

INTERRELATED LIPID ALTERATIONS AND THEIR INFLUENCE ON THE PROLIFERATION AND FUSION OF CULTURED MYOGENIC CELLS

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ABSTRACT

We have cultured myogenic cells derived from primary explants and a cell line (L_6) in a lipid-depleted medium (LDM) and produced large alterations of the fatty acyl and polar headgroup composition and of the cellular sterol levels. These alterations were produced by altering the composition of the media as follows: removing biotin and providing exogenous fatty acid; removing choline and providing exogenous ethanolamine or choline analogues; and by adding 25-OH cholesterol, an inhibitor of 3-hydroxy-3-methylglutarate (HMG)-CoA reductase. Relatively small, secondary alterations of other lipid classes accompany the large primary alteration. In general, they are not obviously compensatory for the primary alteration by retaining some physical property.

We have explored the influence of these lipid alterations on myoblast proliferation and fusion into myotubes. In general, considerable variability appears tolerated, but there also appear to be limits. Long-term cultures grown in media containing a single fatty acid do not proliferate indefinitely, and the fatty acid does not become the sole fatty acyl component of the phospholipids. This phenomenon is also observed for cultures enriched in phosphatidylethanolamine (PE) or phosphatidylidimethylethanolamine (PDME).

The influence of the lipid alterations on fusion is particularly interesting. The inclusion of 25-OH cholesterol inhibits fusion. Enrichment of the fatty acyl chains with elaidate or the polar headgroups with PE also inhibits fusion, but in contrast to that by 25-OH cholesterol, a significant fraction of the myoblasts are aligned and interacting with each other. Oleate enrichment enhances the rate of fusion.

KEY WORDS membranes · lipids · fusion

Myogenic cells derived from primary explants or available as continuous cell lines can be grown in culture while retaining many of their differentiated membrane properties. When grown under suitable culture conditions, for example, they

sort out and fuse into multinucleate myotubes, synthesize acetylcholine receptors, become electrically and chemically excitable, cluster their receptors into "hot spots", and form synapses with appropriate neurons (17-20, 22, 25, 34, 37, 41, 43, 50, 56, 57, 59, 61). It is likely that the synthesis and assembly of the relevant molecular

components as well as the mechanisms of some of these phenomena directly or indirectly involve the membrane lipids. One approach to demonstrate such a lipid involvement and elucidate the nature of its coupling to a membrane process is via select alterations of the synthesis and composition of membrane components (11, 26, 27, 39, 54).

We reported previously that it is possible to exert such control on the fatty acyl chains of 3T3 and SV 101-3T3 cells and retain some of their characteristic properties, e.g., differential agglutinability (23, 28). Using L cells which do not require serum, Williams et al. (60) and Ferguson et al. (16) have also reported procedures for altering the fatty acyl composition. More recently, Glaser et al. (21) and Blank et al. (5) have demonstrated alterations in the phospholipid composition of L cells by growing them in the presence of choline analogues, and Chen et al. (10, 31) have reported changes in their levels of sterol induced by inhibitors of 3-hydroxy-3-methylglutaryl (HMG)¹-CoA reductase. In this communication, we extend these methods to include myogenic cells derived from primary explants of chick pectoral muscle and L₆, a myogenic cell line, and describe procedures for altering the composition of their fatty acyl chains, the ratio of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), and the levels of cellular sterol. We also show that large, secondary lipid alterations do not accompany the primary alterations. In addition, we present our observations on the influence of altered lipid compositions on myoblast proliferation and fusion into multinucleate myotubes. Preliminary accounts of this work have appeared elsewhere (26, 27, 29). During the

¹ *Abbreviations used in this paper:* AraC, cytosine arabinoside; CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; DME, dimethylethanolamine; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate; HMG, 3-hydroxy-3-methylglutarate; LDM, lipid-depleted medium (comprised of DMEM and 13% lipid-extracted fetal calf serum); LDMA, lipid-depleted medium containing 2 μg/ml avidin; LDMB, lipid-depleted medium containing biotin—1.25 μg/ml (primaries) or 1.5 μg/ml (L₆); PBS, phosphate-buffered saline (35, 42); PC, phosphatidylcholine; PDME, phosphatidyl dimethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PPLO, pleuropneumonia-like organisms; PS, phosphatidylserine; Sph, sphingomyelin.

course of these studies, Schroeder et al. (49) reported an analogous procedure for altering the ratio of PE to PC in LM cells.

MATERIALS AND METHODS

Preparation of Lipid-Depleted

Fetal Calf Serum

Lipid-depleted fetal calf serum was prepared as described previously for calf serum except that 100 ml of serum was used instead of 55 ml, and the dried residue (~4.8 g) was resuspended into 100 ml of distilled water (28). Both fetal calf serum and the extracted and reconstituted fetal calf serum contain ~18 mg/ml protein (40). The extracted serum contains <0.5% (0.18 nmol/ml) of the original phospholipid which consists of lecithin (57.4%), sphingomyelin (Sph) (24.3%) and lysolecithin (18.2%); 0.06% (1.3 μg/ml) of the original sterol which is almost exclusively cholesterol; and 2.5% (5.0 μg/ml) of the original fatty acids and esters which are primarily 16:0 (24.3%), 16:1 (4.0%), 18:0 (16.2%), 18:1 (25.7%), 18:2 (4.0%), and 20:4 (24.3%). The relative amounts of the residual fatty acids and esters present in the extracted serum are 16:0 (41.7%), 16:1 (4.2%), 18:0 (37.5%), and 18:1 (12.5%).

Cell Growth

Primary myogenic cultures were prepared by the method of Bischoff and Holtzer (4) with several minor modifications. Pectoral muscle was dissected from 11-day chick embryos, placed into calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), and the connective tissue was removed. The muscle was then teased into small pieces and placed into a sterile tube containing 0.25% trypsin in CMF-PBS for 30–45 min. When the tissue appeared dissociated, the growth medium (with serum) was added. The cell suspension was pipetted several times to disperse the cells and then spun at 900–1000 rpm for 2–3 min in a desk top centrifuge (HN-S, IEC Corp., Austin, Tex.). The supernatant was removed and 10 ml of growth medium (with serum) was added. The pellet was then resuspended and filtered through a Swinney filter apparatus (Millipore Corp., Bedford, Mass.) containing a double thickness of uncoated lens paper (Arthur H. Thomas Co., Philadelphia, Pa., no. 6325). An aliquot of the cell suspension was diluted ten-fold and counted. The cells were then diluted to 0.25–0.5 × 10⁶ cells/ml (depending on the experiment) and plated into collagen-coated plastic tissue culture dishes (35 mm, 2 ml; 100 mm, 16 ml).

All manipulations were performed under sterile conditions, and CMF-PBS as well as the final growth medium contained 1% penicillin-streptomycin-fungizone (antibiotic-antimycotic mixture, Grand Island Biological Co. (GIBCO), Grand Island, N.Y. [100 ×]).

As estimated by the final extent of fusion, the fraction of myogenic cells was usually between 60 and 90%.

Primary myoblasts were prepared by growing the explanted myogenic cells in regular medium (or lipid-depleted medium [LDM]) made without exogenous calcium and containing 200–300 μM ethylene glycol-bis (β -aminoethyl ether) N,N,N',N' -tetraacetate (EGTA) (62). The EGTA was added 3 h after plating. Cytosine arabinoside (AraC, 1 $\mu\text{g}/\text{ml}$) was added on the 2nd day after plating (18), and the cells were harvested for analysis on day 3.

Myotubes were obtained from myogenic cells grown in regular medium or LDM containing the indicated supplements. AraC (1 $\mu\text{g}/\text{ml}$) was added on day 2, and the cells were harvested for analysis or scored for fusion on day 3, unless otherwise indicated. Control cultures contained ~70% of the nuclei in myotubes, and in the lipid-altered cells the extent of fusion depended on the nature of the supplement.

Preformed myotubes were obtained by growing myogenic cells in LDM containing 2 $\mu\text{g}/\text{ml}$ avidin (LDMA) (for fatty acyl alterations) or LDM containing 1.25 $\mu\text{g}/\text{ml}$ biotin (LDMB) (for sterol and polar headgroup alterations) for 3 days. At this time, the designated supplements were added and the cells were harvested for analysis after two additional days of growth (day 5) for fatty acid and polar headgroup alterations, or three additional days growth (day 6) for sterol alterations.

Primary myogenic cells with altered fatty acyl compositions were prepared by terminating the trypsin dissociation of the primary explant with LDMA. The cells were then centrifuged and resuspended into LDMA at a concentration of 0.25×10^6 cells/ml and pipetted into tissue culture plates as described above. Fatty acid or biotin was added at the indicated concentration (5–50 $\mu\text{g}/\text{ml}$ or 1.25 $\mu\text{g}/\text{ml}$, respectively) within 3–4 h after plating or on day 3 (for alterations of preformed myotubes). For polar headgroup alterations, the primary myogenic cells were plated in LDMB at a concentration of 0.25×10^6 cells/ml. Ethanolamine or dimethylethanolamine (DME) was added at the indicated concentration (2.8–100 $\mu\text{g}/\text{ml}$ or 4 $\mu\text{g}/\text{ml}$, respectively) 2–4 h after plating. For sterol alterations, the primary myogenic cells were plated at a concentration of 0.5×10^6 cells/ml in LDMB, and after 3–4 h, 0.63 $\mu\text{g}/\text{ml}$ 25-OH cholesterol was added. When cholesterol was also present, it was added at a final concentration of 5 $\mu\text{g}/\text{ml}$ either simultaneously with the 25-OH cholesterol or at the stated time. For sterol alterations of preformed myotubes, the 25-OH cholesterol was added on day 3 at a concentration of 1.2 $\mu\text{g}/\text{ml}$.

L₆

Stock cultures of *L₆* (61), a rat myogenic cell line obtained from D. Schubert (Salk Institute, La Jolla, Calif.), or a clone derived from it were grown in tissue

culture flasks containing Dulbecco's Modified Eagle's medium (DMEM) and 10% fetal calf serum (termed regular medium). The stocks were subcultured with trypsin and EDTA well before confluence every 3rd day. The cells were free of pleuropneumonia-like organisms (PPLO) as assayed by a fluorescence technique and by incorporation of [^3H]thymidine followed by autoradiography (38, 47).

For growth curves and compositional analyses, cells were plated into tissue culture dishes containing 2 ml (35 mm), 5 ml (60 mm), or 15 ml (100 mm), respectively, of regular medium or DMEM and 13% lipid-depleted fetal calf serum (termed LDM). For experiments involving fatty acyl synthesis and composition, avidin was present in LDM at a concentration of 2 $\mu\text{g}/\text{ml}$ (LDMA). Additional supplements were added 2–4 h after plating. The cells were grown at 37°C in an atmosphere containing 10% CO_2 and counted in a hemacytometer after removal from the substratum with trypsin and EDTA. A factor of 28.3 $\text{cm}^2/60\text{-mm}$ plate was used to calculate the cell density. The cell density is expressed as the mean with standard deviation of the number of determinations shown by the integer in parentheses.

Long-Term Growth

L₆ was plated at a density of 6×10^4 cells/60-mm plate or at 1.8×10^5 cells/75 cm^2 flask (oleate), and additions were made as described above. On day 4–5, the cells were removed from the substratum with trypsin-EDTA, and LDMB (LDM + 1.50 $\mu\text{g}/\text{ml}$ biotin) (for sterol and polar headgroup modifications) or LDMA (LDM + 2 $\mu\text{g}/\text{ml}$ avidin) (for fatty acyl alterations), was added. The cells were counted and replated at 6×10^4 or 1×10^5 cells/60-mm plate or at 1.8×10^5 cells/75 cm^2 -flask, and additions were made as described above. This process was repeated through the indicated number of passages.

Lipid Analyses

Cultured plates washed once with versine and harvested with EDTA and trypsin were washed 2 \times in phosphate-buffered saline (PBS) or versine by centrifugation and extracted by the method of Bligh and Dyer (6). The chloroform was evaporated under a stream of argon, and the residue was resuspended into ~20 μl of chloroform and applied to Absorbil 5 prekotes (Applied Science Laboratories, State College, Pa.). The thin-layer plate was developed with a solvent of chloroform:methanol:acetic acid:water (75:45:8:2) (55), and the two-dimensional system of Glaser et al. (21) was used to resolve phospholipids derived from monomethylethanolamine and dimethylethanolamine.

