig. 7. Arrhenius plot of glucose transporter function for 18:0-18:1*c*- (a) and 18:0- (b) supplemented cells. First-order rate constants were obtained and plotted as a function of temperature through the range of 20–42°C as detailed in Materials and Methods (———). Included are the respective differential scanning calorimetric scans illustrated in Fig. 6, inverted for clarity (- - - - -).

pholipid thermal phase transition. Thus, the transporter appears to reveal a thermal phase transition, undetectable calorimetrically, in intact membranes. Furthermore, preferential partitioning of the transporter into the more crystalline regions of the bilayer is observed, consistent with reconstituted transporter/model membrane systems [11].

Discussion

The data presented strongly indicate that the sharp temperature dependence exhibited by 18:0-enriched cell growth is mediated by the physical state of the bilayer. Dual fatty acid supplementation data support this contention, since only those fatty acids known to fluidize bilayers (18:1*c*, 18:1*t*, dihydrosterculate) are capable of preventing 18:0-induced cell death at 37°C. Moreover, this 'saving' effect is potentiated by only small quantities of unsaturate (or unsaturate analog) relative to media 18:0 content. These data are consistent with Doi et al. [4] and suggest a minimum bilayer fluidity requirement for proper cell growth. Further support is gained through growth temperature drop-down studies, since the membranes would tend to become more crystalline as the temperature was decreased from 41 to 25°C. In these studies, the rapid onset of degenerative changes are indicative of significant alterations in cell permeability, consistent with recent reports demonstrating major distrubances in cation transport as membranes become more crystalline [6,10,32].

Examination of membrane structure by both physical and chemical means reveals no significant temperature-dependent compositional alterations suggestive of an adaptive response to 18:0 enrichment at 41 while not at 37°C. The only structural modifications resulting from fatty acid enrichment occur in phospholipid head group composition (Table II). Even in this case, no differences were observed between 18:0-supplemented cells grown at 37 and 41°C. Moreover, the trend toward increased PE content upon 18:0 enrichment would tend to augment rather than compensate for increases in bilayer crystallinity.

It is noteworthy that neither fatty acid enrichment nor growth temperature affected membrane

cholesterol content in these studies. The demonstration by Rintoul et al. [33] that decreased membrane sterol content is accompanied by an increased state of fatty acid unsaturation suggests that as fatty acid unsaturation is increased, cholesterol levels would correspondingly decrease. In our studies, however, while bilayer 18 : 1*c* content was enriched to 67% no changes in cholesterol levels were observed. Conversely, the concept that cholesterol functions as a bilayer plasticizer [34,35] suggests that as bilayers become more crystalline, cholesterol levels would increase. Again, this appears not to be the case, since 18 : 0 enrichment at either temperature failed to effect a change in cholesterol content. While no alterations in cholesterol content are apparent, the trend toward increased PE content in 18 : 0-enriched membranes might enhance cholesterol partitioning between sphingomyelin and PC over PE as suggested by Demel et al. [36] and Barenholz and Thompson [37] and/or association with lower melting phospholipids [35]. One consequence of any preferential association could be an alteration in the distribution of cholesterol (e.g., non-random) within the bilayer.

Enrichment of the exogenously supplied fatty acid is reflected in the bilayer physical state as assessed by fluorescence anisotropy and differential scanning calorimetry. Both perylene and diphenylhexatriene yield similar data on relative bulk membrane fluidity, demonstrating that the fluid state of the bilayer can be manipulated by incorporation of specific fatty acids. The 4°C difference in growth temperature, in agreement with biochemical data, does not result in differences in membrane fluid state when assayed isothermally at 25°C. In addition, the fluorescence anisotropy measurements in Fig. 5 (inset) reveal that 18 : 0-enriched membranes are essentially as fluid at 41°C as 18 : 0-18 : 1*c* controls at 37°C. Thus, while the extent of 18 : 0 incorporation is unaffected by growth temperature, the 4°C difference has profound effects on the physical state of the bilayer at the respective growth temperatures. While these differences are clearly evident in the data, it must be kept in mind that they reflect the 'opinion' of these probes as they are arranged within the bilayer.

While fluorescence data indicate increased membrane crystallinity with 18 : 0 enrichment, calorimetric data on similar (in fact, on the same) membrane preparations failed to detect any lipid endotherm (Fig. 6). However, phospholipids isolated from these membranes exhibit dramatically altered thermal phase transitions as compared with control supplements. Again, a great deal of similarity exists between 37 and 41°C phospholipid phase transitions consistent with the structural data presented. The inability to demonstrate a detectable endotherm in native membranes is related to the diverse population of lipids, the presence of protein and cholesterol. As has been demonstrated by Mabrey et al. [38], high cholesterol levels substantially decrease calorimetrically detectable thermal phase transitions in homogeneous lipid bilayer. In native membranes with highly heterogeneous lipid populations, thermal phase transitions are broader in range and lower in enthalpy [39] and hence the presence of cholesterol in these membranes would make a bilayer transition harder to detect.

