Apparent Coordination of the Biosynthesis of Lipids in Cultured Cells: Its Relationship to the Regulation of the Membrane Sterol:Phospholipid Ratio and Cell Cycling

ROSEMARY B. CORNELL and ALAN F. HORWITZ
Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT The coordination of the syntheses of the several cellular lipid classes with one another and with cell cycle control were investigated in proliferating L_6 myoblasts and fibroblasts (WI-38 and CEF). Cells cultured in lipid-depleted medium containing one of two inhibitors of hydroxymethylglutaryl-CoA reductase, 25-hydroxycholesterol or compactin, display a rapid, dose-dependent inhibition of cholesterol synthesis. Inhibition of the syntheses of each of the other lipid classes is first apparent after the rate of sterol synthesis is depressed severalfold. 24 h after the addition of the inhibitor, the syntheses of DNA, RNA, and protein also decline. The inhibition of sterol synthesis leads to a threefold reduction in the sterol: phospholipid ratio that parallels the development of proliferative and G_1 cell cycle arrests and alterations in cellular morphology. All of these responses are reversed upon reinitiation of cholesterol synthesis or addition of exogenous cholesterol.

A comparison of the timing of these responses with respect to the development of the G_1 arrest indicates that the primary factor limiting cell cycling is the availability of cholesterol provided either from an exogenous source or by *de novo* synthesis. The G_1 arrest appears to be responsible for the general inhibition of macromolecular synthesis in proliferating cells treated with 25-hydroxycholesterol. In contrast, the apparent coordinated inhibition of lipid synthesis is not a consequence of the G_1 arrest but may in fact give rise to it.

Sequential inhibition of lipid syntheses is also observed in cycling cells when the synthesis of choline-containing lipids is blocked by choline deprivation and is observed in association with G_1 arrests caused by confluence or differentiation. In the nonproliferating cells, the syntheses of lipid and protein do not appear coupled.

The study of the mechanism and control of membrane biogenesis is still in its infancy. One of the many unexplored areas is the coordination between the synthesis of membranes and the synthesis of DNA and cell cycling. We have begun to study the coupling between membrane lipid and macromolecular syntheses by culturing cells in lipid-depleted medium (LDM)[1] containing inhibitors of the synthesis of selective lipid pathways. In this way the cells are deprived of both the exogenous and endogenous source of a particular lipid.

By this approach evidence for coordinated control of membrane and DNA syntheses has been found (7). The lipid

[1] *Abbreviations used in this paper:* LDM, lipid-depleted medium; 25-OH, 25-hydroxycholesterol; CMF-PBS, calcium, magnesium-free, phosphate-buffered saline; Versene, 0.02% disodium EDTA in CMF-PBS; DMEM, Dulbecco's minimal essential medium; GLC, gas-liquid chromatography.

THE JOURNAL OF CELL BIOLOGY · VOLUME 86 SEPTEMBER 1980 810–819

requirement for cell cycling was investigated with WI-38 fibroblasts and L_6 myoblasts. When the synthesis of cholesterol, choline-containing lipids, or fatty acids was inhibited in the absence of exogenous lipid, these cells were arrested in the G_1 phase of the cell cycle. Cell cycling could be restimulated by restoring synthesis or by adding the limiting lipid.

The generation and maintenance of distinctive membrane lipid compositions is another fundamental area of membrane biogenesis that remains to be explored. In proliferating cells the maintenance of a preferred phospholipid class distribution and cholesterol:phospholipid ratio over many generations, regardless of the source of lipid (1, 11), indicates that the production of the various lipids and/or their assembly into membrane is well coordinated.

In this paper, we focus primarily on the metabolic consequences of inhibiting the synthesis of cholesterol with 25-hydroxycholesterol (25-OH) and compactin, two inhibitors of hydroxymethylglutaryl-CoA reductase (6, 14). Three objectives are addressed: (*a*) the primary change that is relayed to the cell cycling control point, (*b*) the coordination of lipid, RNA, and protein synthesis, and (*c*) the coordination of the synthesis of the various lipid classes.

MATERIALS AND METHODS

Cell Culture and Lipid Synthesis Blocks

L_6 myoblasts, a cell line derived from neonatal rat skeletal muscle, obtained from Dr. D. Schubert (Salk Institute), and WI-38 human fibroblasts from embryonic lung, obtained from Dr. V. Cristofalo (Wistar Institute), were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum in a 10% CO_2, humidified incubator. Subconfluent L_6 were passaged every 3 d at a split ratio of 1:15. New vials of L_6 were thawed approximately every 6 mo. WI-38 were passaged every 7 d at a split ratio of 1:8; SV-40-transformed WI-38 were split 1:3 every 3 d. Primary myogenic cultures were prepared by the method of Bishoff and Holtzer (4) with minor modifications (11). Primary cultures of chick embryo fibroblasts were prepared as described by Rein and Rubin (23).

Lipid-depleted fetal calf serum and LDM containing biotin were prepared as described previously (11). Dehydrated lipid-depleted serum protein was reconstituted at 46 mg protein per milliliter. In experiments with cell lines, cultures were prepassaged for 3–4 d in LDM (containing 100 U/ml each of penicillin and streptomycin.) This resulted in an eightfold activation of total endogenous lipid synthesis. For L_6 the seeding density was $5.2–5.8 \times 10^3$ cells/cm^2; this was the highest density that would permit logarithmic growth for 4 d. 25-OH or compactin was added 12 h after seeding. To remove the inhibitors from the cultures, the medium was aspirated, the cells were washed once with DMEM, and conditioned LDM lacking inhibitor was transferred from cultures set up in parallel.

Radioactive Labeling and Harvesting

The rates of lipid, protein, DNA, and RNA syntheses were measured at each time point by adding the appropriate radioisotope to cell cultures in a total of 3.0 ml (60-mm plate) or 8.0 ml (100-mm plate) of growth medium. At the end of the labeling period, the medium was removed, the plates were washed three times with ice-cold calcium, magnesium-free, phosphate-buffered saline (CMF-PBS), and the cells were removed from the dish either with warm trypsin (0.05% in 0.2% disodium EDTA in CMF-PBS [Versene]) or with Versene. The cells were then washed with ice-cold CMF-PBS and pelleted (1,500 rpm, 4 min) in 12-ml conical centrifuge tubes. The pelleted cells were resuspended, counted with a hemacytometer, washed, and centrifuged again. Samples were stored under argon at −20°C.

Extraction and Separation of Lipids

Lipids were extracted by the method of Bligh and Dyer (5). The chloroform layer (98% recovery) was evaporated under argon or nitrogen and redissolved in 1.0 ml of toluene. Duplicate 0.1-ml aliquots were transferred to scintillation vials and the radioactivity incorporated into total lipid was counted. The remaining lipid was fractionated by TLC either on 20-cm Adsorbosil 5 Precoats (Applied Science Labs., Inc., State College, Penn.) using a solvent system consisting of petroleum ether:diethyl ether:acetic acid, 75:25:1, or on 25-cm plates spread with silica gel G (Applied Science) using the solvent systems of Freeman and West (8). The latter systems separate diglyceride from sterol; however, free fatty acids frequently migrate with sterol. In the former system, the separation of sterol and diglyceride was improved by chromatographing twice and letting the solvent run nearly to the top of the plate. Phospholipid classes were separated with chloroform:methanol:H_2O:acetic acid, 75:45:8:2 (25). The lipids were visualized by I_2 vapors. Standard Mixes 1, 3, and 8 (Applied Science Labs., Inc.) were used to identify the spots, which were then scraped and counted in Quantifluor (Scientific Products, McGraw Park, Ill.).

Determination of Radioactivity in DNA, RNA, and Protein

Protein, DNA, and RNA were precipitated with TCA by one of two methods. (*a*) The cells were harvested and stored at −20°C. Pellets were thawed and resuspended in 0.4–0.5 ml of H_2O or CMF-PBS, and an aliquot was removed for protein determination. An equal volume of cold 10% TCA was added (total vol ~1 ml), and the samples were precipitated in the cold for ≥2 h. The precipitate was collected on 0.45-μm Millipore filters with a Millipore filtering manifold (Millipore Corp., Bedford, Mass.). Filters were washed first with cold 5% TCA, then with ethanol; they were dried and counted in Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.). (*b*) Alternatively, TCA precipitation was carried out directly on the culture plates with 2 ml of cold 5% TCA for a 5-cm plate. After 30–60 min, the plates were washed with cold 5% TCA followed by ethanol; they were then dissolved in 1 ml of 0.2% SDS in 0.1 N NaOH. Aliquots were removed for counting in Scintiverse.

