Control of Lipid Metabolism in Cultured Cells[1]

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ABSTRACT

Several studies are presented which indicate that composition of cell lipid is regulated by interaction between intracellular metabolism and lipid transport processes. When the fatty acid composition of cells cultured in essential fatty acid deficient conditions was studied, activation of synthesis of unusual polyunsaturated fatty acids was observed for a number of cell lines. In addition cells contained persistent residual amounts of linoleic acid, presumably owing to efficient scavenging mechanisms. The source of cell lipids was studied in both chemically defined and serum-supplemented media. In the absence of exogenous lipid, cells synthesize lipids from simple precursors, a process which is inhibited by adding serum. When serum lipid is present, cells preferentially utilize fatty acids as a source of nonsterol lipid. These are subsequently esterified intracellularly to make glycerides and phospholipids. When triglyceride is utilized as a source of cell lipid, it is first hydrolyzed before being taken up. By use of a nonhydrolyzable cholesterol ester analog, it is confirmed that both free and ester cholesterol are taken up and excreted by cells. Intracellular cholesterol content is thus regulated by rates of uptake, hydrolysis and excretion as well as by biosynthesis.

INTRODUCTION

Cultured cells provide a promising system for studying the regulation of lipid metabolism. In the first place their rather homogeneous character allows the examination of events separated from the complex physiological interactions encountered in vivo. Moreover nutritional and environmental conditions can be more easily manipulated to facilitate examination of molecular mechanisms. When studying lipid metabolism in cultured cells, several factors must be considered. Lipid composition is affected by the interaction and regulation of the individual pathways for biosynthesis and degradation. In addition cultured cells like cells in vivo grow in an environment containing available lipid in the form of the serum liproteins. The contribution of this to the lipid content of the cell is affected by interaction of extracellular lipid and lipoprotein with cell surface lipoprotein, and upon mechanisms of selective transport and excretion. These latter mechanisms appear to regulate at the level of permeability. In the present paper studies are presented on three areas of lipid metabolism—the contribution of exogenous lipid, the regulation of de novo lipid synthesis and the transport processes for lipids across the cell membrane. In each case our results indicate that lipid composition is regulated by an interaction between intracellular metabolism and trans-membrane permeability processes.

MATERIALS AND METHODS

Lipid extracts for routine measurement of lipid content were prepared by the chloroform-methanol extraction procedure of Folch et al. (1), and the individual lipid classes were separated by thin layer chromatography by the method of Stahl (2). For experiments in which essential fatty acids (EFA) were to be measured, lipid extracts were prepared overnight by extraction at 4 C with 5 ml or 20 volumes, whichever was greater, of ethanol-diethyl ether 1:1. α-Tocopherol (0.2 mg) in ethanol-ether 1:1 was added to prevent oxidation of polyunsaturated fats. Ethanol-ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness at room temperature in a stream of nitrogen. The lipid residue was redissolved in hexane. It has been shown previously that these relatively mild extraction procedures are necessary for quantitative recovery of polyunsaturated fatty acids (3). Methyl esters of the fatty acids were prepared by transesterification of the lipid extracts in methanol with boron trifluoride (14%) as a catalyst (4), and analyzed by gas liquid chromatography using a 1662 series gas chromatograph (HCL Scientific Inc., Chicago, Ill.) equipped with a hydrogen flame ionization detector. The chromatograph was fitted with glass columns packed with ethylene glycol-succinate polyester (15%) on a 100-120 mesh Gas Chrom Q.

[1]One of 13 papers presented at the symposium "Lipid Metabolism in Cells in Culture," AOCS Meeting, Houston, May 1971.

TABLE I

Distribution of Fatty Acids in Cells as Compared to Their Culture Medium[a]

Fatty acid	Calf serum[b]	WI-38 cells	WI-38VA13A cells	Mouse blood	Ehrlich Ascites cells	Sarcoma 180 cells
14:0	0.9 ± 0.2	1.6 ± 4.2	1.8 ± .11	n.d.	n.d.	n.d.
14:1	n.d.[c]	Trace	Trace	n.d.	n.d.	n.d.
16:0	26.6 ± 2.0	22 ± 1.2	25 ± 1.6	23.0 ± 1.3	14.8 ± 0.5	14.7 ± 3.0
16:1	3.5 ± 0.3	.38 ± .06	Trace	1.3 ± 0.5	1.7 ± 0.1	2.2 ± 0.3
18:0	20.8 ± 1.6	18 ± 1.3	15 ± 1.6	10.0 ± 0.6	18.2 ± 0.3	16.0 ± 1.4
18:1	29.2 ± 1.4	32 ± 1.2	39 ± 2.1	23.7 ± 2.6	25.0 ± 0.9	22.4 ± 0.8
18:2	12.6 ± 1.6	6.6 ± .71	10.1 ± 1.8	18.4 ± 1.0	25.0 ± 0.4	20.0 ± 1.2
18:3	n.d.	1.9 ± .20	Trace	1.4 ± 0.3	2.2 ± 0.2	1.6 ± 0.2
20:2	n.d.	n.d.	n.d.	1.5 ± 0.3	1.7 ± 0.2	1.2 ± 0.2
20:4	5.1 ± 3.5	18 ± 1.6	8.7 ± 2.1	12.8 ± 0.6	11.0 ± 0.6	17.1 ± 2.5
22:0	n.d.	n.d.	n.d.	n.d.	1.6 ± 0.2	1.8 ± 0.3