For phospholipid analyses, the chromatograms were visualized by exposure to iodine vapors, and the spots were identified by comparison to a set of standards and to published R_f values. The regions of the silica gel

corresponding to phospholipid were collected, 0.5 ml of 10 N H₂SO₄ was added, and the mixture was heated to 160°C for 3 h. After cooling, 0.5 ml of H₂O₂ (30%) was added, and the samples were heated at 160°C for 2 h. After cooling again, 0.2 ml of ammonium molybdate (5% in H₂O), 0.2 ml of Fiske-Subbarow reagent, and 4.4 ml water were added; the tubes were covered with aluminum foil, and the mixture was heated for 7 min at 100°C. The OD was read at 802 Å against appropriate blanks and compared to standards carried out through the same procedure (1; G. Khuller and H. Goldfine, personal communication).

For fatty acyl analyses, the lipids were chromatographed as described above, visualized by a water spray, and the regions of the silica gel containing phospholipid were collected and eluted with chloroform:methanol (1:1) followed by chloroform:methanol (2:1). The extract was dried under a stream of argon and transesterified by resuspension into BF₃-methanol reagent (Applied Science Laboratories) followed by an incubation for 10 min in boiling water. After cooling on ice, 1 ml each of pentane and water were added, the mixture was vortexed, and the upper phase was removed. The extraction was repeated twice, and the upper (pentane) phases were collected and concentrated. The methyl esters were analyzed by gas chromatography on a 6-foot column of EGSS-x or a 20-foot column of Silar 10c on 100/120 mesh Gas Chrom Q (Applied Science Laboratories) using nitrogen as the carrier gas. In some experiments, the former column was temperature-programmed between 180° and 205°C and the latter run at 220°C. The Silar 10c column was used to separate cis and trans isomers. The fatty acid peaks were identified by comparing their retention times to those of standards. Peaks corresponding to unusual fatty acids, those that varied from determination to determination, and those with retention times longer than that of 20:4 were not determined. The relative areas under the peaks were estimated using the peak height by retention time method along with triangulation.

The ratio of 16/18 carbon fatty acids was determined by dividing the fraction of 16-carbon fatty acids by the fraction of 18-carbon fatty acid. The percentage of unsaturated fatty acids is defined as the fraction of fatty acids possessing one or more double bonds multiplied by 100. The number of double bonds is determined by adding together the percent of each type of unsaturated fatty acid multiplied by its number of double bonds.

The levels of cellular sterol were determined as follows (2): Pellets of harvested cells were sonicated for 20 s on ice, an aliquot was removed for protein determination (40), and coprostanol was added as an internal standard. The sonicate was extracted by the method of Bligh and Dyer (6), and the chloroform phase was evaporated under a stream of nitrogen or argon. The residue was taken up into CS₂ and analyzed by gas chromatography using a 6-foot glass column containing OV-17 on 100/120 mesh Gas Chrom Q.

The column was run at 250°C using N₂ as the carrier gas. The sterols were identified by comparing their retention times to those of standards. In some cases, ³/₄ of the chloroform phase was removed for determination of total phospholipid, leaving the remainder for sterol analysis.

All analyses are presented as the mean with standard deviation of the number of determinations shown by the integer in parentheses.

Fusion

3-day-old cultures were generally fixed and stained to facilitate scoring fusion. Two methods were used. In the initial experiments, the culture plates were washed 2× with PBS, aspirated well, and 100% methanol was added for 15 min. The methanol was aspirated and replaced in 5-min intervals by 30, 70, and 100% ethanol. After the last ethanol treatment, 10% aqueous Giemsa's (obtained as a concentrated stock from Harleco [Gibbstown, N. J.], diluted to 10% and filtered through Whatman no. 2 filter paper) was added for 30 min. The plate was rinsed in deionized water and covered with glycerol. In later experiments, a different procedure was used. The cells were washed 2× with PBS and then fixed and stained in methanol containing 0.05% orcein (J. T. Baker Chemical Co., Phillipsburg, N. J.) for 10 min. The plates were aspirated and glycerol was added (K. Knudsen, unpublished communication). All operations were performed at room temperature.

Fusion was scored in 10 fields/plate along a single diameter using a phase microscope at × 200. In general, there were between 50 and 100 nuclei/field. The number of nuclei in myotubes, interacting cells, and single cells was determined and the results were expressed as a percent of the total nuclei present in the field. Each nucleus was counted only once, and hence the sum of the nuclei in myoblasts, myotubes, and interacting cells accounted for all the nuclei in the field. We operationally defined single cells, myotubes, and interacting cells as follows: Single cells are discrete, individual cells that contain only one nucleus and possess clear boundaries. Myotubes are cells containing two or more nuclei in a cluster or a line within a single cytoplasm. Interacting cells are those cells that are in close association with each other such that their boundaries are not distinguishable. (Two interacting myoblasts are scored as two interacting cells; a myoblast interacting with a myotube is scored as one interacting cell.)

The number of myotubes was estimated by scoring all individual myotubes within a field and any fractions of myotubes originating within the field but extending beyond it.

The percent fusion and number of nuclei and myotubes per field are expressed as the mean with standard deviation of the number of determinations shown by the integer in parentheses.

Materials

Fatty acids were purchased from Nu Chek Prep (Elysian, Minn.) and Applied Science Laboratories; fatty methyl esters, gas chromatography columns, phospholipid standards, thin-layer chromatography plates, and BF_3 -methanol reagent, from Applied Science Laboratories; Lipopure solvents from Burdick & Jackson Laboratories (Muskegon, Mich.); steroids from Steraloids, Inc. (Wilton, N. H.); media from GIBCO (or made from components purchased from Sigma); serum from GIBCO or Flow Laboratories, Inc. (Rockville, Md.); DME from Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, N. Y.); ether analogues of lecithin² from Calbiochem (San Diego, Calif.); and mevalonate, biotin, avidin, and other reagents from Sigma Chemical Co. (St. Louis, Mo.). Phosphorylcholine was a generous gift of T. Henderson (University of Illinois Medical School), and the phosphonate and phosphinate analogues of lecithin,³ generous gifts of A. F. Rosenthal (Long Island Jewish-Hillside Medical Center, New Hyde Park, N. Y.). Tissue culture plates were manufactured by BioQuest, BBL & Falcon Products, Becton Dickinson & Co. (Cockeysville, Md.) and Corning Glass Works, Science Products Div. (Corning, N. Y.). The azido analogues of palmitate and stearate were synthesized as described by Chakrabarti and Khorana (9).

Fatty acid and sterol stocks were made up in ethanol at concentrations such that $\leq 3 \mu\text{l}$ (L_6) or $\leq 7 \mu\text{l}$ (primaries) was added for each ml of growth medium. Biotin was stored in ethanol: H_2O (1:1) at a concentration of 0.5 mg/ml, and 3 μl (for L_6) or 2.5 μl (for primaries) was used for each ml of the growth medium. Other stocks were made up in PBS at concentrations such that the volume added was $< 0.5\%$ of the growth medium. The formulations of the buffers and reagents used for cell culture are given in Paul (42). All stock solutions were stored at 15°C , and the fatty acid and cholesterol stocks were kept under argon.

RESULTS

Proliferation

The proliferation of L_6 grown under conditions used to alter the fatty acyl composition is shown in Fig. 1. The growth rate and saturation density of cells grown in LDM containing different fatty acids and/or biotin depend on the particular supplement. Cells grown without biotin or exog-

² 1-octadec-9-enyl, 2-hexadecyl ether-DL-lecithin and dihexadecyl ether-L- α -lecithin.

³ Phosphorylcholine; 1,2-dipalmitoyl-sn-glycerol-3-(2'-trimethyl ammoniummethyl)phosphate; and DL-2-hexadecyloxy-3-octadecyloxy-propyl[2-(trimethylammonium)ethyl]phosphinate.

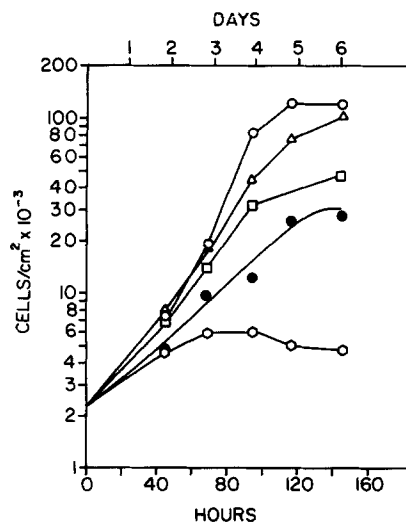


FIGURE 1 The growth of L_6 myoblasts in regular medium (○) and in LDMA containing biotin (1.5 $\mu\text{g}/\text{ml}$) (△), oleate (12 $\mu\text{g}/\text{ml}$) (□), elaidate (12 $\mu\text{g}/\text{ml}$) (●), or no additional supplement (◇). The cells were plated at a concentration of 6×10^4 cells/5 ml.

enous lipid do not proliferate appreciably. Of the fatty acids tested, septadecanoate, oleate, linoleate, elaidate, linolenate, and azido derivatives of stearate and palmitate support proliferation but to different extents (Table I). Oleate gives the fastest growth rate and highest saturation density of the fatty acids tested, although it is less effective than biotin. Biotin and linoleate when supplemented together result in proliferation exceeding that obtained with linoleate or biotin alone. The addition of biotin to the cultures grown with some other fatty acids, e.g. elaidate, enhances their proliferation as well, but not to the extent observed for linoleate-biotin-supplemented cells. Nonadecanoate, when used at similar concentrations, is toxic, but a twofold reduction in concentration does support significant proliferation. A cyclopropane fatty acid (cis 9,10 methylene octadecanoic acid) is either toxic or supports no net proliferation through day 4, depending on the concentration used; in some experiments, cell doubling commenced after 4–5 days. In addition to fatty acids and biotin, dipalmitoyllecithin supports proliferation; however, ether analogues² of lecithin do not, unless biotin or fatty acid is present (Table I).

We have attempted to maintain cultures of L_6 grown on LDMA containing fatty acid and/or biotin through multiple subculturings (Table II).

TABLE I
Growth of *L*₆ Myoblasts in Medium Containing Fatty Acid and Polar Headgroup Supplements

Medium supplement	Cells/cm ² (day 4)	Medium supplement	Cells/cm ² (day 4)
Regular medium (3)	7.0 ± 2.1 × 10 ⁴	Octadecenyl, hexadecylether- lecithin (20 μg/ml) (3)	5.1 ± 2.6 × 10 ³
LDMA (17)	5.6 ± 1.8 × 10 ³	Octadecenyl, hexadecylether- lecithin (20 μg/ml) + biotin (1.5 μg/ml) (3)	1.0 ± 0.4 × 10 ⁴
LDMA +:		Octadecenyl, hexadecylether- lecithin (20 μg/ml) + oleate (12.5 μg/ml) (2)	1.7 ± 0.5 × 10 ⁴
Biotin (1.5 μg/ml) (21)	4.6 ± 1.4 × 10 ⁴	LDMB +:	
Heptadecanoate (4.2 μg/ml) (3)	4.0 ± 0.5 × 10 ⁴	Dipalmitoylphosphorylcholine (20 μg/ml) (4)*	1.7 ± 0.5 × 10 ⁴
Heptadecanoate (8.3 μg/ml) (3)	3.0 ± 1.5 × 10 ⁴	Dipalmitoylphosphorylcholine (20 μg/ml) + choline (4 μg/ ml) (2)*	5.5 ± 2.3 × 10 ⁴
Stearate (8.3 μg/ml) (2)	3.5 ± 0.8 × 10 ⁴	Hexadecoxy, octadecoxypro- pylphosphinate (20 μg/ml) (2)*	1.3 ± 0.6 × 10 ⁴
6-azido Stearate (8.3 μg/ml) (3)	1.5 ± 0.3 × 10 ⁴	Phosphorylcholine (8 μg/ml) (4)*	1.3 ± 0.2 × 10 ⁴
9-azido Stearate (8.3 μg/ml) (3)	2.5 ± 0.2 × 10 ⁴	Phosphorylcholine (8 μg/ml) + choline (4 μg/ml) (2)*	5.7 ± 1.0 × 10 ⁴
12-azido Stearate (8.3 μg/ml) (3)	2.1 ± 0.5 × 10 ⁴	DME (4 μg/ml) (2)*	2.5 ± 0.1 × 10 ⁴
9-azido Palmitate (8.3 μg/ml) (3)	1.4 ± 0.1 × 10 ⁴	LDMA +:	
Elaidate (8.3 μg/ml) (2)	2.4 ± 0.5 × 10 ⁴	Dipalmitoylphosphatidylcholine (8.3 μg/ml) (2)	1.3 ± 0.1 × 10 ⁴
Elaidate (8.3 μg/ml) + biotin (1.5 μg/ml) (4)	3.8 ± 0.5 × 10 ⁴	Egg lecithin (8.3 μg/ml) (2)	1.7 ± 0.3 × 10 ⁴
Linoleate (8.3 μg/ml) (4)	2.3 ± 0.6 × 10 ⁴	Cis-methyleneoctadecanoate (12.5 μg/ml) (2)	5.0 ± 0.8 × 10 ³
Linoleate (8.3 μg/ml) + biotin (1.5 μg/ml) (4)	5.2 ± 1.4 × 10 ⁴		
Linolenate (8.3 μg/ml) (2)	1.7 ± 0.1 × 10 ⁴		
Nonadecanoate (4.2 μg/ml) (3)	1.4 ± 0.2 × 10 ⁴		
Dihexadecylether-lecithin (20 μg/ml) (3)	4.9 ± 2.2 × 10 ³		
Dihexadecylether-lecithin (20 μg/ml) + biotin (1.5 μg/ml) (3)	2.1 ± 1.2 × 10 ⁴		
Dihexadecylether-lecithin (20 μg/ml) + oleate (12.5 μg/ml) (2)	2.1 ± 0.8 × 10 ⁴		

*L*₆ cultures were plated at a concentration of 6 × 10⁴ cells/5 ml and counted on day 4.