Other Analyses and Preparations

Cholesterol was measured by gas-liquid chromatography, (GLC) (11) and phospholipid phosphorus by the method of Bartlett (3) as described previously. Protein was measured by the procedure of Lowry et al. (19).

A crude membrane fraction of chick myotubes (containing nuclear, microsomal, mitochondrial, and plasma membranes) was obtained using the protocol of Kent et al. (17).

Liposomes were prepared by sonicating 10 μmol each of egg lecithin (purified by chromatography [24]) and cholesterol in 1 ml of Hanks' balanced salt solution (BSS) for 1 h on ice with a Branson sonicator (Branson Sonic Power Co., Danbury, Conn.) at a power setting of 3. The large multilamellar vesicles and titanium debris were sedimented at 5,000 rpm in the Sorvall SS-34 rotor (DuPont Instruments-Sorvall, DuPont Co., Newton, Conn.) for 10 min. The liposomes in the supernate were sterilized with a Millipore filter (0.2 μm), flushed with argon, and used within 2 d.

Low density lipoprotein (LDL), density 1.006–1.063, was isolated by standard flotation procedures (9, 20). It was dialyzed against saline for 2 d, sterilized by Millipore filtration, and aliquots were taken for determination of protein and sterol content. 75% of the sterol present was esterified, and the protein:cholesterol ratio was one.

Chemicals

25-hydroxycholesterol, coprostanol, and desmosterol were obtained from Steraloids, Inc., Wilton, N. H; dolichol and dolichol phosphate from Calbiochem-Behring Corp., San Diego, Calif.; biotin, cholesterol, and mevalonolactone from Sigma Chemical Co., St. Louis, Mo.; [2-^{14}C]acetate (40–60 mCi/mmol), L-[3,4,5,-^{3}H]leucine (>100 Ci/mmol), [5,6-^{3}H]uridine (40 Ci/mmol), [*methyl*-^{3}H]thymidine (2 Ci/mmol), D-[^{14}C(U)]glucose (>300 mCi/mmol), [2-^{3}H]glycerol (200 mCi/mmol), [*methyl*-^{3}H]choline (60–90 Ci/mmol), D,L-[2-^{14}C]mevalonolactone (46 mCi/mmol), ^3H_2O (1 mCi/g) [1-^{14}C]fucose (40–55 mCi/mmol), and D-[U-^{14}C]glucosamine (355 mCi/mmol) were purchased from New England Nuclear, Boston, Mass.

RESULTS

Effects of Inhibitors of Cholesterol Synthesis on Proliferation

The growth of L_6 cultured under a variety of conditions is presented in Fig. 1. Cells treated with 0.16 μg/ml 25-OH double approximately twice before the culture density plateaus. Appreciable cell death becomes apparent only after 3.5 d exposure to this concentration of inhibitor, as indicated by the decline in cell density and trypan blue inclusion. The inhibitor concen-

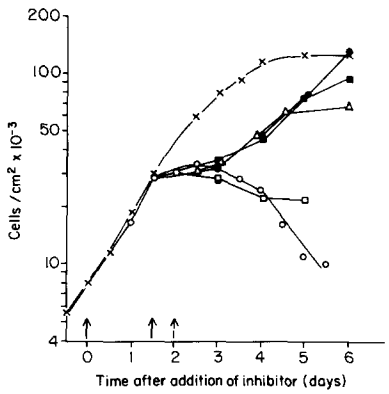

FIGURE 1 L_6 growth in response to inhibitors of sterol synthesis and subsequent reversals of inhibition. L_6 cells were seeded at 5.8 × 10^3 cells/cm^2 on 60-mm plates, in LDM (×) and in LDM containing 0.15 μg/ml 25-OH (○) or 0.66 μg/ml compactin (□). The inhibitors were added 12 h after seeding. The solid arrow at 36 h indicates the time at which the 25-OH containing medium was replaced with conditioned LDM (●) or at which 2 mg/ml mevalonate (△) was added to 25-OH containing cultures. The broken arrow at 48 h indicates the time at which 47 μg/ml LDL-cholesterol (■) was added to cultures containing compactin. The growth curves for the LDM control and for LDM + 25-OH are the averages of three and six experiments, respectively. The other growth curves are from one representative experiment each.

tration and seeding density chosen for experimentation were those that gave log phase growth of controls for 4 d and maximum inhibition of sterol synthesis without appreciably affecting cell viability for the first 2 d.

Proliferation resumes ~36 h after addition of mevalonic acid, the product of the inhibited step, or replacement of the medium with conditioned medium lacking 25-OH. The latter method results in a growth rate and a saturation density comparable to those of the control. Although the restimulation of growth with mevalonate under these conditions is not complete, the culture density clearly doubles.

The synthesis of dolichol and ubiquinone share a common pathway with cholesterol before farnesyl phosphate. Thus, the 25-OH-induced inhibition of growth might be caused in part by the failure to make dolichol or ubiquinone. However, the growth arrest cannot be reversed with exogenous dolichol, dolichol phosphate, or ubiquinone, and when cholesterol and dolichol are added together growth is not stimulated more than when cholesterol is added alone.

Compactin, a nonsteroid inhibitor of HMG-CoA reductase, inhibits cell proliferation with minimal toxicity, as shown in Fig. 1. The growth curve indicates that there is very little cell death up to day 5 at compactin concentrations between 0.33 and 0.66 μg/ml, whereas proliferation ceases after ~2.5 doublings. Exogenous cholesterol consistently reverses this growth inhibition when supplied as LDL. When supplied as an ethanol solution at low doses (2–4 μg/ml), the ability of cholesterol to restimulate growth varies among experiments. This is likely caused by the nonphysiological manner of presentation.

Effects of 25-Hydroxycholesterol and Compactin on Lipid Synthesis

The major lipid classes of L_6 synthesized from acetyl CoA are phospholipid, sterol, triglyceride, free fatty acid, monoglycerides and diglycerides, and cholesteryl esters. The fraction of total [^{14}C]acetate label incorporated into the lipids of log phase cells growing in LDM after a 2-h pulse is: phospholipid, 0.50; sterol, 0.20; triglyceride, 0.20; diglyceride, 0.07; free fatty acid, 0.02; and others, <0.01.

The incorporation rate of [^{14}C]acetate into these lipid classes responds to 25-OH addition in the following sequence (Fig. 2, left panel). Uptake into sterol declines to <10% of control within 12 h and is eventually completely inhibited. The synthesis of all other lipid classes analyzed continues at control levels for 6 h. Thereafter, a concerted inhibition in their rates of synthesis appears. The rate of phospholipid synthesis is reduced 75% over the course of 2 d. There is a three- to fivefold reduction in synthesis rates for each of the five phospholipid species. Synthesis of free fatty acids and cholesteryl esters is also inhibited at least 50%. The rate of synthesis of triglyceride steadily increases in LDM; 25-OH prevents this twofold increase. Similarily, incubation of L_6 with compactin, at a concentration that depresses sterol synthesis 94%, also results in a decreased rate of incorporation of [^{3}H]choline, [^{3}H]glycerol, and [^{14}C]acetate into phospholipid (50–60% inhibition). Triglyceride synthesis is not depressed, however.