The glucose transport system was chosen in these preliminary studies of membrane function to assess the relative effects of 18 : 0 enrichment on bilayer function, since it had been demonstrated by Melchior and Czech [11] and Pilch et al. [12] to be highly sensitive to changes in bilayer fluid state. As demonstrated in Fig. 7b, transitions in Arrhenius plot of glucose transport coincide with the onset and midpoint of the main endotherm of phospholipids isolated from 18 : 0-enriched membranes. Since the native membranes do not display any overall thermal phase transition, it appears that cholesterol may not be modulating (plasticizing) the microenvironment of the transporter. This conclusion is consistent with findings of Carruthers and Melchior [40] using a reconstituted transporter system and is based on the coincidence of the two transitions, pure phospholipid and transporter, the major difference being the absence of cholesterol. As such, the transporter is detecting an event undetectable by calorimetric means, strongly suggesting lipid microheterogeneity within the bilayer. Moreover, the temperature parameters of the sigmoidal Arrhenius plot suggest that the transporter is partitioning into the more cystalline regions of the membrane. These data and interpretations are consistent with that

of Melchior and Czech [11] and Thilo et al. [41]. Moreover, a similar transition in $(Na^+ + Mg^{2+})$-ATPase activity was observed in 18 : 0-enriched *A. laidlawii* [6]. While the glucose transporter function would not of itself explain cell death at 37 and not at 41°C, other systems (e.g. Ca^{2+}-ATPase) similarly affected might significantly alter cell physiology and homeostasis. A recent study by Yeagle [42] suggested that cholesterol interacts with the $(Na^+ + K^+)$-ATPase within human erythrocyte membranes; however, in this chick fibroblast system Arrhenius plot of ouabain-sensitive $(Na^+ + K^+)$-ATPase activity in 18 : 0-enriched membranes mimic the glucose transport data reported here (Tourtellotte, M.E. and Chester, D.W., unpublished observation). There is ample evidence that different membrane-associated enzymes require different lipid environment for optimal activity [6,8–10,43,44].

While the plasticizing effects of cholesterol have been established in model membrane systems [34,35], the function of cholesterol in native bilayers remains unanswered. Clear indications of lateral phase separations and/or regional domains have been reported by Sklar et al. [45] and Klausner et al. [46]. Moreover, Van Winkle and co-workers [47] have demonstrated phospholipid compositional dissimilarities between sarcoplasmic reticulum subpopulations in rabbit muscle. Sterol binding experiments have demonstrated the absence of cholesterol from coated pits and acetylcholine receptor patches in *Xenopus* [48–50]. As such, a non-random distribution of cholesterol could be anticipated.

A function of cholesterol in native bilayers is alluded to in sterol mutant studies using LM cells, where sterol depletion resulted in an increased bilayer 18 : 1*c* content [33], indicating that cholesterol functions mainly to fluidize bilayers. This contention is inconsistent with the data herein where 18 : 1*c* enrichment does not alter cholesterol levels. Within biological membranes there appear to be three parameters affecting cholesterol distribution. Demel and De Kruyff [34] and Calhoun and Shipley [35] have demonstrated, in model systems, that cholesterol tends to preferentially associate with lower melting phospholipids. Demel et al. [36] and Barenholz and Thompson [37] have further demonstrated that cholesterol preferentially interacts with sphingomyelin > PC > PE. Both observations are consistent with a geometric argument for cholesterol/phospholipid interactions. Cullis and De Kruyff [51] and Israelachvilli et al. [52] have proposed, based on molecular shape, motion and bilayer orientation, that cholesterol geometry is most readily accommodated by larger head group phospholipids. This has been demonstrated in *A. laidlawii* where cholesterol incorporation increases the diglucosyldiacylglycerol content (larger head group) over that of the smaller monoglucosyldiacylglycerol [30].

From these data it appears that cholesterol may function, primarily, to facilitate optimization of phospholipid packing within the bulk lipid phase. As such, cholesterol would not tend to induce a homeoviscous state within the bilayer. As demonstrated herein, the cholesterol content of native 18 : 0-enriched membranes, while abrogating any calorimetrically detectable thermal phase transition, does not affect glucose transporter detection of the main endotherm of the pure phospholipid transition. These data are consistent with the existence of regions of microheterogeneity within the bilayer. These regions, devoid of cholesterol, when sufficiently modified, could affect integral enzyme function, cell physiology, homeostasis and, ultimately, cell viability.

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