* For these experiments, choline was not present in the LDM unless specifically indicated.

Oleate-supplemented cells can be subcultured, but proliferation decreases substantially. If stearate or biotin is added to these arrested cultures, proliferation resumes. Biotin-, stearate-oleate-, and linoleate-biotin-supplemented cultures continue to proliferate at normal rates through several subculturing. Attempts to maintain long-term cultures of *L*₆ on septadecanoate are consistently successful only when subculturing is done at higher cell densities.

The supplementation of LDMB with 25-OH cholesterol has a marked, dose-dependent effect on *L*₆ proliferation. The data presented in Fig. 2 show that proliferation is inhibited when the amount of inhibitor approaches or exceeds 0.1 μg/ml. A marked change in cellular morphology accompanies the proliferative inhibitions. When viewed in the phase-contrast microscope, the cells appear less spread out and triangular in

shape. These effects of 25-OH cholesterol on proliferation and morphology are reversed by the addition of cholesterol or mevalonate (the end product of the inhibited pathway and reaction, respectively [12]) and are prevented by the inclusion of these compounds at the time of plating (Fig. 2). Desmosterol, an analogue of cholesterol, was also tested for its ability to support the proliferation of *L*₆ grown in the presence of 25-OH cholesterol. The inclusion of desmosterol results in cell densities comparable to those of cholesterol-supplemented cells. *L*₆ grown in LDMB containing 25-OH cholesterol and mevalonate or desmosterol proliferate at nearly normal rates through several subculturing (Table II).

When grown in LDM, *L*₆ displays a choline requirement for growth (Fig. 3). In addition to choline, lecithin and DME support proliferation,

TABLE II
Long-Term Culture of *L*₆ Myoblasts

Medium supplement	Pass 1	Pass 2	Pass 3
	Cells/cm ²	Cells/cm ²	Cells/cm ²
A*			
LDMA + biotin (1.5 μg/ml) (5)	3.0 ± 1.2 × 10 ⁴	4.4 ± 1.2 × 10 ⁴	4.7 ± 2.1 × 10 ⁴
LDMA + oleate (8.3 μg/ml) (3)	2.9 ± 1.0 × 10 ⁴	4.7 ± 2.2 × 10 ³	3.9 ± 1.4 × 10 ³
To stearate (4.2 μg/ml)-oleate (4.2 μg/ml) (3)‡	—	—	1.6 ± 0.3 × 10 ⁴
To biotin (1.5 μg/ml) (3)‡	—	—	2.1 ± 1.3 × 10 ⁴
LDMA + linoleate (8.3 μg/ml) + biotin (1.5 μg/ml) (2)	2.9 ± 2.0 × 10 ⁴	8.3 ± 0.4 × 10 ⁴	8.1 ± 1.6 × 10 ⁴
B§			
LDMA	6.8 ± 1.6 × 10 ³	5.8 ± 0.3 × 10 ³	7.3 ± 2.4 × 10 ³
LDMA + biotin (1.5 μg/ml) (2)	4.9 ± 0.1 × 10 ⁴	2.8 ± 0.1 × 10 ⁴	9.0 ± 0.0 × 10 ⁴
LDMA + stearate (4.2 μg/ml) + oleate (4.2 μg/ml) (2)	5.4 ± 0.6 × 10 ⁴	2.8 ± 0.2 × 10 ⁴	3.7 ± 2.5 × 10 ⁴
LDMA + septadecanoate (4.2 μg/ml) (2)	4.0 ± 0.7 × 10 ⁴	2.0 ± 1.1 × 10 ⁴	2.6 ± 0.0 × 10 ⁴
LDMA + 25-OH cholesterol (0.1 μg/ml) + mevalonate (1 mg/ml) (2)	4.4 × 10 ⁴ , 4.8 × 10 ⁴	3.7 ± 0.7 × 10 ⁴	5.2 ± 0.8 × 10 ⁴
LDMA + 25-OH cholesterol (0.1 μg/ml) + desmosterol (2 μg/ml) (2)	3.2 × 10 ⁴ , 5.7 × 10 ⁴	2.5 ± 0.1 × 10 ⁴	4.6 ± 1.6 × 10 ⁴

* The cells were initially plated and subsequently subcultured at a concentration of 6 × 10⁴ cells/5 ml every 4–5 days; the values presented are the cell densities at the time of subculturing.

‡ *L*₆ was plated and subcultured in oleate-supplemented LDMA, and, after the second pass, replated into LDMA supplemented with stearate and oleate.

§ The cells were initially plated and subsequently subcultured at a concentration of 6 × 10⁴ and 1 × 10⁵ cells/5 ml, respectively. The values presented are the cell densities at the time of subculturing.

|| In this experiment, *L*₆ was initially plated at 6 × 10⁴ and 1 × 10⁵ cells/5 ml and both values are presented here; the first corresponds to cells plated at the lower density.

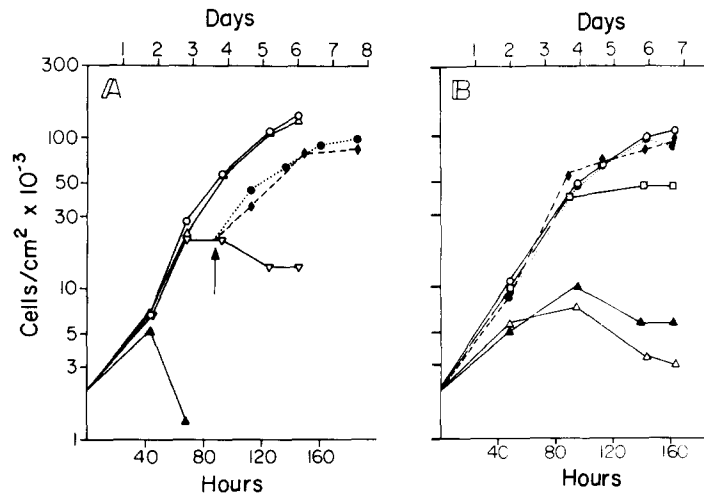


FIGURE 2 (A) The growth of *L*₆ myoblasts in LDMA (○) and in LDMA containing 25-OH cholesterol—0.06 μg/ml (△), 0.1 μg/ml (▽), or 0.3 μg/ml (▲). The arrow shows the time at which mevalonate (1 mg/ml) (●) or cholesterol (2 μg/ml) (◆) was added to the cultures growing in LDMA containing 25-OH cholesterol (0.1 μg/ml). The cells were plated at a concentration of 6 × 10⁴ cells/5 ml. (B) The growth of *L*₆ myoblasts in LDMA (○), in LDMA containing 0.1 μg/ml 25-OH cholesterol (△), and in LDMA containing 25-OH cholesterol (0.1 μg/ml) and cholesterol—1 μg/ml (□), 2 μg/ml (◆) or mevalonate—100 μg/ml (▲), 1 mg/ml (●). The cells were plated at a concentration of 6 × 10⁴ cells/5 ml.

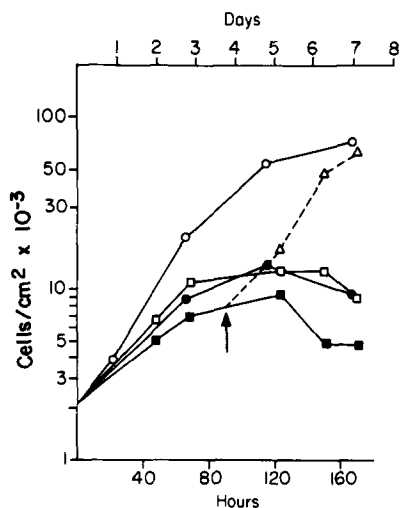


FIGURE 3 The growth of L_6 myoblasts in LDMB (4 $\mu\text{g/ml}$ choline) (\circ), choline-free LDMB (\bullet), and in choline-free LDMB containing ethanolamine—2 $\mu\text{g/ml}$ (\square), 20 $\mu\text{g/ml}$ (\blacksquare). The arrow shows the time at which exogenous choline (4 $\mu\text{g/ml}$) (Δ) was added to cultures growing in LDMB containing 20 $\mu\text{g/ml}$ ethanolamine. The cells were plated at a concentration of 6×10^4 cells/5 ml.

whereas a phosphonate analogue of choline and phosphonate and phosphinate analogues³ of lecithin do not (at comparable concentrations) unless choline is present (Table I). We have also studied the growth of L_6 in LDMB containing ethanolamine since it can be used to synthesize PE, a principal, natural phospholipid found in these cells. As shown in Figs. 3 and 4, low concentrations of ethanolamine produce little or no further effect on proliferation compared to that of cells grown in choline-free LDMB, whereas at relatively higher concentrations there are changes in morphology as well as a decrease in proliferation (or attachment to the substratum). Even when grown in the presence of relatively high amounts of ethanolamine, a substantial fraction of the cells remains viable and rapidly grows to confluence upon addition of choline (Fig. 3). L_6 grown in choline-free LDMB containing DME does not proliferate appreciably after subculturing (data not shown).

Primary Lipid Alterations

Table III contains fatty acyl analyses of the phospholipids present in L_6 grown in regular medium and in LDMA containing the indicated supplements and analyzed on day 4. There are only small differences between the fatty acyl

composition of cells grown in regular medium, LDMA containing biotin, and LDMA without biotin. These occur mostly in the amounts of 16:1 and 18:1 and result in changes in the proportion of 16- and 18-carbon fatty acids but maintain a normal fraction of unsaturated fatty acids. L_6 grown in LDMA (no biotin) supplemented with select fatty acids displays fatty acyl compositions reflecting the supplement and its metabolic derivatives. In cells grown in septadecanoate-supplemented media, for example, 17:0 and 17:1 occupies ~45% of the total. This enrichment results only in a small change in the fraction of unsaturated fatty acyl chains compared to that of the control (regular medium) or biotin-supplemented cultures. Both the nonadecanoate- and septadecanoate-derived enrichments occur primarily at the expense of both stearate and palmitate and their mono-unsaturated derivatives. The resulting ratio of 16-carbon fatty acids to 18-carbon fatty acids increases almost twofold.

Supplementation with unsaturated lipids produces an enrichment in the supplements and their metabolic derivatives in the cellular phospholipids and produces large increases in both the fraction of unsaturated fatty acyl chains and the fraction of 18-carbon fatty acids. In oleate-supplemented L_6 , up to 75% of its fatty acyl positions are occupied by oleate. There is no indication of appreciable further metabolism into longer or polyunsaturated fatty acid. In linoleate- and elaidate-supplemented cells, ~55% of the fatty acyl positions are occupied by linoleate and arachidonate, and 65–80% by elaidate, respectively. These enrichments are accompanied by a parallel decrease in oleate and produce a large decrease in the ratio of 16-carbon to 18-carbon fatty acids. The supplementation of LDMA containing linoleate or elaidate with biotin somewhat lowers the extent of exogenous fatty acid incorporation and shifts the ratio of 18:2/20:4 derived fatty acyl chains to favor 20:4. In the range of fatty acid concentrations used for these experiments, there is little, if any, dose dependence on the resultant incorporation of oleate and elaidate. Using NMR and IR, we have obtained evidence for incorporation of 12-azido stearate into phospholipids (data not shown). Attempts to enrich L_6 with linolenate were not always successful. In three attempts, two were successful and showed incorporation of 18:3 and its metabolism to 20:5; these data are presented in Table III.