The lipid synthesis response to increasing concentrations of 25-OH is shown in Fig. 3. The rate and magnitude of the

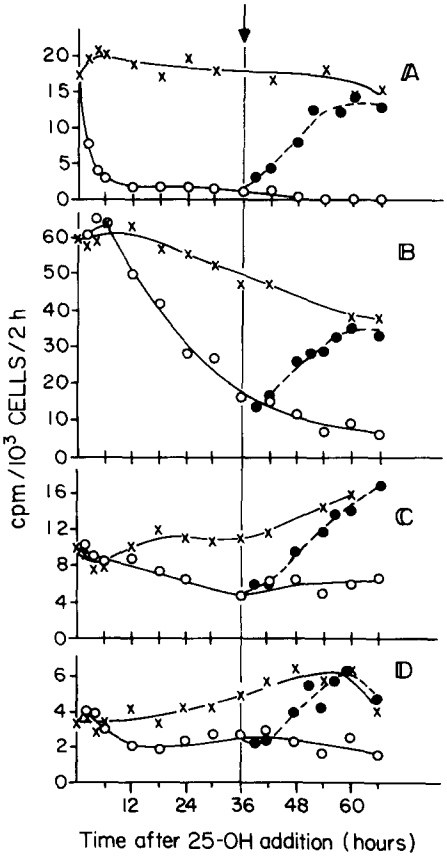

FIGURE 2 Effect of 25-OH on [^{14}C]acetate incorporation into L_6 lipids. (*A*) Sterol. (*B*) Phospholipid. (*C*) Triglyceride. (*D*) Free fatty acids and cholesteryl esters. Cells were seeded in LDM at 5.8 × 10^3 cells/cm^2 on 60-mm plates. 25-OH was added 12 h after seeding to a final concentration of 0.16 μg/ml. 0.33 μCi/ml [^{14}C]acetate was added to cultures 2 h before harvesting. After 36 h the medium was aspirated from some of the 25-OH-inhibited cultures, the plates were washed with DMEM, and 5 ml of conditioned LDM lacking the inhibitor was added from cultures set up in parallel. (×) LDM. (○) LDM + 0.16 μg/ml 25-OH. (●) LDM reversal. The points in this figure are averaged from three separate experiments. The error is indicated by the scatter of the points.

inhibition are dose dependent, not only for sterol synthesis but also for phospholipid and triglyceride synthesis. The response of phospholipid and triglyceride syntheses to increasing inhibitor concentration gives the impression that the inhibition of phospholipid synthesis precedes the inhibition of triglyceride synthesis by 3–5 h (Fig. 3).

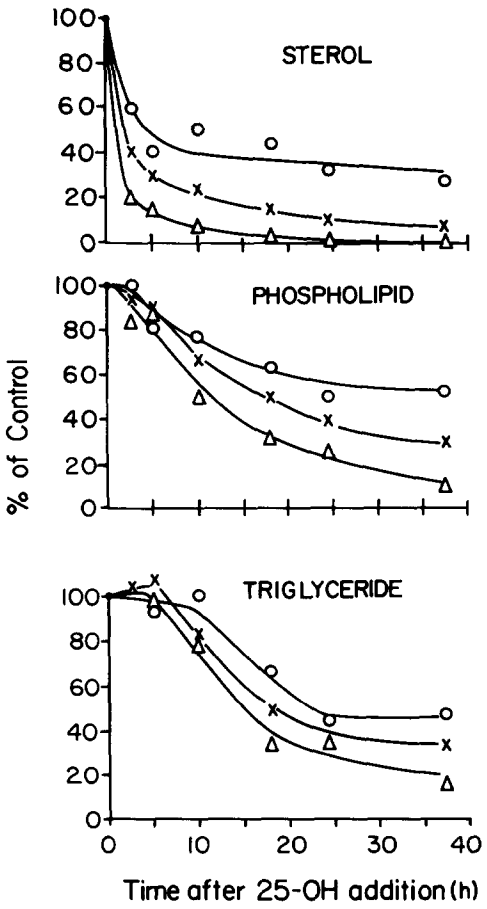

FIGURE 3 Dose dependence of the inhibition of lipid synthesis by 25-OH. L_6 cells were seeded in LDM at 5.8×10^3 cells/cm^2 on 60-mm plates. Incorporation of [^{14}C]acetate (0.33 μCi/ml) into L_6 lipids after 2-h pulse was measured at the times indicated after addition of 0.05 μg/ml (○), 0.17 μg/ml (×), or 1.0 μg/ml (△) 25-OH to cells cultured in LDM. Control values are [^{14}C]acetate incorporated per cell by LDM cultures receiving no inhibitor (average total cpm per control sample per 2 h = 5×10^5; average standard deviation = 8.2%). The points in this figure are averaged from two separate experiments.

The effect of 25-OH on dolichol synthesis was tested indirectly by measuring both [^{14}C]fucose and [^{14}C]glucosamine incorporation into total cellular proteins. 25-OH (0.16 μg/ml) had no appreciable effect on the rate of incorporation of these isotopes into protein before the general inhibition of protein synthesis (see below) as monitored by [^{3}H]leucine incorporation. These results suggest that the availability of dolichol for protein glycosylation is not significantly affected by 25-OH at the concentration we have used to inhibit growth and lipid synthesis.

The possibility that these decreases in [^{14}C]acetate incorporation into nonsterol lipid might arise from fluctuations in the lipid turnover rates or acetyl CoA pool size was investigated. Fig. 4 shows that the lipid classes that are labeled during the standard 2-h pulse are not degraded at a measurable rate. The turnover rate as measured by a pulse-chase experiment after

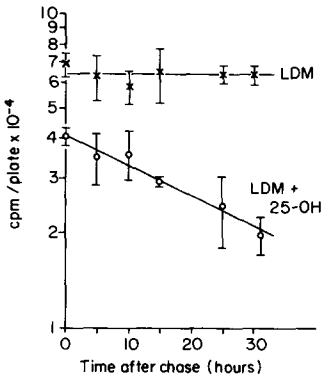

FIGURE 4 Turnover of nonsterol lipids. L_6 was seeded in LDM at 5.8×10^3 cells/cm^2 on 60-mm plates. After 24 h of culturing in LDM (×) or LDM + 0.16 μg/ml 25-OH (○), L_6 cultures growing in 60-mm plates were pulsed for 2 h with 0.33 μCi/ml [^{14}C]acetate. At the end of 2 h, the radioactive medium was removed and replaced with unlabeled conditioned medium from parallel cultures (LDM or LDM + 25-OH) containing 0.6 mM sodium acetate. Two plates of each treatment were harvested immediately and the others returned to the incubator. Cultures were harvested, including those cells that had detached into the medium after the time intervals shown. The decrease in stability of unfractionated glycerolipids in the presence of 25-OH is primarily caused by an increase turnover rate of phospholipid. The data are from one representative experiment that was performed three times.

TABLE I

Effect of 25-OH Cholesterol on Lipid Synthesis Rates of L_6. Comparison of the Incorporation of Various Labeled Precursors after 36 h of 25-OH Cholesterol Incubation

	No. of determinations	PL*	C*	TG*
[^{14}C]Acetate (0.33 μCi/ml) (6.24 μM)	(5)	27.6 ± 4.8	7.6 ± 0.7	43.1 ± 11.7
[^{14}C]Acetate (2 μCi/ml) (12.3 mM)	(2)	42.8 ± 3.1	11.2 ± 0.8	47.3 ± 3.4
[^{14}C]Glucose (2 μCi/ml)	(4)	73.3 ± 4.2	28.7 ± 1.6	51.5 ± 2.8
[^{3}H]Choline (2 μCi/ml)	(4)	31.8 ± 4.9	NA	NA
[^{3}H]Glycerol (6 μCi/ml)	(4)	56.2 ± 0.35	NA	58.7 ± 8.4
^3H_2O (83–250 μCi/ml)	(4)	60.5 ± 44.0	25.8 ± 15.2	72.2 ± 45.0

L_6 cells were seeded at a density of 5.8×10^3 cells/cm^2 in LDM. 25-OH cholesterol (0.16 μg/ml) was added 12 h after seeding. For all precursors other than ^3H_2O the incorporation of radioactivity into lipids was measured after a 2-h pulse. The final concentration of isotope is given in parentheses. The radioactivity incorporated into total lipids of control cultures ranged between 7×10^3 and 2×10^5 cpm per sample per 2 h. The labeling period for ^3H_2O was 24 h, during which an average of 2×10^3 cpm per sample was incorporated. NA, the percent of total cpm was <5%.

PL, phospholipid.

C, cholesterol.

TG, triglyceride.

* The data are means ± standard deviations expressed as percent of control (LDM without 25-OH cholesterol).

24 h of 25-OH treatment was 4% per 2 h. This decrease in stability is clearly not sufficient to account for the 40% reduction in counts incorporated into total nonsterol lipid during the 2-h pulse after 24 h in the presence of 25-OH. The turnover rate would have to be 10-fold higher for the reduced incorporation to be entirely attributable to changes in lipid stability.