[a]Values are mean % ± S.E. For analysis of the fatty acids of cell cultures, lipid was saponified at 70 C for 4 hr in 4 N Ethanolic NaOH. After removal of nonsaponifiables and acidification, fatty acids were extracted with ethyl ether and converted to methyl esters by reaction with diazomethane (16). For mouse blood and ascites tumor cells, lipids were extracted and fatty acid composition determined by gas liquid chromatography as described in Methods.

[b]Data of Spitzer et al. (24).

[c]n.d. = None detected.

Column temperature was 165 C; injector temperature was 268 C; and the carrier gas (helium) flow rate was 75 ml/min.

Cultures were grown as monolayers using conventional sterile techniques. They were supplemented with serum and an antibiotic mixture of penicillin, streptomycin, mycostatin and achromycin. When chemically-defined media was used, the antibiotics were omitted. Incubation temperature was 37 C, and the gas phase and pH in cultures were adjusted by flushing with filtered 5% CO_2 in air before closing the bottles. Solutions for addition to experimental cultures were sterilized by autoclaving or, for thermolabile components, by filtration through Millipore bacteriological filters. After being washed twice with 2 volumes of balanced salt solution, cells were harvested from the glass by trypsinization or by scraping with a rubber policeman.

Chemically defined media and sera were obtained from Microbiological Associates, Bethesda, Md. Human serum was inactivated by heating at 57 C for 1 hr before use to destroy toxic factors. Stock L-2071 strain mouse fibroblast cells adapted to growth on synthetic medium for a number of years were supplied by Virginia Evans' laboratory, National Cancer Institute, and MBIII cells by G.O. Gey, Finney Howell Cancer Research Laboratory, Johns Hopkins Hospital. ^{14}C-labeled compounds ([2-^{14}C] acetate, [2-^{14}C] mevalonolactone, [U^{14}C] glucose, 1-^{14}C palmitic and oleic acids, tripalmitin and 4-^{14}C cholesterol), were from Nuclear Chicago Inc., and were checked for purity by paper and silicic acid chromatography. Purified lipids for addition to cultures were from California Biochemicals and Nutritional Biochemicals Companies. Silicic acid, "chromatography grade," was from Mallinckrodt Chemicals and was sieved between 100 and 200 mesh screens before use.

Cholesterol-4-^{14}C α-α methyl ethyl caproate was synthesized according to the method of Swell and Treadwell (5), and purified by chromatography on silicic acid. Cholesterol in lipid extracts was precipitated for radioactivity determinations by the digitonin-$AlCl_3$ procedure (6).

Radioactive lipids were added to the medium, dissolved in small amounts of ethanol, and sterilized by filtration. Water soluble radioactive compounds were added as a sterile saline solution. Radioactivity determinations were made by liquid scintillation counting, with appropriate corrections for sample quenching.

RESULTS

EFA in Cultured Cells

The fatty acid composition of cells cultured in the presence of normal serum usually reflects the fatty acid composition of the culture medium. Table I shows examples of the similarity between the fatty acids of a number of different kinds of cells and their culture media. The fatty acids in WI-38 and WI-38VA13A cells are similar, and resemble the fatty acids of calf serum which was used in the culture medium; similarly the fatty acids of two strains of ascites tumor cells (the Ehrlich Ascites and an ascitic form of Sarcoma 180) closely paralleled the

TABLE II

Fatty Acid Biosynthesis by L-Strain Mouse Fibroblasts From Various ^{14}C Precursors in Lipid Free Synthetic Medium[a]

	^{14}C Oleic acid		^{14}C Glucose		^{14}C Acetate	
Fatty acid	Mass	Isotope	Mass	Isotope	Mass	Isotope
16:0	24	0	26	31	24	39
16:1	11	0	8	6	10	7
18:0	22	0	16	8	21	22
18:1	36	100	38	42	37	20
18:2	6	0	10	0	7	0

[a]Monolayers of L 2071 strain mouse fibroblasts were incubated in serum free culture medium (NCTC 135) containing either 1-^{14}C oleic acid, U-^{14}C glucose or 2-^{14}C acetate, for 96 hr. Lipid was extracted and methyl esters prepared as described in Methods. Extracts were analyzed for both chemical composition (listed under the column heading "Mass" in the table) and for radioactivity in the individual fatty acids (listed as "Isotope") using a 1662 series gas chromatograph fitted with a stream splitter and a radioactivity monitor (H.C.L. Scientific). Note that the small amounts (8-10%) of linoleic acid found in cells cultured in lipid free chemically defined medium are not synthesized de novo from any of the tested precursors. Note that also oleic acid supplied exogenously in the culture medium is incorporated intact into the cellular lipids without apparent interconversion to other fatty acids.