TABLE III
Fatty Acyl Alterations in Myogenic Cells

Medium supplement	Fatty acyl composition (% by weight)					
	14:0	16:0	16:1	17:0	17:1	18:0
Regular Medium						
L ₆ (3)	1.8 ± 0.5	24.1 ± 2.3	10.1 ± 2.7	—	—	19.6 ± 2.6
PMB (2)	2.2 ± 0.7	34.9 ± 0.6	2.6 ± 1.1	—	—	31.4 ± 6.7
PMT (3)	1.2 ± 0.2	25.3 ± 5.0	4.2 ± 1.4	—	—	24.1 ± 3.6
PPT (2)	1.0 ± 0.0	21.4 ± 2.0	5.2 ± 0.8	—	—	25.9 ± 0.5
LDMA						
L ₆ (4)	7.6 ± 1.5	22.1 ± 2.3	21.9 ± 4.8	—	—	14.0 ± 3.1
PMB (2)	0.8 ± 0.2	19.6 ± 1.2	6.8 ± 0.2	—	—	20.5 ± 0.2
PMT (2)	1.3 ± 0.8	23.9 ± 7.2	10.4 ± 1.4	—	—	16.0 ± 0.3
PPT (2)	3.5 ± 0.1	28.4 ± 6.5	6.8 ± 6.1	—	—	24.9 ± 3.3
LDMA + biotin						
(1.5 μg/ml) L ₆ (5)	2.1 ± 0.2	22.6 ± 4.9	12.0 ± 2.3	—	—	17.3 ± 2.3
(1.25 μg/ml) } PMB (2)	0.7 ± 0.1	18.3 ± 1.2	6.5 ± 1.4	—	—	20.7 ± 3.7
(1.25 μg/ml) } PMT (2)	1.4 ± 0.4	29.0 ± 1.6	4.6 ± 0.2	—	—	20.8 ± 1.9
(1.25 μg/ml) } PPT (2)	1.1 ± 0.1	17.7 ± 1.7	12.7 ± 0.7	—	—	20.3 ± 2.1
LDMA + heptadecanoate						
(8.3 μg/ml) L ₆ (2)	2.4 ± 0.6	6.1 ± 1.3	5.2 ± 0.8	22.4 ± 2.7	49.5 ± 0.4	3.5 ± 0.3
(5.2 μg/ml) } PMB (2)	0.9 ± 0.5	7.9 ± 2.6	5.4 ± 1.5	23.2 ± 0.9	29.5 ± 2.9	6.4 ± 0.9
(5.2 μg/ml) } PMT (2)	1.1 ± 0.3	12.4 ± 0.5	5.9 ± 1.0	22.3 ± 0.7	29.5 ± 3.7	7.6 ± 0.2
(10.4 μg/ml) PPT (3)	1.5 ± 1.3	9.6 ± 1.2	6.0 ± 1.0	29.9 ± 10.6	23.3 ± 5.0	8.6 ± 2.4
LDMA + nonadecanoate						
(4.2 μg/ml) L ₆ (3)	3.3 ± 1.4	13.0 ± 7.3	6.9 ± 0.3	2.1 ± 0.8	4.3 ± 1.9	5.8 ± 3.7
(5.2 μg/ml) PMT (2)	1.2 ± 0.2	11.9 ± 1.1	5.6 ± 0.5	2.0 ± 0.1	2.9 ± 0.1	4.5 ± 0.5
LDMA + oleate						
(8.3 μg/ml) L ₆ (3)	3.3 ± 0.7	9.3 ± 1.1	5.3 ± 1.5	—	—	6.6 ± 1.6
(16.7 μg/ml) L ₆ (2)	2.1 ± 0.0	8.9 ± 2.2	4.6 ± 0.9	—	—	7.1 ± 0.8
(10.4 μg/ml) } PMB (2)	1.0 ± 0.1	17.7 ± 5.5	2.4 ± 0.9	—	—	11.6 ± 1.1
(10.4 μg/ml) } PMT (2)	1.1 ± 0.1	16.9 ± 1.2	2.0 ± 0.2	—	—	9.3 ± 1.1
(62.5 μg/ml) PPT (2)	1.2 ± 1.0	12.2 ± 9.1	2.0 ± 0.5	—	—	11.2 ± 5.9
LDMA + elaidate						
(8.3 μg/ml) L ₆ (3)	2.9 ± 2.3	5.7 ± 2.3	10.3 ± 4.4	—	—	2.2 ± 0.9
(16.7 μg/ml) L ₆ (2)	1.4 ± 0.7	3.8 ± 1.0	3.3 ± 2.0	—	—	2.1 ± 0.3
(10.4 μg/ml) } PMB (2)	—	4.0 ± 1.0	2.7 ± 1.5	—	—	2.8 ± 0.3
(10.4 μg/ml) } PMT (2)	0.4 ± 0.2	4.7 ± 2.8	7.5 ± 3.1	—	—	1.8 ± 0.5
(62.5 μg/ml) PPT (2)	—	4.1 ± 0.6	3.9 ± 0.8	—	—	3.1 ± 0.5
LDMA + biotin (1.5 μg/ml) + elaidate (8.3 μg/ml)						
L ₆ (2)	1.9 ± 0.1	12.5 ± 1.3	10.9 ± 2.9	—	—	7.3 ± 0.9
LDMA + linoleate						
(8.3 μg/ml) L ₆ (2)	4.1 ± 1.3	21.9 ± 2.1	1.5 ± 0.3	—	—	10.0 ± 0.2
LDMA + biotin (1.5 μg/ml) + linoleate (8.3 μg/ml)						
L ₆ (4)	3.2 ± 0.3	25.1 ± 4.3	3.3 ± 0.9	—	—	24.9 ± 1.1
LDMA + linolenate						
(8.3 μg/ml) L ₆ (2)	4.5 ± 1.3	19.3 ± 0.5	2.4 ± 0.0	—	—	15.5 ± 9.4

L₆ myoblasts were plated at a concentration of 1.8×10^6 cells/15 ml and harvested for analysis on day 4. Primary myoblasts (PMB), myotubes (PMT), and preformed tubes (PPT) were plated at a concentration of 4×10^6 cells/16 ml. The primary preformed myotubes were formed by growing the explanted cells in LDMA for 3 days, at which time the stated additions were made; the cells were harvested for analysis on day 5 (~57 h after the additions).

The fatty acyl analyses of L₆ cultures grown through several subcultures on LDM containing biotin or fatty acids are presented in Table IV. When compared to the control or cells grown in biotin-supplemented LDM, long-term cultures supplemented with oleate or stearate-oleate (1:1) show a continued, marked enrichment in oleate and in the fraction of unsaturated fatty acids. However, at early times, the enrichment is somewhat larger than at the later times. In septadecanoate-supplemented cultures, the extent of enrichment also does not increase with subsequent subculturing. In stearate-oleate-

(1:1) supplemented cells, there is little, if any, net enrichment in stearate.

Though not studied in comparable detail, primary explants of chick pectoral muscle grown under similar conditions show fatty acyl compositions analogous to those just described for L₆ (Table III). Three types of primary muscle culture were studied: myoblasts, prepared by growth in low calcium media; myotubes, prepared by growth in a medium containing normal calcium levels; and preformed myotubes, prepared by allowing myotubes to form before fatty acyl supplementation. The myoblasts grown in

TABLE III (CONTINUED)

Fatty acyl composition (% by weight)								% Un-saturated	16c/18c	Double bonds
18:1c	18:1t	18:2	18:3	19:0	19:1	20:4	20:5			
35.5 ± 0.9	—	2.2 ± 0.5	—	—	—	6.8 ± 1.4	—	54.6	0.60	77.2
20.2 ± 2.3	—	3.5 ± 0.3	—	—	—	5.3 ± 2.2	—	31.6	0.68	51.0
31.1 ± 7.1	—	3.0 ± 1.9	—	—	—	11.1 ± 2.8	—	49.4	0.51	85.7
30.6 ± 1.6	—	2.5 ± 0.1	—	—	—	13.3 ± 3.3	—	51.6	0.45	94.0
28.7 ± 1.5	—	—	—	—	—	5.6 ± 1.9	—	56.2	1.03	73
42.3 ± 1.0	—	2.4 ± 0.0	—	—	—	7.7 ± 0.1	—	59.2	0.40	84.7
40.0 ± 9.1	—	2.0 ± 0.3	—	—	—	6.0 ± 0.0	—	58.4	0.58	78.4
31.8 ± 0.5	—	1.3 ± 1.8	—	—	—	3.3 ± 2.4	—	43.2	0.58	54.4
42.3 ± 7.8	—	—	—	—	—	3.6 ± 2.7	—	58.1	0.58	68.9
43.4 ± 3.1	—	2.6 ± 0.1	—	—	—	7.6 ± 0.2	—	60.1	0.37	85.5
27.1 ± 3.5	—	4.5 ± 0.8	—	—	—	12.6 ± 0.5	—	48.8	0.64	91.1
41.7 ± 0.6	—	—	—	—	—	6.5 ± 1.0	—	60.9	0.49	80.4
9.6 ± 1.9	—	—	—	—	—	1.2 ± 1.8	—	65.5	0.86	69.1
14.5 ± 0.2	—	2.4 ± 0.2	—	—	—	9.9 ± 1.4	—	61.7	0.57	93.8
16.1 ± 0.8	—	—	—	—	—	5.1 ± 2.7	—	56.6	0.77	71.9
15.2 ± 8.7	—	—	—	—	—	13.4 ± 11.6	—	57.9	0.66	98.1
16.0 ± 3.9	—	—	—	21.5 ± 2.3	24.0 ± 8.0	2.9 ± 2.6	—	54.1	0.91	62.8
16.6 ± 0.8	—	—	—	18.3 ± 0.3	29.9 ± 2.0	7.1 ± 1.2	—	62.1	0.83	83.4
73.0 ± 1.9	—	—	—	—	—	2.5 ± 0.3	—	80.8	0.18	88.3
76.3 ± 1.1	—	—	—	—	—	1.1 ± 1.5	—	82.0	0.16	85.3
62.1 ± 6.6	—	—	—	—	—	5.1 ± 1.1	—	69.6	0.27	84.9
67.2 ± 0.3	—	—	—	—	—	3.5 ± 0.7	—	72.7	0.25	83.2
70.0 ± 8.1	—	2.8 ± 3.9	—	—	—	1.6 ± 2.3	—	76.4	0.17	84.0
13.4 ± 4.4	65.5 ± 13.8	—	—	—	—	—	—	89.2	0.20	89.2
7.4 ± 2.4	81.9 ± 3.8	—	—	—	—	—	—	92.6	0.08	92.6
11.2 ± 1.4	79.3 ± 1.6	—	—	—	—	—	—	93.2	0.07	93.2
13.5 ± 2.3	72.1 ± 9.0	—	—	—	—	—	—	93.1	0.14	93.1
11.3 ± 0.2	77.6 ± 0.0	—	—	—	—	—	—	92.8	0.09	92.8
24.6 ± 1.5	42.4 ± 1.4	0.4 ± 0.6	—	—	—	—	—	78.3	0.31	78.7
6.7 ± 0.1	—	43.8 ± 1.8	—	—	—	12.0 ± 1.5	—	64.0	0.39	143.8
9.2 ± 1.6	—	13.7 ± 7.4	—	—	—	20.7 ± 6.1	—	46.9	0.59	122.7
6.7 ± 2.5	—	—	17.7 ± 10.6	—	—	7.6 ± 3.7	26.1 ± 4.4	60.5	0.54	223.1

control media have a somewhat more saturated fatty acyl composition than does L₆ and the other primary cell types. This arises primarily from differences in the amounts of 16:0 and 18:1. In cells grown in oleate-supplemented media, oleate occupies between 62 and 70% of the fatty acyl positions. Supplementation with elaidate results in 72–77% of the acyl positions being occupied by elaidate. This enrichment is accompanied by a marked decrease in relative amount of oleate. In both oleate- and elaidate-enriched cells, the fraction of saturated fatty acids and the ratio of 16- to 18-carbon fatty acids decreases. In myotube cultures produced by fusion of elaidate-enriched myoblasts, a substantial fraction of my-

oblasts are present along with the myotubes (see Fusion section below).