Possible reduction of [^{14}C]acetyl CoA specific activity caused by an expanded pool(s) has been assessed by a variety of approaches. The problem of pool fluctuation might not be resolved by a direct measurement of the specific activity of the intracellular acetate pool because it is not known whether the syntheses of cholesterol and glycerolipids stem from a common acetyl CoA pool. Two observations suggest that pool expansion does not contribute significantly to the decreases in [^{14}C]acetate incorporation. In the first place, the effect of 25-OH on the pulse-labeling of lipids with several other isotopes is analogous to the effect seen when [^{14}C]acetate is used as the label. The data in Table I show that 25-OH reduces the incorporation of [^{14}C]glucose, [^{3}H]glycerol, and $^{3}H_2O$, thus increasing the probability that the reduction measured with [^{14}C]acetate is caused by the inhibition of synthesis rates. It is not uncommon to see the greatest change in synthesis rate with [^{14}C]acetate (2). Effects on [^{3}H]glycerol and [^{3}H]choline labeling of lipids should be independent of fluctuations in acetate pool size. When [^{14}C]glucose is used to label lipids, the specific activity of the pool of acetyl CoA is dictated by the rate of its formation from glucose, the primary carbon source for lipid synthesis. Equilibration of the label into the acetyl CoA pool occurs on a time scale that is short compared with the pulse length.[2] Thus the specific activity of the acetyl CoA pool would not change despite changes in pool size. Hence, even if 25-OH treatment were to cause pool expansion, [^{14}C]glucose incorporation should not be affected. An analogous condition can be created for [^{14}C]acetate incorporation by raising the external acetate concentration, thereby increasing the contribution of acetate as carbon source for lipid synthesis (12). The effect of 25-OH on [^{14}C]acetate incorporation is similar, whether acetate is present externally at a high or tracer concentration or not (Table I). $^{3}H_2O$ is the isotope of choice for demonstrating the absence of pool size fluctuations because the pool size is effectively infinite. Although the data for $^{3}H_2O$ incorporation in Table I are consistent with the other data appearing there, in general the lipids were labeled with <4,000 cpm per sample, and the results were highly variable.

Secondly, the uptake of [^{14}C]acetate into whole cells and its incorporation into lipids were compared, the initial difference being a measure of the internal acetate pool plus incorporation into other macromolecules. An expanded pool would delay the incorporation of [^{14}C]acetate into lipids. Fig. 5 shows that there is no delay in incorporation into lipids in either control or 25-OH-treated cells. Both the uptake rate into the cell and that into lipids are depressed from the first time point in 25-OH-treated cells. The graphs demonstrate that the decrease in incorporation into lipid at the end of a 2-h pulse is caused by a true rate depression rather than a delay followed by incorporation at the control rate that would indicate an expanded pool (22). The time-courses of uptake of the label into lipids as a function of inhibitor concentration show that lipid synthesis rates decline in proportion to the 25-OH dose (data not shown). These two lines of evidence suggest that the inhibition of [^{14}C]acetate incorporation into nonsterol lipid primarily reflects inhibition of synthesis rates.

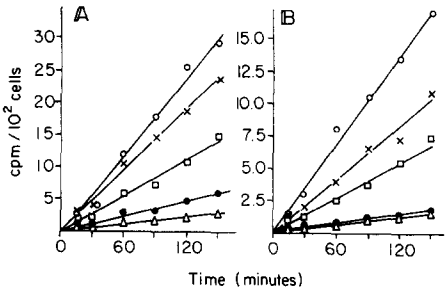

FIGURE 5 Comparison of the uptake of [^{14}C]acetate into whole cells and its incorporation into lipid classes. L_6 cells were seeded in LDM at 5.8 × 10^3 cells/cm^2 on 60-mm plates. 0.33 μCi/ml [^{14}C]acetate was added to 60-mm plates of L_6 cultured for 24 h in (*A*) LDM, or (*B*) LDM + 0.16 μg/ml 25-OH. At the time indicated, cultures were harvested. An aliquot of the cell suspension was removed for counting whole cell radioactivity. The remainder was extracted for determination of radioactive lipids. (○) Whole cell. (×) Total lipid. (□) Phospholipid. (●) Sterol + diglyceride. (△) Triglyceride and free fatty acid. These data are the average of three experiments. Note the difference in scale for *A* and *B*.

Restimulation of Lipid Synthesis

The restimulation of lipid synthesis by removing the medium containing 25-OH is shown in Fig. 2, right panel. The rate of sterol synthesis has clearly doubled before the syntheses of the other lipids is affected. A delay of 6 h between the effects on sterol and nonsterol lipid syntheses is seen for both the inhibition and stimulation responses. The synthesis rates of all lipid classes increase to control levels within 24 h after the medium change. These restimulations occur before the reinitiation of DNA synthesis (~24 h after removal of 25-OH) and cell division (>36 h).

When mevalonic acid is added to 25-OH-inhibited cultures at 36 h, lipid synthesis rates remain depressed for ~24 h. Thereafter [^{14}C]acetate incorporation into all lipid classes (except cholesterol) accelerates and reaches control levels within 24 h. [^{14}C]mevalonate-labeling experiments verified the resumption of sterol synthesis from mevalonate. Addition of cholesterol at 36 h, either as 1:1 cholesterol:egg lecithin vesicles (38.7 mg/ml cholesterol) or LDL (44 mg/ml cholesterol), restimulates cell division without stimulating the synthesis of any lipids.

Effect of Choline Removal on Lipid Synthesis

Sequential inhibitions of lipid synthesis are observed in response to blocks in the synthesis of other lipid pathways.[3] The synthesis of phosphatidylcholine, the major membrane phosphatide, is inhibited by eliminating choline from the medium. Proliferation is inhibited after an average of 1.5 divisions (36 h) in choline-deficient medium. Under these circumstances, an inhibition of [^{14}C]acetate incorporation extends to other

[2] Linear incorporation of [^{14}C]glucose into lipid is achieved in <15 min. The standard pulse length is 2 h.

[3] Treatment of cells cultured in biotin-free LDM with avidin, a protein that binds biotin, a coenzyme in fatty acid synthesis, causes a threefold and sevenfold reduction in [^{14}C]acetate incorporation into free fatty acids and triglycerides, respectively. The synthesis rates of sterol and phospholipid, on the other hand, are substantially stimulated; sterol synthesis doubles, leading to an increase in the sterol mass per milligram of protein. These affects of avidin were also seen with $^{3}H_2O$.

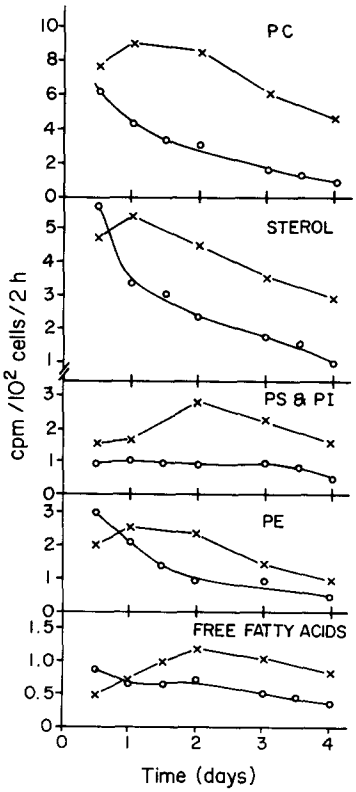

FIGURE 6 Effects of choline removal on lipid synthesis. L_6 was seeded at 5.8 × 10^3 cells/cm^2 on 60-mm plates in LDM (×) or LDM deficient in choline (○). 0.33 μCi/ml [^{14}C]acetate was added 2 h before harvesting at the times indicated. This is the average of two experiments. The standard deviations ranged from 16 to <1%.

phospholipids and also to sterol and free fatty acids (Fig. 6). The synthesis of storage lipids is not significantly altered.

Effects of Inhibitors of Cholesterol Synthesis on Lipid Content

The effect of the inhibition of cholesterol synthesis on total sterol mass per cell is shown in Fig. 7. 25-OH could not be detected by GLC analysis of the sterols from 25-OH-treated cells; thus, it is not incorporated significantly into membranes as a structural component. 24 h after addition of 25-OH or compactin, the sterol content (μg/cell) is reduced 50%. Many of the cells proceed to divide once more, resulting in a further reduction of the sterol mass per cell. The phospholipid content, in contrast to sterol content, does not decline significantly over a 4-d period of treatment with 25-OH (Fig. 7 *C*); thus, the time-course of change in sterol mass per cell is equivalent to the change in sterol:phospholipid mole ratio.[4] The sterol to protein mass ratio, however, falls no more than 50% because the average protein mass per cell is reduced by approximately one-third as the cells accumulate in G_1 and protein synthesis declines. Increasing the concentration of inhibitor reduces the time required to reach the lower limit but does not affect the value of this minimum level.

Removal of 25-OH after 36 h results in a fairly rapid increase in the sterol content per cell (Fig. 7 *A*). A 50% increase is seen as early as 9 h. DNA synthesis is reinitiated 20–24 h after the reversal, at which time the sterol content has increased twofold but is still just two-thirds of the uninhibited level. Addition of mevalonic acid, cholesterol-lecithin vesicles (1:1 M/M), or LDL-cholesterol will also raise the cholesterol content per cell by two- or threefold. Cells containing as low as 60% of the sterol content of control LDM grown cells (i.e., mevalonate-reversed cells) can still proliferate at the control rate.

Effects of Sterol Synthesis Inhibitors on Morphology

25-OH and compactin have a pronounced effect on the morphology of L_6 myoblasts that coincides temporally with the reduction in cholesterol content. The cells gradually reduce their surface area and assume the condensed shapes shown in Fig. 8. This change begins about 24 h after treatment with 0.16 μg/ml, or sooner, if the inhibitor concentration is increased. After 3 d, many cells round up and detach from the dish. 25-OH-treated cells with drastically altered appearance aggregate very tightly when harvested with trypsin-EDTA but not with EDTA alone.

Effects of 25-OH on Macromolecular Synthesis

Rates of RNA and protein synthesis were measured after treatment with 25-OH (Fig. 9). The syntheses of DNA and RNA decline simultaneously from near 100% at 24 h to 20 or 30% at 48 h.[5] Protein synthesis responds to the general inhibition of RNA synthesis after a 6–9-h delay. These inhibitions lag 18–24 h behind the inhibition of lipid synthesis and begin after the sterol content is 50% of control.

Lipid and Protein Synthesis and the Response to 25-OH in Other Cell Types

We have explored lipid synthesis rates in other systems to examine the generality of coupling among lipid synthesis pathways and the coupling between lipid and macromolecular syntheses. Rates of lipid synthesis in differentiated myotubes of chick primaries were measured after 4 d in culture. At this time the cultures are composed almost entirely of multinucleated myotubes. (Inclusion of Ara-C in the medium kills the fibroblasts in the culture.) Over a 3-d period, total lipid synthesis is reduced nearly sevenfold (Fig. 10). This decline in synthesis rate is reflected in each of the lipid classes. Although lipid synthesis declines 85% from day 4 to day 7, protein synthesis continues at a steady rate (Table II).

The addition of 25-OH to chick myotube cultures has a small inhibitory effect on total lipid synthesis. This inhibition can be accounted for by the decrease in the fraction of total label incorporated into cholesterol and diglyceride. Cholesterol labeling declines ninefold, from 23% to 2.5%, within 18 h after 25-OH addition. There is no difference in the synthesis rates of phospholipid and triglyceride attributable to 25-OH. The selective effect on cholesterol synthesis results in a 40–50% de-

[4] In contrast to the threefold reduction in sterol content resulting from the rapid decline in the sterol synthesis rate (Fig. 2 *A*), the gradual decline in the phospholipid synthesis rate (Fig. 2 *B*) should, in theory, yield a <20% reduction in mass. This degree of change is within the experimental error of the phosphate determination (Fig. 7 *C*).

[5] The decline in [^{3}H]thymidine incorporation into DNA is not a reflection of a decreased rate of DNA synthesis, rather it reflects the passage of fewer cells into S phase. Autoradiographic analysis demonstrated a decrease in the percent of [^{3}H]thymidine-labeled nuclei from 40 to 10% within 3 d after addition of 25-OH. Those nuclei that were labeled by a 1-h pulse had the same grain density as S-phase nuclei of control cells, hence the *rate* of DNA synthesis had not been altered (7).

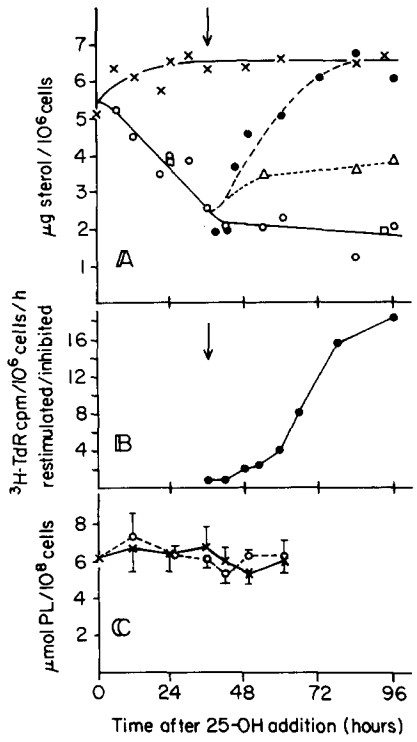

FIGURE 7 Effects of inhibition and restimulation of sterol synthesis on (*A*) sterol mass per cell, (*B*) DNA synthesis, and (*C*) phospholipid mass per cell. (*A*) L_6 was seeded in LDM at 5.8×10^3 cells/cm^2 on 100-mm plates. 25-OH (0.16 μg/ml) was added 12 h after seeding. Cells were harvested at each time point for determination of sterol mass. (×) LDM sterol. (○) LDM + 0.16 μg/ml 25-OH. (□) LDM + 0.33 mg/ml compactin. After 36 h (solid arrow), 25-OH-containing LDM was replaced with conditioned LDM lacking inhibitor (●), or 2 mg/ml mevalonate was added (△). Each curve is the composite of 2–10 separate experiments. (*B*) L_6 was seeded in LDM at 5.8×10^3 cells/cm^2 on 60-mm plates. 25-OH (0.16 μg/ml) was added 12 h later. After 36 h the medium containing 25-OH was replaced with conditioned LDM lacking the inhibitor, and cultures were pulsed for 1 h with 1.0 μCi/ml [^{3}H]thymidine. The data (composite of three experiments) are presented as the ratio of [^{3}H]thymidine incorporated into the restimulated cultures over incorporation into inhibited cultures. (*C*) L_6 were cultured on 100-mm plates containing LDM (×), or LDM containing either 0.16 μg/ml or 1.25 μg/ml 25-OH (○). Cells were harvested at each time point for determination of phospholipid content. These plots are the average of seven separate experiments.

crease in the sterol:phospholipid ratio. 25-OH addition affects further fusion, and hence, the myotubes do not increase in size (see Fig. 8). The amount of protein per nucleus is not altered by 25-OH treatment. A 10–15% decline in protein synthesis rates accompanies the addition of 25-OH after 1 d of incubation (Table II). This decline does not appear to be selective for the synthesis of either membrane or cytosolic proteins as measured by [^{3}H]leucine incorporation.[6]

We compared the time-course of lipid synthesis in differentiated chick myotubes with that in other cells in a variety of states. The results are shown in Fig. 11. Lipid synthesis in confluent WI-38 and L_6 myotubes undergoes sharp declines reminiscent of that seen in chick primary myotubes. Low serum-arrested, sparse WI-38 cultures have a very low but constant rate of synthesis that is about one-onehundredth the rate of proliferating WI-28. Proliferating WI-38, chick fibroblasts, and L_6 myoblasts synthesize total lipids at the same rate within a factor of two. Chick primary cultures of postmitotic myoblasts synthesize lipids at one-tenth the rate of proliferating chick fibroblasts. Lastly, confluent, transformed WI-38 that have a doubling time of 48 h maintain a high rate of total lipid synthesis. These results suggest that time in culture and confluence are not necessary determinants of the lipid synthesis rate; they also indicate that a G_1 arrest is normally accompanied by a depression in lipid synthesis. Only in the proliferating cultures and in differentiating chick myoblasts can an inhibition of glycerolipid synthesis caused by 25-OH be detected, regardless of the culture density.