Evidence for the incorporation of fatty acid supplements into a plasma membrane preparation of L₆ is presented in Table V.⁴ The plasma

⁴ Using the fractionation procedure of Schimmel et al. (40), we also find plasma membrane enrichments in elaidate- and oleate-supplemented chick myotube cultures comparable to those observed in analyses of the total cellular phospholipids. After 3 days in culture and starting at an initial cell density of 0.5×10^6 cells/ml, plasma membranes from control cultures (regular fetal calf serum) have $26.6 \pm 2.9\%$ (three determinations) of their fatty acyl positions occupied by oleate. In oleate- (10 $\mu\text{g/ml}$) supplemented cultures, this value increases

TABLE IV
Fatty Acyl Alterations in Long-Term Myoblast Cultures

Medium supplement	Fatty acyl composition (% by weight)									
LDMA + biotin (1.5 µg/ml)*										
Pass no.	14:0	16:0	16:1	18:0	18:1	% Unsat.	16c/18c	Double bonds		
1 (3)	2.1 ± 0.8	23.8 ± 4.9	7.6 ± 2.2	22.0 ± 7.4	44.4 ± 5.4	52.0	0.47	52.0		
2 (2)	2.2 ± 0.8	18.6 ± 0.1	7.3 ± 0.0	21.9 ± 0.9	49.2 ± 1.1	56.5	0.36	56.5		
3 (5)	2.4 ± 0.4	25.0 ± 4.1	8.3 ± 2.0	19.0 ± 4.9	45.0 ± 3.8	53.3	0.52	53.3		
4 (2)	2.1 ± 0.6	24.7 ± 4.7	7.9 ± 0.3	23.9 ± 1.0	41.3 ± 5.9	49.2	0.50	49.2		
5 (2)	3.1 ± 1.3	28.8 ± 9.5	6.9 ± 2.3	24.9 ± 5.6	35.6 ± 13.4	42.5	0.59	42.5		
LDMA + oleate (8.3 µg/ml)*										
Pass no.	14:0	16:0	16:1	18:0	18:1	18:2	20:4	% Unsat.	16c/18c	Double bonds
1 (2)	4.6 ± 1.8	13.1 ± 2.5	4.2 ± 1.0	6.5 ± 1.5	71.7 ± 4.0	—	—	75.9	0.22	75.9
2 (2)	3.9 ± 1.3	14.2 ± 1.4	5.2 ± 0.8	8.5 ± 1.6	65.7 ± 8.5	1.2 ± 0.3	2.2 ± 1.7	74.3	0.26	82.1
3 (2)	6.1 ± 1.2	20.6 ± 3.2	6.7 ± 0.9	10.1 ± 1.6	54.3 ± 1.5	2.9 ± 0.1	—	63.9	0.41	66.8
LDMA + stearate (4.2 µg/ml) + oleate (4.2 µg/ml)‡										
Pass no.	14:0	16:0	16:1	18:0	18:1	20:4	% Unsat.	16c/18c	Double bonds	
1 (3)	2.6 ± 0.5	5.8 ± 1.7	4.2 ± 0.1	24.7 ± 3.0	61.4 ± 3.1	2.0 ± 0.3	67.6	0.12	73.6	
3 (2)	4.4 ± 1.8	11.3 ± 6.4	5.3 ± 3.5	26.3 ± 17.8	52.5 ± 6.3	—	57.8	0.21	57.8	
LDMA + septadecanoate (4.2 µg/ml)‡										
Pass no.	14:0	16:0	16:1	17:0	17:1	18:0	18:1	% Unsat.	16c/18c	Double bonds
3 (2)	6.8 ± 1.7	26.0 ± 4.7	6.6 ± 0.1	14.5 ± 3.2	21.0 ± 4.7	12.6 ± 0.8	12.3 ± 3.9	39.9	1.31	39.9
LDMA + septadecanoate (8.3 µg/ml)‡										
Pass no.	14:0	16:0	16:1	17:0	17:1	18:0	18:1	% Unsat.	16c/18c	Double bonds
3 (2)	4.5 ± 2.1	17.0 ± 7.4	5.4 ± 0.6	20.7 ± 4.4	33.3 ± 15.2	8.4 ± 1.2	10.5 ± 0.6	49.2	1.19	49.2
5 (2)	4.2 ± 0.8	20.0 ± 0.9	5.5 ± 0.6	18.0 ± 10.5	33.0 ± 3.3	7.8 ± 0.6	11.2 ± 4.4	49.7	1.34	49.7
LDMA + linoleate (8.3 µg/ml) + biotin (1.5 µg/ml)*										
Pass no.	14:0	16:0	16:1	18:0	18:1	18:2	20:4	% Unsat.	16c/18c	Double bonds
1 (2)	3.0 ± 1.3	31.1 ± 10.9	2.9 ± 0.4	25.9 ± 0.4	13.3 ± 5.1	6.8 ± 6.8	16.9 ± 11.5	39.9	0.74	97.4
2 (2)	2.5 ± 0.1	29.5 ± 2.8	3.5 ± 0.7	23.3 ± 2.1	20.0 ± 8.0	5.3 ± 0.4	15.7 ± 3.4	44.5	0.68	96.9
3 (2)	3.1 ± 1.1	35.1 ± 14.7	3.7 ± 1.7	23.2 ± 1.4	20.1 ± 11.5	4.1 ± 0.4	10.6 ± 3.5	38.5	0.82	74.4

L₆ cultures were plated at a concentration of 6 × 10⁴ cells/5 ml and subcultured at a concentration of 6 × 10⁴ (*) or 1 × 10⁶ (‡) cells/5 ml every 4–5 days. At the time of subculturing an aliquot was removed for analysis.

membrane preparation was described previously for this cell type, and our morphologic observations (unpublished) are in accord (8). Supplementing the growth medium (LDMA) with oleate or elaidate produces clear enrichments of oleate and elaidate, respectively, in the plasma membrane lipids. The variability, however, is large. In contrast, linoleate-, linoleate-biotin-,

and elaidate-biotin-supplemented cultures are relatively less enriched.

The growth of L₆ under conditions reported to inhibit sterol synthesis results in a decrease in the level of cellular sterol. Analyses of the levels of cellular-free sterol presented in Table VI show a small diminution resulting from the shift from regular media to LDMA and an additional two-fold decrease when grown in LDMA containing 25-OH cholesterol. Comparable or somewhat larger changes are seen when the sterol level is normalized to phospholipid rather than protein. This decrease in sterol appears to be the minimum attainable with this protocol, since increasing the amounts of inhibitor or decreasing the density of the cells does not produce a further

to 74.4 ± 2.8% (three determinations), and in elaidate-(10 µg/ml) supplemented cultures it decreases to 5.8 ± 2.8% and is replaced by elaidate—84.4 ± 2.4% (two determinations). In a single experiment, we saw no significant effect of the presence of biotin on the extent of elaidate enrichment (W. T. Krussman and A. Horwitz, unpublished observations).

diminution. The sterol level of cells grown in LDMB containing 25-OH cholesterol and mevalonate or cholesterol is between that of inhibited and that of control (uninhibited) cells (Table VI). This lowered level is maintained through five passages. We point out that the levels of inhibitor used in these studies are only a small fraction (~1%) of the total cellular sterol. In general, L₆ when grown in LDM has variable amounts of desmosterol, the immediate precursor of cholesterol, and no detectable cholesterol esters contributing to the total sterol.

We have tried to replace cholesterol with sitosterol or desmosterol using this method and to increase the level of cholesterol by increasing the amount of exogenous cholesterol added. We see only slight incorporation of sitosterol at the concentration assayed. The desmosterol level of L₆ grown in LDMB supplemented with 25-OH cholesterol and desmosterol is the same as that of cells grown in LDMB alone. However, the cholesterol level is lower, approaching that of the inhibited cells. These sterol levels are still observed after five subculturings in the desmosterol-containing media. We do find that increasing the amount of external cholesterol does increase the level of both cholesterol and cholesterol esters; however, the possible uptake of "microcrystals" of cholesterol has not been excluded (data not shown).

Analogous changes are seen in myogenic cells derived from primary explants. These data are also presented in Table VI. In primary chick myoblasts, the initial sterol level (normalized to protein) is lower than that of L₆, and decreases ~25% to a value similar to that of L₆ grown in the presence of 25-OH cholesterol. The level of cellular sterol in primary myotubes is higher than that of L₆ or primary myoblasts. It decreases approx. twofold when 0.63 μg/ml 25-OH cholesterol is added to the growth medium (LDMB). Higher concentrations of 25-OH cholesterol are lethal.

The analyses of the membrane phosphatides presented in Table VII show that the ratio of PE/PC changes with alterations in the growth medium. When L₆ is grown in choline-free LDM, the ratio of PE/PC increases twofold from 0.70 to 1.44. The presence of low concentrations of ethanolamine produces little further change in this ratio, whereas at higher concentrations the ratio increases. This increase appears to parallel roughly the effect of ethanolamine on prolifera-

tion (or attachment to the substratum) (Fig. 4). The sum of PE and PC always accounts for ~80% of the total phospholipid; the remaining 20% is divided equally between sphingomyelin (Sph) and phosphatidylserine (PS) and phosphatidylinositol (PI). We are unable to detect appreciable amounts of phospholipid derived from either DME or monomethylethanolamine in these cells or large secondary changes in the other phospholipid classes except perhaps for Sph (data not shown). When L₆ is grown in LDM lacking choline and containing DME under the conditions described, the phospholipids derived from it occupy ~55% of the total membrane phospholipid. In contrast to these enrichments, the growth of L₆ (or primary myoblasts) in a 100-fold excess of choline produces no significant change in the PE/PC ratio compared to that of the control.

Supplementing the growth medium of primary cells with ethanolamine or DME produces enrichments comparable to those just described for L₆. In primary myotubes grown in regular medium a larger fraction of their phospholipids is derived from choline than in L₆ (60% in primaries vs. 44% in L₆). This accounts for the somewhat lower initial PE/PC ratio of 0.53 (compared to 0.70 in L₆); this relative increase in PE is accompanied by lower levels of PS and/or PI, with little change in Sph. The addition of ethanolamine to the growth medium produces myoblasts and myotubes (see section on Fusion) with an increased PE/PC ratio whose magnitude depends on the ethanolamine concentration. As discussed for L₆, the relative amounts of PS and/or PI and Sph do not appear to change appreciably (data not shown). Supplementing the growth medium with DME produces myotubes in which ~70% of the phospholipids are phosphatidyl dimethylethanolamine (PDME).

Secondary Lipid Alterations

We have analyzed the cellular membranes of L₆ for possible secondary alterations that accompany the primary alterations of fatty acyl chains, polar headgroups, and levels of sterol. An inspection of the data presented in Tables X-XII reveals that many secondary changes in the lipids do in fact occur; however, in general, they are not of a magnitude comparable to that of the primary alteration. For example, the PE/PC ratio in fatty acyl altered cells (Table VIII) is significantly lower than in control cultures, and in 25-OH cholesterol-grown cells this ratio is signifi-

TABLE V
Fatty Acyl Alterations in the Plasma Membrane of *L₆* Myoblasts

Medium supplement	Fatty acyl composition (% by weight)			
	14:0	16:0	16:1	18:0
Regular medium (4)	3.4 ± 2.4	41.8 ± 12.1	6.2 ± 1.0	22.9 ± 5.6
LDMA +:				
Biotin (1.5 μg/ml) (3)	4.0 ± 0.3	33.1 ± 10.5	5.7 ± 1.7	21.3 ± 7.8
Oleate (16.7 μg/ml) (2)	2.4 ± 1.4	14.0 ± 4.2	3.8 ± 0.8	7.8 ± 1.8
Elaidate (8.3 μg/ml) (2)	2.6 ± 2.2	19.0 ± 10.3	3.8 ± 2.0	13.1 ± 0.1
Biotin (1.5 μg/ml) + elaidate (8.3 μg/ml) (2)	5.0 ± 0.8	38.2 ± 3.8	1.6 ± 2.3	28.1 ± 5.8
Linoleate (8.3 μg/ml) (3)	4.1 ± 1.1	32.1 ± 2.0	5.1 ± 2.3	23.7 ± 5.7
Biotin (1.5 μg/ml) + lin- oleate (8.3 μg/ml) (2)	3.5 ± 1.3	24.6 ± 7.8	1.4 ± 0.4	23.3 ± 10.4

L₆ cultures were plated at a concentration of 1.8×10^6 cells/15 ml and harvested for analysis on day 4. Membranes were prepared by the method of Carlsen and Till (8).

cantly higher. The relative amounts of PC and Sph remain roughly constant, and hence the alterations reflect changes in the relative amounts of the other phospholipid classes, e.g. PS and PI. There is little, if any, difference in the phospholipid composition of oleate- and elaidate-enriched cultures.

A comparison of the fatty acyl composition of cells possessing altered sterol levels and PE/PC ratios reveals several small differences (Table IX). When the data are expressed as the fraction of unsaturated fatty acids, there are only slight variations. PE-enriched cells have a smaller fraction of unsaturated fatty acyl positions. This

TABLE VI
Sterol Alterations in Myogenic Cells

Medium supplement	Free sterol	Cholesterol	Desmosterol	Sitosterol	Cholesterol (mol)/ phospholipid (mol)
<i>μg/mg protein</i>					
<i>L₆</i> Myoblasts					
Regular medium	23.3 ± 1.7 (10)	23.3 ± 1.7 (10)	—	—	—
LDMB	20.8 ± 2.3 (8)	18.6 ± 2.5 (14)	*	—	0.25 ± 0.05 (3)
LDMB + 25-OH cholesterol:					
0.10 μg/ml	11.6 ± 1.4 (4)	11.6 ± 1.4 (4)	—	—	—
0.17 μg/ml	10.2 ± 1.1 (12)	10.2 ± 1.1 (12)	—	—	0.087 ± 0.025 (12)
0.23 μg/ml	9.1 ± 0.9 (3)	9.1 ± 0.9 (3)	—	—	—
LDMB + 25-OH cholesterol (0.17 μg/ml) +:					
Mevalonate (1 mg/ml)	17.5 ± 2.0 (8)	9.8 ± 2.5 (10)	8.4 ± 2.1 (10)	—	—
Cholesterol (4 μg/ml)	14.1 ± 2.0 (8)	13.8 ± 2.4 (8)	‡	—	—
Sitosterol (4 μg/ml)	11.9 ± 1.1 (2)	10.3 ± 0.4 (2)	0.2 ± 0.3 (2)	1.5 ± 1.2 (2)	—
Desmosterol (4 μg/ml)	11.4 ± 1.0 (2)	10.0 ± 0.7 (2)	1.5 ± 0.3 (2)	—	—
Primary myoblasts					
LDMB	14.9 ± 2.3 (3)	14.3 ± 1.8 (3)	<1.0 (3)	—	—
LDMB + 25-OH Cholesterol (0.63 μg/ ml)	11.0 ± 1.1 (4)	10.4 ± 1.5 (4)	<1.0 (4)	—	—
Primary preformed myotubes					
LDMB	35.6 ± 3.6 (6)	31.1 ± 4.0 (6)	4.1 ± 1.0 (6)	—	—
LDMB + 25-OH cholesterol (1.2 μg/ml)	18.8 ± 0.5 (7)	17.4 ± 0.9 (7)	1.7 ± 0.14 (7)	—	—

L₆ myoblasts were plated at a concentration of $0.7-1.0 \times 10^6$ cells/15 ml and harvested for analysis on day 3; the concentration of biotin in the LDM was 1.5 μg/ml. The explanted primary cells were plated at a concentration of 8×10^6 cells/16 ml. The myoblasts were harvested for analysis on day 3; the preformed myotubes were prepared by growing the explanted cells in LDMB for 3 days, at which time the 25-OH cholesterol was added; the cells were harvested for analysis 3 days after the addition (day 6).

* Variable amount of desmosterol between 4 and 30%.

‡ Variable amount of desmosterol between 0 and 16%.

TABLE V (CONTINUED)

Fatty acyl composition (% by weight)				% Unsat.	16c/18c	Double bonds
18:1c	18:1t	18:2	20:4			
16.0 ± 7.1	—	4.5 ± 3.7	3.2 ± 6.5	29.9	1.11	44.0
31.5 ± 3.2	—	3.6 ± 0.7	—	40.8	0.69	44.4
66.9 ± 6.2	—	2.4 ± 3.4	2.7 ± 3.8	75.8	0.23	86.3
9.4 ± 0.9	52.1 ± 15.5	—	—	65.3	0.31	65.3
10.7 ± 3.9	13.0 ± 4.3	—	—	25.3	0.77	25.3
19.6 ± 1.2	—	14.2 ± 3.0	1.1 ± 1.9	40.0	0.65	57.5
40.1 ± 20.1	—	7.1 ± 0.1	—	48.6	0.37	55.7

decrease which arises from the low levels of 18:1 found in PE-enriched cells is one of the larger changes. When data are expressed as the ratio of 16-carbon to 18-carbon fatty acids, the data vary greatly without an obvious trend.

The sterol levels of L_6 possessing altered fatty acyl and phospholipid composition are presented in Table X. As with the alterations just discussed, the fluctuations are relatively small. Cells enriched in cis-unsaturated fatty acids have the lowest sterol levels, whereas enrichment in ethanolamine or alteration of the fatty acids produces the higher values.

Fusion

The data presented in Table XI and Fig. 5 show the modulations of myoblast fusion into multinucleate myotubes that accompany altered lipid compositions. (The myoblasts derived from primary chick explants are better suited for studying fusion than is L_6 , which normally requires confluence before fusion commences. The fusion of primary myoblasts is largely independent of cell density; hence the majority of our reported studies focuses on this cell type.) A comparison of the extent of the fusion of myoblasts enriched in oleate to that of control cells (LDMA and LDMB) or to those enriched in 19:0 or elaidate (18:1_t) reveals differences. The oleate-enriched myoblasts show a slightly enhanced extent of fusion while the elaidate- and 19:0-enriched myoblasts show a marked decrease. A parallel increase in the fraction of interacting cells, i.e., those cells that are touching one another

but are neither obviously fused nor single cells (see Materials and Methods and Fig. 5) accompanies this decrease in fusion. These modulations of fusion are even more striking and are apparent at lower levels of supplementation when the myoblasts are grown in a low calcium medium for 52 h, and the rate of fusion is then measured after calcium addition (A. Wight and A. Horwitz, unpublished observations). The inhibition is reversible since the addition of regular medium (containing AraC) to elaidate-enriched cultures on day 2 enhances the extent of fusion. We have observed also that L_6 enriched in oleate fuses as does L_6 grown for several passages in LDMA containing biotin, linoleate-biotin, or stearate-oleate. In addition, the fusion of L_6 enriched in elaidate is inhibited (data not shown).

Alterations in the extent of fusion also accompany changes in the polar headgroup composition. As the ratio of PE/PC in primary cultures increases, the fraction of fused cells decreases. This decrease is accompanied by an increase in the fraction of interacting cells; however, the magnitude of the increase does not appear as large as that observed for elaidate- and 19:0-enriched cells. The addition of choline to the ethanolamine-containing growth medium at any time through day 2 reverses the inhibition. In contrast to the marked inhibition produced by PE enrichment, the inhibition produced by PDME is only slight in primary myoblasts. In L_6 , we have not observed fusion of cells grown in LDMB supplemented with either DME or ethanolamine.

TABLE VII
Phospholipid Alterations in Myogenic Cells

Medium supplement	PE (mol)/PC (mol)
L₆ Myoblasts	
Regular medium (4 μg/ml choline) (3)	0.70 ± 0.05
Regular medium + choline (40 μg/ml) (2)	0.73 ± 0.04
Regular medium + choline (400 μg/ml) (2)	0.68 ± 0.02
LDMB + choline (4 μg/ml) (14)	0.76 ± 0.07
LDMB (no choline) (7)	1.44 ± 0.20
LDMB + ethanolamine (6 μg/ml) (5)	1.72 ± 0.14
LDMB + ethanolamine (20 μg/ml) (5)	2.45 ± 0.6
Primary myotubes	
LDMB + choline (4 μg/ml) (2)	0.53 ± 0.01
LDMB + ethanolamine (3.75 μg/ml) (2)	1.11 ± 0.25
LDMB + ethanolamine (0.1 mg/ml) (2)	1.54 ± 0.13
L₆	
LDMB + DME (4 μg/ml) (2)	% PDME 56.7 ± 1.8
Primary myotubes	
LDMB + DME (3.75 μg/ml) (2)	71.4 ± 1.1

L₆ myoblasts were plated at a concentration of 5×10^5 cells/15 ml and harvested for analysis on day 4; the primary myogenic cells were plated at a concentration of 4×10^6 cells/16 ml and harvested on day 3. In this table, LDM refers to lipid-depleted medium without exogenous choline.

Primary myoblasts grown in LDMB containing 25-OH cholesterol show the most dramatic inhibition of fusion (Fig. 5 and Table XI). This inhibition is reversed by the addition of cholesterol at any time through day 2. L₆ cells, when grown under analogous conditions, also do not fuse. And in long-term or 4-day cultures of L₆ grown in LDMB containing both 25-OH cholesterol and mevalonate the cells do fuse, whereas in those grown in LDM containing 25-OH cholesterol and desmosterol the cells do not fuse. In contrast to the inhibitions that accompany the other lipid alterations, those by 25-OH cholesterol are not accompanied by an increase in the fraction of interacting cells.

DISCUSSION

Regulation of the Cellular Lipid Composition

The data presented show clearly that large and

somewhat selective perturbations in the fatty acyl, phospholipid headgroup, and sterol composition can be produced in cultured myogenic cells from a cell line, L₆, and primary explants from chick embryos. Using the primaries, we have produced alterations in two cell types, myoblasts and myotubes. In the myotubes, we have produced the alterations by fusion of altered myoblasts and by addition of medium supplements to preformed myotubes. We chose to explore lipid alterations with L₆ in greater detail since relatively large quantities of cells are more easily prepared. However, the extent of alterations in the different systems is comparable. These alterations also compare favorably with those described for other cell types (16, 28, 60).

The nature and extent of the secondary alterations that accompany the primary alterations are of some importance since they are relevant to possible regulatory mechanisms that maintain constant physical properties of the membrane lipids. In general, large primary alterations of any one lipid type are not accompanied by large changes of the other principal lipid types. Furthermore, the secondary changes do not obviously compensate for the primary change. For example, the enrichment of the fatty acyl chains with oleate is accompanied by a decrease in the PE/PC ratio and a decrease in cellular sterol; and enrichment in PE is accompanied by a decrease in the fraction of unsaturated fatty acids and little change in sterol. These changes, if they would have an effect, are opposite to those expected to preserve physical properties of the membrane,

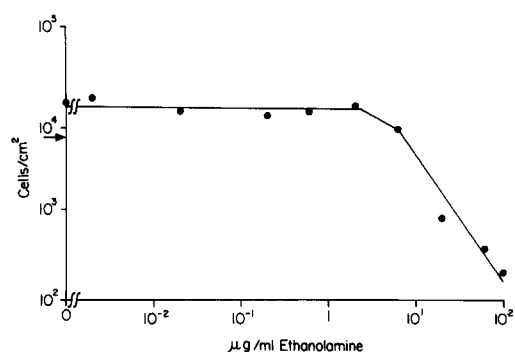


FIGURE 4 The effect of various concentrations of ethanolamine on the growth of L₆ myoblasts in choline-free LDM. The cells were plated at a concentration of 1.8×10^5 cells/5 ml (indicated by the arrow) and were counted on day 4.

TABLE VIII
Phospholipid Composition of L_6 Myoblasts Possessing Altered Levels of Sterols and Fatty Acyl Compositions

Medium supplement	Phospholipids				
	PE	PS + PI	PC	Sph	PE/PC ratio
	<i>relative %</i>				
Regular serum (3)	32.2 ± 3.0	11.4 ± 0.8	45.5 ± 1.2	10.7 ± 1.1	0.71
LDMA + biotin (1.5 µg/ml) (3)	29.6 ± 2.2	16.5 ± 2.4	44.1 ± 3.1	9.8 ± 1.9	0.67
LDMA + oleate (16.7 µg/ml) (2)	29.1 ± 0.5	12.2 ± 0.4	48.1 ± 1.6	10.4 ± 1.6	0.60
LDMA + elaidate (8.3 µg/ml) (2)	28.6 ± 1.1	16.5 ± 1.1	45.2 ± 2.0	9.6 ± 2.0	0.63
LDMA + biotin (1.5 µg/ml) + linoleate (8.3 µg/ml) (2)	30.2 ± 0.4	11.3 ± 2.2	46.8 ± 0.8	11.6 ± 1.0	0.64
LDMB + 25-OH cholesterol (0.17 µg/ml) (3)*	39.8 ± 1.8	7.5 ± 1.1	44.5 ± 2.4	8.3 ± 0.3	0.89

The L_6 cell cultures, except for those supplemented with 25-OH cholesterol, were plated at a concentration of 1.8×10^5 cells/15 ml and harvested for analysis on day 4.

* The cultures grown in LDMB containing 25-OH cholesterol were plated at a concentration of 7.0×10^5 cells/15 ml and harvested for analysis on day 3.