[6] The ratio of the specific activity of soluble protein:total protein was LDM, 1.42; LDM + 25-OH, 1.41. 25-OH does not alter the protein mass in either fraction.

DISCUSSION

The maintenance of a balanced state of growth requires that the syntheses of membrane components be coordinated with one another and with the syntheses of other macromolecular components of the cell. The experiments in this paper suggest that such balanced growth occurs in dividing, quiescent, and postmitotic differentiated mammalian cultured cells. They provide evidence for coupling between (*a*) the syntheses of cholesterol, phospholipid, and triglyceride, and (*b*) lipid supply and DNA synthesis.

Coordination of Lipid Synthesis Pathways in Proliferating L_6

The changes in the lipid synthesis rates in response to 25-OH, compactin, and choline deprivation imply coordinated regulation of sterol and glycerolipid pathways. This coordination could serve as one way to regulate the lipid composition. Experiments have been presented in Results that indicate the minimal influence of changes in turnover rates or pool size on the apparent coordination. The concerted inhibition of cholesterol and glycerolipid syntheses in response to 25-OH and compactin occurs in healthy cells, as judged by their rates of growth, cell cycling (7), and macromolecular synthesis. The rapid restimulation of DNA synthesis and cell cycling when the inhibitor is removed also indicates that the cells are healthy for at least 36 h at the low inhibitor doses used in these experiments.

The apparent coupling of phospholipid and sterol syntheses provides an explanation for the observation that the ratio of these membrane lipids does not change more than threefold in inhibited cells. The inhibition of phospholipid synthesis in the presence of 25-OH is several hours later than the inhibition of sterol synthesis, and its synthesis rate declines more slowly than does that of cholesterol. Thus, during one division cycle, less sterol than phospholipid is generated. The ratio of cholesterol to phospholipid stabilizes because the rates of sterol and phospholipid synthesis reach new steady-state levels. Whereas the rates of synthesis of membrane phospholipid and sterol are always coordinated in their response, we have observed that the syntheses of triglyceride and membrane lipids are not always coupled (e.g., the responses to choline or biotin deprivation).

We have few clues concerning the mechanism of the coordination of lipid syntheses. We can dismiss the hypothesis that the coupled responses of lipid synthesis are part of a general metabolic depression accompanying the G_1 arrest because they precede the effects on cell cycling. Models requiring large and

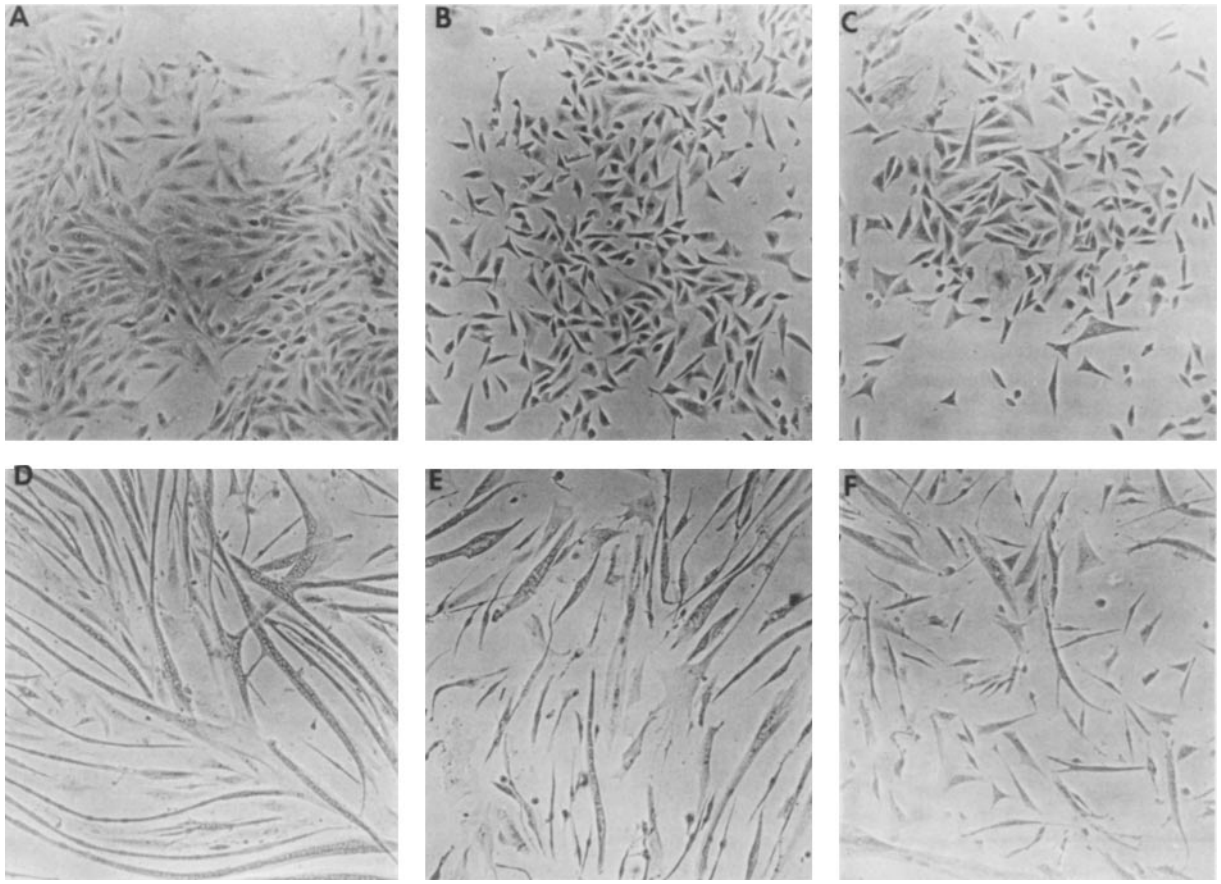

FIGURE 8 Effect of inhibitors of cholesterol synthesis on cell morphology. (*A–C*) L_6 Myoblasts. (*D–F*) Chick primary myotubes. L_6 myoblasts were cultured for 2.5 d in LDM (*A*); LDM + 0.10 μg/ml 25-OH (*B*); or LDM + 2.5 μg/ml compactin (*C*). Chick primary myotubes were cultured for 7 d in LDM (*D*); LDM + 3.8 μg/ml 25-OH added on day 4 (*E*); or LDM + 25 μg/ml compactin added on day 4 (*F*). Cells were fixed and stained with methyl alchohol-Giemsa and rehydrated for photography. Bar, 100 μm. Culture conditions were as described in the legends to Figs. 1 and 10.

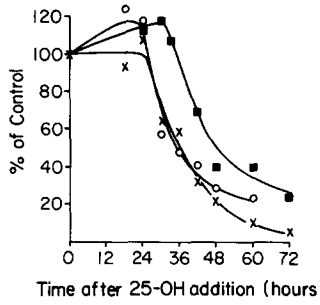

FIGURE 9 Effect of 25-OH on macromolecular synthesis. L_6 was seeded in LDM at 5.8×10^3 cells/cm^2 on 60-mm plates. 25-OH (0.16 μg/ml) was added 12 h after seeding. At the times indicated, cultures labeled with 1 μCi/ml [^{3}H]thymidine for 2 h (×), with 2 μCi/ml [^{3}H]uridine for 30 min (○), or with 2 μCi/ml [^{3}H]leucine for 2 h (■) were harvested and frozen until the completion of the experiment. The radioactivity incorporated into TCA-precipitable material was determined as described in Methods. Controls varied from 3×10^3 cpm/sample ([^{3}H]uridine) to 2×10^4 cpm/sample ([^{3}H]thymidine). The DNA curve is the composite of six separate experiments, and the RNA and protein curves are composites of four experiments each.

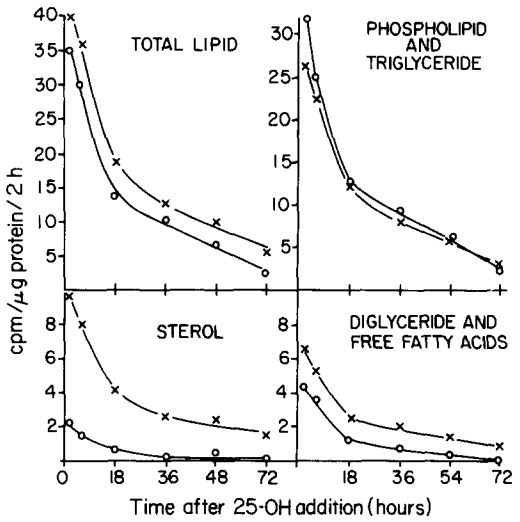

FIGURE 10 Lipid synthesis rates in chick primary myotubes. Cells from breast muscle of 11-d chick embryos were plated in LDM at a density of 1.3×10^4 cell/cm^2 on 60-mm plates. EGTA (300 μM) was added at 6 h, Ara C (1.2 μg/ml) at 29–30 h, and Ca^{+2} (1.8 mM) at 52 h. After 92 h in culture, the medium was replaced with fresh LDM containing Ara C. 25-OH (3.8 μg/ml) was added 30 min later. Plates were pulsed at intervals thereafter with 1.3 μCi/ml [^{14}C]acetate. These plots are from one representative experiment. (×) LDM. (○) LDM + 25-OH.

general perturbations of structure or physical state also seem unlikely: the synthesis of glycerolipids responds after a fivefold inhibition or restimulation of the rate of sterol synthesis but after only a >10% change in sterol content. A first step toward

TABLE II

Rates of Protein Synthesis in Chick Myotubes

Time after 25-OH addition	LDM*	LDM + 25-OH*
h		
25	12.6 ± 1.4 (4)	11.1 ± 0.9 (4)
40	12.2 ± 0.4 (2)	10.4 ± 0.6 (2)
48	10.9 ± 1.6 (4)	10.5 ± 0.3 (4)
60	11.3 ± 1.2 (4)	9.5 ± 1.3 (3)
72	10.2 ± 1.7 (2)	9.5 ± 0.04 (2)

Cells were cultured as described in the legend to Fig. 10.

* Data are expressed as cpm [^{3}H]leucine incorporated per microgram of protein per 2-h pulse. The data are presented as mean with standard deviation. The number of determinations is given in parentheses.

the discovery of the mechanism of this coordination would be to determine what step(s) in phospholipid and triglyceride synthesis are sensitive to the availability of cholesterol and vice versa. Preliminary experiments toward this end reveal a decrease in the specific activity of cholinephosphate cytidylyltransferase, the rate-limiting enzyme in phosphatidylcholine synthesis (26), which parallels the inhibition of of [^{3}H]choline incorporation into phospholipid after 25-OH addition (R. Cornell and H. Goldfine, unpublished observations).

Lipid Synthesis in Response to Growth Demands

Our studies on lipid synthesis in a variety of cell types show that in general the rate of lipid synthesis varies proportionally with the growth rate and that when the rate of total lipid synthesis declines, equally reduced rates of synthesis are seen for all lipid classes. Thus, these studies offer further support for the notion of coordinated control of lipid synthesis. The depressions of synthesis are not primarily responses to the length of time spent in culture or to cell density, since confluent transformed WI-38 cells maintain a constant high rate of lipid synthesis for 6 d (provided the medium is changed to ensure continued proliferation). Rates of lipid synthesis decrease when cells are arrested in G_1. Myogenesis may also involve the depression of lipid synthesis as part of its program or, alternatively, this response may result from the G_1 arrest that accompanies myoblast differentiation.

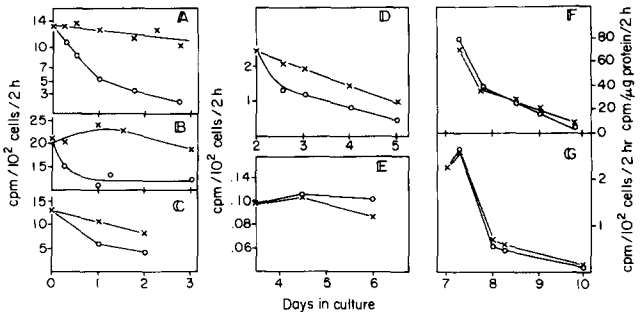

FIGURE 11 Lipid synthesis rates compared in proliferating, G_1-arrested, and postmitotic differentiating myoblasts and myotubes. Note the differences in ordinate scales and abscissa of *D*. All rates are determined from 2-h pulses of 0.66 μCi/ml [^{14}C]acetate. (×) LDM. (○) LDM + 25-OH. (*A*) Proliferating L_6, [25-OH] = 0.16 μg/ml. (*B*) Proliferating WI-38. Cells were seeded at 5.2 × 10^3/cm^2 [25-OH] = 0.15 μg/ml. (*C*) Proliferating chick embryo fibroblasts. Secondary cultures were seeded at 7.9 × 10^3/cm^2. [25-OH] = 1.3 μg/ml. (*D*) Postmitotic chick myoblasts. Primary cultures were grown in Ca^{+2}-deficient medium as described in the legend to Fig. 9. [25-OH] = 1.3 μg/ml. (*E*) Low serum-arrested WI-38. WI-38 were seeded at 5.2 × 10^3/cm^2 in medium containing 0.05% lipid-depleted serum. [25-OH] = 0.15 μg/ml. (*F*) L_6 myotubes. Cells were seeded at 5.8 × 10^3/cm^2. [25-OH] = 1.0 μg/ml. (*G*) Confluent WI-38. Cells were seeded at 5.2 × 10^3/cm^2. [25-OH] = 0.15 μg/ml.

In general, 25-OH treatment results in the inhibition of the synthesis of the three major lipid classes when added to cells, such as proliferating cells, whose lipid synthesis rates are relatively constant and high. In contrast, the addition of 25-OH to cells whose lipid synthesis rates are already declining because of differentiation or the G_1 arrest, depresses sterol synthesis an additional fivefold without affecting long-term rates of phospholipid or triglyceride synthesis. In spite of this less tightly coupled response of lipid synthesis to 25-OH, the sterol:phospholipid ratio drops no more than twofold before a new steady state in lipid synthesis rates is reached.

Further studies of the mechanism of coordination of cholesterol, phospholipid, and triglyceride syntheses using the response to inhibitors of synthesis and other biochemical and genetic approaches may point to a general mechanism for the regulation of the lipid composition of cell membranes. Independent evidence for coordination of sterol and fatty acid biosyntheses has come from work on a recently isolated mutant from Chinese hamster ovary cells (18).

Cholesterol Availability and Cell Cycling

We have shown that the inhibition of cholesterol synthesis leads to a proliferative arrest in the G_1 phase of the cell cycle (1) and depresses the synthesis of other lipid classes and macromolecules. We can dispense with two arguments for the origin of these inhibitions after 25-OH treatment, namely, that the cellular responses are related to (*a*) secondary sites of action unrelated to the depression of lipid synthesis of (*b*) an inhibition of dolichol synthesis. First, compactin, an inhibitor bearing no structural resemblance to the steroid inhibitor, mimics its effect on growth, the cell cycle, sterol and glycerolipid syntheses, morphology, and sterol mass. Second, exogenous cholesterol can reverse the cell cycle arrest resulting from incubation with either 25-OH or compactin. Last, the availability of dolichol for glycosylation of protein appears not to be limited by treatment with 25-OH at concentrations that result in inhibition of lipid and macromolecular syntheses. James and Kandutsch (13) have also observed that at a concentration of 25-OH that inhibits sterol synthesis 85% inhibits the incorporation of [^{14}C]acetate into dolichol in mineral oil-induced plasmacytoma 104E cells only 9%. Similar responses to 25-OH have been observed in L cells and aortic smooth muscle cells (14, 21).

Thus, it appears that these inhibitions occurring in the presence of 25-OH are the consequence of the decrease in the rate of cholesterol synthesis or availability. Our findings suggest that cholesterol availability rather than *de novo* synthesis is the key factor. First, exogenously supplied cholesterol will stimulate 25-OH-arrested cells to enter S phase without restimulating the rate of cholesterol synthesis. Second, when cultures are presented with 25-OH, sterol synthesis decreases 80% within the first 4 h, and yet the cells divide at least twice more. L cells also appear to lack a sterol synthesis requirement for cell cycling; they proliferate in the presence of 25-OH when either cholesterol or desmosterol, its immediate precursor, is provided exogenously (16).