TABLE IX
Fatty Acyl Composition of L_6 Myoblasts Possessing Altered Levels of Cellular Sterol and Ratios of Phospholipid Classes

Medium supplement								% Un- sat.			Double bonds
	14:0	16:0	16:1	18:0	18:1	18:2	20:4	16c/	18c		
LDMB + 25-OH cholesterol*											
(0.1 µg/ml) (2)	2.4 ± 1.0	24.4 ± 6.6	11.8 ± 1.9	18.8 ± 0.3	38.5 ± 3.3	1.2 ± 1.7	2.9 ± 0.8	54.0	0.63	64.3	
(0.2 µg/ml) (2)	2.7 ± 0.8	26.6 ± 0.4	15.0 ± 1.0	18.4 ± 0.5	33.7 ± 0.6	—	3.6 ± 0.2	52.3	0.80	63.1	
LDMB + 25-OH cholesterol (0.2 µg/ml)*											
+ Mevalonate (1 mg/ml) (2)	3.0 ± 0.3	23.1 ± 0.4	16.7 ± 2.4	18.3 ± 2.9	34.1 ± 2.3	1.4 ± 2.0	3.3 ± 0.5	55.5	0.74	66.8	
+ Cholesterol (2 µg/ml) (2)	2.5 ± 0.8	23.8 ± 3.5	18.3 ± 2.1	16.6 ± 1.5	32.7 ± 4.0	2.6 ± 0.3	3.4 ± 1.1	57.0	0.81	69.8	
LDMB + choline (4 µg/ml) (2)‡	2.1 ± 0.3	21.3 ± 2.2	10.9 ± 3.5	21.0 ± 0.8	41.4 ± 0.8	2.8 ± 0.8	1.1 ± 1.5	56.2	0.51	62.3	
LDMB (no choline)‡	2.1 ± 1.0	22.7 ± 6.0	12.2 ± 1.0	27.3 ± 1.1	29.8 ± 1.2	3.3 ± 2.3	2.6 ± 3.6	47.9	0.61	59.0	
LDMB + ethanolamine (20 µg/ml) (2)‡	2.6 ± 1.1	24.1 ± 3.5	13.9 ± 2.2	23.9 ± 1.7	27.0 ± 0.8	6.0 ± 2.6	2.3 ± 3.3	49.2	0.67	62.1	

* For sterol alterations, L_6 myoblasts were plated at a concentration of 1.8×10^5 cells/15 ml in LDMB and harvested for analysis on day 4.

‡ For polar headgroup alterations, L_6 myoblasts were plated at a concentration of 5×10^5 cells/15 ml in LDMB containing no exogenous choline and were harvested for analysis on day 4.

i.e., bilayer order, phase equilibria, and self diffusion rates (27). One possible exception to this conclusion is the marked increase in the PE/PC ratio that accompanies lowered levels of sterol.

In this study, we have not explored possible secondary changes in the minor lipids and membrane protein. Such an analysis would require excessive amounts of material. It seems unlikely that minor lipids would contribute significantly to the bulk bilayer properties; however, they might be an important determinant of localized membrane properties. On the other hand, membrane proteins can contribute substantially to

some bulk membrane properties such as acyl chain ordering, and their omission poses a limitation on our conclusions (30, 51, 52).

In the context of the preceding discussion, it is interesting that L_6 cells, possessing altered lipids, remain viable and in many cases proliferate at appreciable rates and express the differentiated property of fusion. Stearate-oleate-supplemented cultures are enriched in oleate; the biotin-supplemented cultures contain no detectable amounts of 18:2 and 20:4, two essential fatty acids; and the linoleate-biotin-supplemented cultures are enriched in these essential fatty acids.

TABLE X
Sterol Levels of L₆ Myoblasts Possessing Altered Phospholipid and Fatty Acyl Compositions

Medium supplement	Free sterol	Desmosterol	Cholesterol
		<i>μg/mg protein</i>	
LDMA + biotin (1.5 μg/ml)*	20.4 ± 1.44 (5)	4.2 ± 2.6 (5)	16.1 ± 2.1 (5)
LDMA + oleate (16.7 μg/ml)*	19.1 ± 3.1 (4)	4.2 ± 1.4 (4)	15.0 ± 2.6 (4)
LDMA + elaidate (8.3 μg/ml)*	24.3 ± 1.8 (3)	3.9 ± 3.6 (3)	20.4 ± 4.7 (3)
LDMA + linoleate (8.3 μg/ml)*	17.5 ± 0.5 (4)	1.5 ± 0.7 (4)	16.0 ± 2.7 (4)
LDMA + linoleate (8.3 μg/ml) + biotin (1.5 μg/ml)*	18.7 ± 3.7 (4)	6.3 ± 1.5 (4)	12.4 ± 3.0 (4)
LDMB (no choline)‡	22.4 ± 1.9 (4)	1.7 ± 0.4 (4)	20.3 ± 2.0 (4)
LDMB (no choline) + ethanolamine (10 μg/ml)‡	23.1 ± 2.9 (4)	1.7 ± 0.5 (4)	21.4 ± 2.7 (4)
LDMB (no choline) + ethanolamine (20 μg/ml)‡	27.2 ± 1.5 (3)	5.9 ± 3.9 (3)	21.3 ± 5.4 (3)

* L₆ myoblasts grown in LDMA containing fatty acids or biotin were plated at a concentration of 1.8×10^5 cells/15 ml and harvested for analysis on days 3-4.

‡ For headgroup alterations, L₆ myoblasts were plated at a concentration of 5.0×10^5 cells/15 ml in LDMB containing no exogenous choline and were harvested for analysis on day 4.

These cells proliferate through several population doublings, if not indefinitely, and fuse to form myotubes. Similarly, mevalonate- or desmosterol-reversed 25-OH cholesterol-supplemented cultures have lowered levels of cholesterol and also proliferate at least through several population doublings, and the mevalonate-reversed cells fuse to form myotubes.⁵

Although L₆ appears to tolerate some variability, there appear to be limits. The cells in desmosterol-reversed 25-OH cholesterol-supplemented cultures, which possess lowered sterol levels and proliferate through several population doublings, do not fuse. Furthermore, the maximum decrease in sterol using the 25-OH cholesterol procedure is two- to threefold. The decrease is accompanied by a cessation of proliferation and an inhibition of fusion. This magnitude of decrease is seen in both proliferating L₆ and resting (myotube) cells and is comparable to that produced by another method (53). Attempts to further decrease the sterol level by using higher levels of inhibitors or lower densities of cells result in decreased viability. It is difficult to ascertain whether these apparent limits arise

from altered critical physical membrane properties since the amount of cholesterol varies among the subcellular membrane fractions and may be disposed asymmetrically in the membrane (7, 46). However, our estimates of the plasma membrane cholesterol content (based on the data of Kent et al. (33) in the altered cells is close to 20%, a value at which the physical properties of the bilayer may well change (J. Sturtevant, unpublished communication).

It is difficult to ascertain whether an upper bound also exists on the sterol level, since the uptake of microcrystalline cholesterol cannot be easily excluded. Others, however, have reported that the levels of cholesteryl ester increase as the cholesterol concentration in the medium increases. This conversion may be a mechanism to buffer the cholesterol level analogous to that of triglyceride formation for fatty acids (44, 45). Van der Bosch et al. (58) have reported that myoblast fusion is inhibited when myoblasts are grown under conditions thought to increase cellular sterol levels. It is also possible that this apparent upper limit arises from altered, critical, physical properties of the membrane.

It is interesting that cells grown in DMEM containing either regular or lipid-depleted serum have comparable cholesterol levels. In one case, the sterol is derived exogenously from a large extracellular pool, and in the other via endogenous pathways. This not only reflects an upper limit or optimal amount of cholesterol in the cell, but it also suggests that without perturbation the cholesterol levels are regulated.

⁵ In L₆ cultures supplemented with mevalonate and 25-OH cholesterol, the cholesterol levels can become equal to or lower than those of the inhibited (25-OH cholesterol supplemented) cultures. However, the total sterol content (cholesterol and desmosterol) is well above that of the inhibited cells. The implication is that desmosterol, like cholesterol, can function as a structural component of the membrane.

The variability in the polar headgroup composition also appears limited and regulated. Whereas PDME can partially replace PC and support proliferation and myoblast fusion, L₆ grown on DME does not survive repeated subculturing and PDME does not totally replace PC (or PE). Furthermore, increasing the amount of ethanolamine produces increasing amounts of PE; however, increments of PE exceeding ~2× that of the control are accompanied by decreased fusion and viability and/or attachment to the substratum. In contrast, the ratio of PE/PC, the two major phospholipid classes, is changed little, if any, by growing L₆ or primaries in media containing regular serum, regular serum containing 100× the normal amount of choline, or LDM. In the first two, choline is available both in the free form and as lecithin while in the latter only as free choline.

The fatty acyl composition appears more tolerant of variation and less precisely regulated; however, we have not yet achieved total replacement by a single fatty acid. The limit for oleate enrichment appears to be ~65–70% of the fatty acyl chains, and that for elaidate slightly higher. Our attempts to enrich further in oleate or septadecanoate by subculturing were not successful.⁶ Whether these apparent limits arise biosynthetically or metabolically or from altered physical properties of the membrane remains to be determined. In elaidate-enriched cells, we have presented correlative evidence consistent with the latter explanation (23, 24). Since the fraction of unsaturated fatty acid in control and saturated fatty acid-supplemented cells is somewhat constant, this parameter appears regulated.

The data on fatty acyl alterations show clear evidence for metabolism of the fatty acid supplements. In nonadecanoate-supplemented cells, appreciable amounts of 19:0, 19:1, and traces of 17:0 and 17:1 are present, providing evidence for elongation and desaturation. In addition, L₆ also elongates 18:2 to form 20:4 since a dramatic increase in 20:4 accompanies enrichment in 18:2. These results contrast with those with L

cells which neither desaturate 19:0 nor elongate 18:2 (14, 60).

Since detailed compositional studies on subcellular membrane fractions are not reported here, we consider our discussion only suggestive. With one exception, the analyses presented are an average from all cellular membranes and may not accurately reflect the composition of any particular membrane fraction. A detailed analysis of membrane fractions is more appropriately pursued using a cell type from which a large number of cells are readily propagated and pure membranes prepared (49). This latter requirement is complicated by the difficulties in preparing pure and intact membranes from cultured cells (13, 48). Studies of analogous modifications in L cells show enrichments in the plasma membrane that parallel those detected in the whole cell analyses, and it seems reasonable to expect a similar parallel in these myogenic cells as well (15, 32, 49).

This expectation is supported by our observations of modulations in fusion that accompany the lipid alterations and by our limited analyses of plasma membrane fractions from fatty acyl altered cells. With respect to the latter, the high level of 16:0 and fraction saturated fatty acid in the control cultures and the relatively low enrichment in the linoleate and especially in the linoleate-biotin and elaidate-biotin supplemented cultures is unexpected. It remains to be established whether these surprising observations are somehow artifactual, by using other membrane preparations, cell types, growth conditions, and analytical procedures. If not, they provide a possible explanation for the effect of biotin in promoting the proliferation of elaidate- and linoleate-supplemented cells. In addition, since the fatty acyl replacement is more complete in whole cell lipids than plasma membrane lipids when cultures are supplemented with both fatty acids and biotin, they also imply that the assembly route for lipids of one plasma membrane may rely on *de novo* synthesis to a greater extent than internal cellular membranes.

Modulations of Fusion by Altered Lipid Compositions

The marked modulations of myoblast fusion that accompany the lipid alterations not only suggest that the plasma membrane lipids are

⁶ Our observation that oleate- and septadecanoate-supplemented cultures exhibit some continued proliferation without a parallel increase in the extent of supplementation can be explained by the utilization of the residual lipid present in the extracted serum (see Materials and Methods).

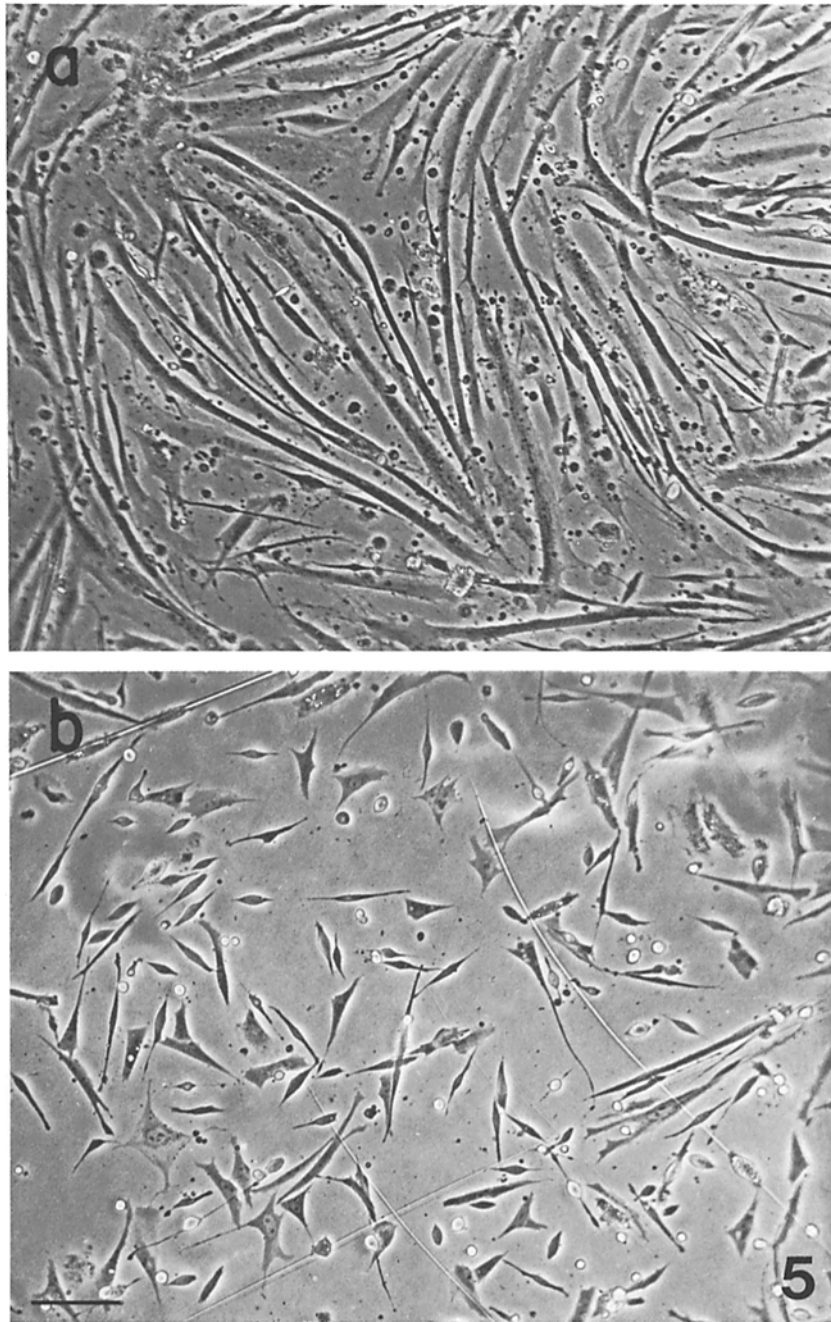
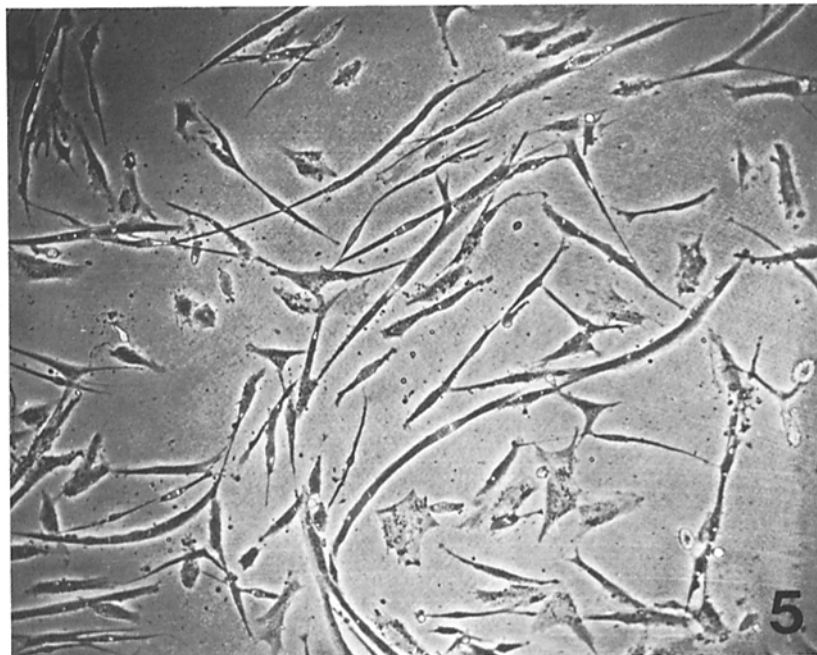
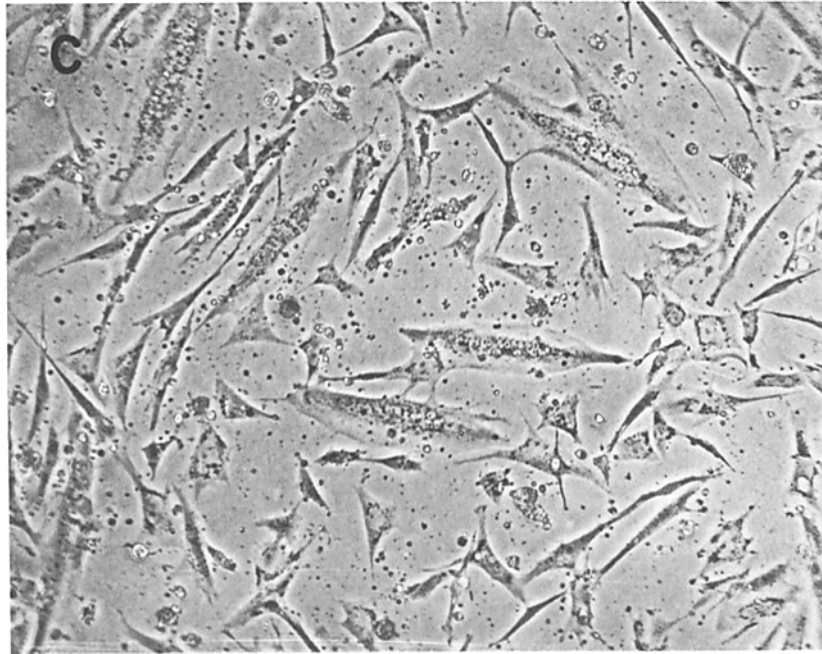


FIGURE 5 The fusion and morphology of primary cultures possessing altered lipid compositions. The cultures were plated at 0.25×10^6 cells/ml (*a*, *c*, and *d*) or 0.5×10^6 cells/ml (*b*) and were cultured in the indicated medium. The phase-contrast pictures were taken of living cultures on day 3. (*a*) LDMA containing biotin ($1.25 \mu\text{g/ml}$). AraC was added on day 2. (*b*) LDMB containing 25-OH cholesterol ($0.63 \mu\text{g/ml}$). AraC was added on day 1. (*c*) LDMA containing oleate ($21 \mu\text{g/ml}$). AraC was added on day 2. (*d*) LDMA containing elaidate ($21 \mu\text{g/ml}$). AraC was added on day 2. Bar, $100 \mu\text{m}$.



altered, but, more important, provide insight into the mechanism of myoblast fusion. The nature of the influence of the lipid alterations on fusion is likely complex. We consider possible toxic effects of the lipid alterations or the lipid-altering procedures an unlikely mechanism for

several reasons: (a) The different lipid alterations influence fusion differently. For example, myoblast fusion is enhanced in oleate-enriched cells and inhibited in cells enriched in elaidate or grown in LDM containing 25-OH cholesterol. In the elaidate-enriched cells, an increase in inter-

TABLE XI
Fusion of Primary Chick Myoblasts

Medium supplement	Fusion	Interacting cells	No. nuclei/tube	No. nuclei/field
	%	%		
LDMA + biotin*				
(1.25 $\mu\text{g/ml}$) (2)	65 \pm 7	2.2 \pm 0.4	12 \pm 4	67 \pm 2
LDMA + oleate*				
(10.4 $\mu\text{g/ml}$) (2)	80 \pm 2	1.9 \pm 1.2	18.6 \pm 0.6	83 \pm 18
(20.8 $\mu\text{g/ml}$) (2)	75 \pm 5	1.1 \pm 1.6	18.8 \pm 3.0	71 \pm 22
LDMA + elaidate*				
(10.4 $\mu\text{g/ml}$) (2)	64 \pm 8	11 \pm 1	9.2 \pm 2.1	76 \pm 6
(20.8 $\mu\text{g/ml}$) (2)	46 \pm 12	19 \pm 5	5.9 \pm 2.2	63 \pm 10
(31.3 $\mu\text{g/ml}$) (2)	23 \pm 2	28 \pm 6	3.5 \pm 0.1	51 \pm 7
LDMA + nonadecanoate*				
(2.6 $\mu\text{g/ml}$) (3)	42 \pm 15	32 \pm 9	5.1 \pm 1.2	61 \pm 7
(5.2 $\mu\text{g/ml}$) (3)	30 \pm 5	37 \pm 4	4.2 \pm 0.7	11 \pm 10
LDMB + 25-OH cholesterol (0.63 $\mu\text{g/ml}$) (2)‡	17 \pm 9	2.0 \pm 1.4	4.9 \pm 1.4	64 \pm 24
(2)‡				
+ Cholesterol (5 $\mu\text{g/ml}$) (day 0) (3)	62 \pm 11	9.0 \pm 8.5	13 \pm 6	108 \pm 38
+ Cholesterol (5 $\mu\text{g/ml}$) (day 2) (2)	43 \pm 3	9.5 \pm 8	7.9 \pm 2.2	55 \pm 31
+ Regular medium (day 2) (2)	51 \pm 5	6.3 \pm 2.5	9.2 \pm 0.7	73 \pm 23
LDMB + ethanolamine (0.1 mg/ml) (2)‡	18 \pm 8	14 \pm 13	4.9 \pm 2.4	37 \pm 6
+ Choline (4 $\mu\text{g/ml}$) (day 0) (2)	75 \pm 6	1.5 \pm 0.7	13 \pm 7	78 \pm 32
+ Choline (4 $\mu\text{g/ml}$) (day 2) (2)	57 \pm 4	7 \pm 1	9.5 \pm 0.2	41 \pm 8
+ Regular medium (day 2) (2)	65 \pm 5	7 \pm 7	11 \pm 3	57 \pm 3
LDMB + DME‡				
(3.75 $\mu\text{g/ml}$) (2)	58 \pm 4	6 \pm 3	9.4 \pm 1.3	65 \pm 8

For studies involving fatty acyl and polar headgroup alterations, the cells were plated at a concentration of 5×10^6 cells/2 ml and for sterol alterations at a concentration of 1×10^6 cells/2 ml. In some experiments, a further addition was made on either day 0 or day 2; this was done by simply adding the agent to the medium in the culture plate, except when regular medium was added, in which case the old medium was first removed.

* LDMA contained 2 $\mu\text{g/ml}$ avidin.

‡ LDMA contained 1.25 $\mu\text{g/ml}$ avidin.

acting cells accompanies the inhibition of fusion; this does not occur in cells with lowered levels of sterol. The presence of interacting cells in the elaidate- (and possibly in the PE-) enriched cells indicates that migratory and adhesive phenomena have occurred.⁷ (b) Although decreases in viability accompany some of the alterations, in general, the extent of fusion does not correlate directly with the remaining number of cells. (c)

⁷ The morphologies seen in Fig. 5 show clear differences between oleate- and elaidate-enriched and control cultures. The myotubes that form in oleate-enriched cultures have a saclike morphology very similar to that of the myosacs formed by colecmid treatment of myotubes (3). Elaidate enrichment, on the other hand, results in relatively thin myotubes. This effect could be direct via a coupling between the lipids and the cytoskeleton and/or a differential adhesion to the substratum, or it could be indirect through any one of a number of mechanisms.

The reversibility of the inhibitions even in the absence of cell division shows that the cells remain viable. The addition of choline or cholesterol to PE-enriched cells or to those with lowered sterol levels, respectively, reverses the inhibition. In sum, a more likely explanation for the influence of the lipid alterations is via changes in the membrane lipids directly influencing the fusion process per se.

As discussed above, the fatty acyl enrichments appear to influence fusion differently than does the lowering of sterol levels by inhibiting sterol synthesis. This implies different mechanisms of inhibition. We have recently provided evidence supporting a model in which fusion proceeds via a sequence of events (35, 36). In the context of this model, the differential influence of the lipid alterations on fusion would arise by inhibiting different stages of the process. Thus, the fatty acyl and PE enrichments would inhibit after

myoblast recognition but before membrane union. The lowered sterol levels would inhibit the recognition process or some event before it; the PE enrichments may have a secondary influence at this stage as well. This interpretation is supported by our recent studies of fusion in suspension. These studies as well as a more complete discussion and presentation of data concerning the role of lipids in fusion will be presented elsewhere.

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