In considering the question of how the availability of cholesterol could influence cell cycling, we have tried to assess the effect of limiting cholesterol on (*a*) RNA and protein syntheses (*b*) the sterol:phospholipid ratio in the membrane, and (*c*) rates

of synthesis of other lipids and their role in the development of the G_1 arrest.

Because large-scale depressions of RNA and protein syntheses follow the inhibition of sterol synthesis, the possibility arises that lipid and protein syntheses are directly coupled and that the G_1 arrest is solely a response to the inhibition of protein synthesis. However, the inhibition of the rates of protein synthesis is delayed 6–9 h after the inhibition of DNA synthesis. We suggest that the inhibition of protein synthesis observed after the addition of 25-OH is not evidence for direct lipid-protein synthesis coupling, but rather that the apparent coupled inhibition of lipid, RNA, and protein syntheses occurs in association with and because of a shift from cycling to G_1 arrested cells. Our suggestion is drawn from observations of lipid-protein synthesis independence in other systems. We find that chick myotubes, i.e., cells that have not been cycling for several days, continue to synthesize protein at a steady rate whereas lipid synthesis declines. RNA and protein syntheses in transformed cells, which do not arrest in G_1 when lipid synthesis is inhibited (10), are not affected by long-term treatment with 25-OH (16).[7] The ability of L_6 and other nontransformed proliferating cells to be arrested in G_1 may be an intrinsic feature in the coordination of membrane lipid and macromolecular syntheses.

Unlike the change in rates of RNA and protein synthesis, a significant shift in the cholesterol:phospholipid ratio always precedes a decline or increment in the number of cycling cells, hence, a shift in this ratio is one possible way in which the availability of cholesterol is communicated to the cell cycle control step. The sterol:phospholipid ratio drops twofold before the decline in DNA synthesis. Passage into S phase resumes after the average sterol content has increased by a factor of 1.8. The rate of cell cycling is independent of the sterol:phospholipid ratio between 0.26 (uninhibited level) and 0.16 (25-OH plus mevalonate), indicating that the growth rate of L_6 can tolerate wide variation in the ratio of membrane lipids. Our findings are consistent with the notion of a minimum sterol content necessary for survival, a narrow range above this limit within which cells are maintained in a G_1 arrested state, and a wide range above this within which the growth rate is unperturbed. More stringent sterol requirements for specific cell functions may well exist.

The cell cycle response to the availability of cholesterol may be mediated via effects on the synthesis of other lipid classes. The changes in the rates of synthesis of phospholipid, triglyceride, and their precursors precede both the arrest in G_1 and the release from G_1 into S when the inhibitor is removed or when mevalonic acid is provided. The G_1 arrest can be overcome by the addition of LDL or liposomes to the inhibited cultures without restimulating lipid synthesis from acetate; however, LDL and liposomes can supply both cholesterol and phospholipid to the cells. A cessation of the assembly of functional membrane is likely to result from the lack of available sterol and phospholipid due to declining synthesis. Under these circumstances, a G_1 arrest from cell cycling would prevent an imbalance in the production of membrane and other macromolecular components.

[7] The superposition of the RNA and DNA inhibition curves could be construed as indicative of a primary lipid requirement for RNA synthesis with the inhibition of DNA synthesis arising from inhibited transcription. This explanation, although possible, seems unlikely. A more plausible explanation is that the inhibitions of DNA, RNA, and protein syntheses all occur in response to the arrest in G_1 (G_0).

Compactin was the generous gift of Dr. K. Mansford, Beecham Pharmaceuticals, London. We thank Dr. S. W. Englander for use of his scintillation counter, Dr. J. Pachence for egg lecithin, and Drs. G. Rothblat and H. Goldfine for many useful discussions.

These studies were supported by National Institutes of Health grant GM-23244 and a grant-in-aid from the American Heart Association. They were performed during the tenure of Dr. Horwitz under the W. D. Stroud Established Investigatorship of the American Heart Association.

Received for publication 14 April 1980.

REFERENCES

1. Bailey, J. M. 1964. Lipid metabolism in cultured cells. V. Comparative lipid nutrition in serum and lipid free chemically defined medium. *Proc. Soc. Exp. Biol. Med.* 115:747–750.
2. Bailey, J. M. 1966. Lipid metabolism in cultured cells. VI. Lipid biosynthesis in serum and synthetic growth media. *Biochim. Biophys. Acta.* 125:226–236.
3. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466–468.
4. Bischoff, R., and H. Holtzer. 1969. Mitosis and the process of differentiation of myogenic cells *in vitro*. *J. Cell Biol.* 41:188–200.
5. Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* 62:983–988.
6. Brown, M. S., J. R. Faust and J. L. Goldstein. 1978. Induction of 3-hydroxy-3-methylglutaryl CoA reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. *J. Biol. Chem.* 253:1121–1128.
7. Cornell, R., G. L. Grove, G. H. Rothblat and A. F. Horwitz. 1977. Lipid requirement for cell cycling: the effect of selective inhibition of lipid synthesis. *Exp. Cell Res.* 109:299–307.
8. Freeman, C. P., and D. West. 1966. Complete separation of lipid classes on a single thin-layer plate. *J. Lipid Res.* 7:324–328.
9. Havel, R. S., H. A. Eder and J. H. Bragdon. 1955. Distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1353.
10. Hatten, M. E., A. F. Horwitz, and M. M. Burger. 1977. The influence of membrane lipids on the proliferation of transformed and untransformed cell lines. *Exp. Cell Res.* 107:31–34.
11. Horwitz, A. F., A. Wight, P. Ludwig and R. Cornell. 1978. Interrelated lipid alterations and their influence on the proliferation and fusion of cultured myogenic cells. *J. Cell Biol.* 77:334–357.
12. Howard, B. V. 1977. Acetate as a carbon source for lipid synthesis in cultured cells. *Biochim. Biophys. Acta.* 488:145–151.
13. James, M. J., and A. A. Kandutsch. 1979. Relationships between dolichol and cholesterol synthesis in cell cultures. *Fed. Proc.* 38:785.
14. James, M. J., and A. A. Kandutsch. 1979. Interelationships between dolichol and sterol synthesis in mammalian cell cultures. *J. Biol. Chem.* 254:8442–8446.
15. Kandutsch, A., and H. Chen. 1973. Inhibition of sterol synthesis in cultured mouse cells by 7α-hydroxycholesterol,7β-hydroxycholesterol, and 7-ketocholesterol. *J. Biol. Chem.* 248:8404–8417.
16. Kandutsch, A. A., and H. Chen. 1977. Consequences of blocked sterol synthesis in cultured cells. *J. Biol. Chem.* 252:409–415.
17. Kent, C., S. D. Schimmel and P. R. Vagelos. 1974. Lipid composition of plasma membranes from developing chick muscle cells in culture. *Biochim. Biophys. Acta.* 360: 312–321.
18. Limanek, J. S., J. Chin, and T. Y. Chang. 1978. Mammalian cell mutant requiring cholesterol and unsaturated fatty acid for growth. *Proc. Natl. Acad. Sci. U. S. A.* 75:5452–5456.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
20. Marsh, J. B. 1974. Lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid Res.* 15:544–560.
21. Mills, J., and A. M. Adamany. 1978. Impairment of dolichyl saccharide synthesis and dolichyl-mediated glycoprotein assembly in the aortic smooth muscle in culture by inhibitors of cholesterol biosynthesis. *J. Biol. Chem.* 253:5270–5273.
22. Mitcheson, J. 1970. *In* Biology of the Cell Cycle. Cambridge University Press, London. 11–14.
23. Rein, A., and H. Rubin. 1968. Local cell concentration and cell growth. *Exp. Cell Res.* 49: 666–678.
24. Singleton, W. S., M. S. Gray, M. L. Brown and J. L. White. 1965. Chromatographically homogeneous lecithin from egg phospholipids. *J. Am. Oil Chem. Soc.* 42:53–56.
25. Skipski, V. P., and M. Barclay. 1969. Thin layer chromatography of lipids. *Methods Enzymol.* 14:530–598.
26. Vance, D. E., and P. C. Choy. 1979. How is phosphatidylcholine synthesis regulated? *Trends Biochem. Sci.* 4:145–148.