fatty acids in the serum of mice in which they were grown. Further evidence that fatty acid composition is usually regulated in a passive manner by uptake of serum fatty acids without extensive modification or interconversion comes from experiments in which a single labeled fatty acid (^{14}C oleic acid) was added to lipid free synthetic media (Table II). The label is found exclusively in the oleic acid of the cell lipids, and no interconversion of fatty acids by these cells was observed over the 4 day growth periods employed, i.e., under conditions in which the serum lipids are not completely depleted. These results imply therefore that under normal (short term) culture conditions, the fatty acid composition of cells is regulated by nonselective uptake of all available serum fatty acids.

However where there is a deficiency of EFA

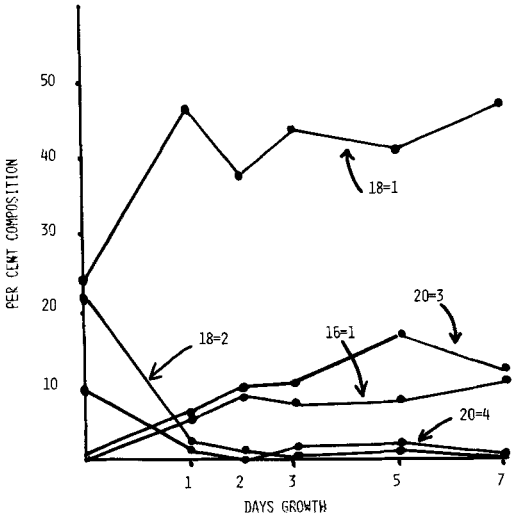

FIG. 1. Changes in fatty acid composition of normal Sarcoma 180 cells grown in EFA deficient mice. Ehrlich Ascites or Sarcoma 180 cells were grown in the peritoneum of CF1 mice fed either normal diet (Purina chow) or fat free diet (Nutritional biochemicals). At the indicated intervals cells were harvested and lipid extracted and fatty acid methyl esters prepared and analyzed as described in Methods.

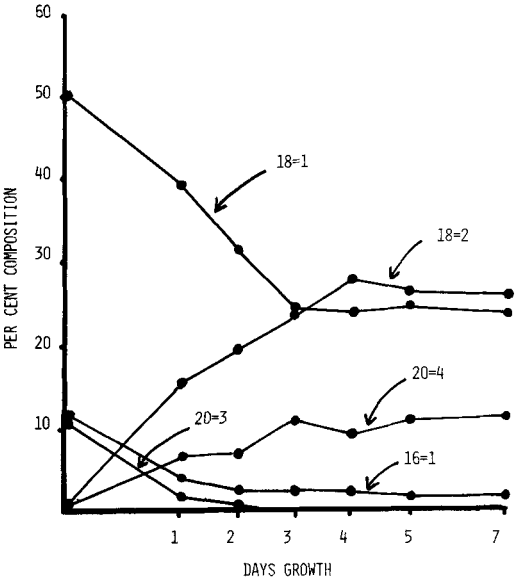

FIG. 2. Changes in fatty acid composition of EFA deficient Ehrlich Ascites cells grown in normal mice. See Figure 1.

TABLE III

Lipid Content of Lipid Free Chemically Defined Media[a]

Medium	Source	Total lipid, mg/100 ml	Linoleic acid, μg/100 ml
NCTC 135	Schwartz	1.12	30
NCTC 135	Microbiological	1.49	39
NCTC 109	GIBCO	1.31	33
7C LF	Custom	0.43	10.3

[a]100 ml quantities of various commercially available synthetic media were dried by lyophilization and the lipids were extracted and analyzed by gas liquid chromatography as described in Methods. The 7CLF medium was custom prepared (using the formula of Gey et al. (17) but omitting lipids), from components which were individually tested for lipid contamination (and if necessary freed from lipid by cold hexane extraction). The figures for 7CLF, showing a residual contamination of some 10 μg/100 ml of linoleic acid, illustrate the difficulty of obtaining a synthetic medium truly free from essential fatty acids.

in the culture medium, the cells compensate in several ways. When L strain mouse fibroblasts were cultured in lipid free medium, i.e., one that had no EFA supply, the cells synthesized most of the cell lipids from simple precursors in the medium. Under these conditions the content of linoleate drops from 25% of the total fatty acids for cells grown on serum to about 5%, but rarely falls below this level. However this residual level is not the result of biosynthesis, for when ^{14}C-glucose or acetate was added to the medium no radioactivity was incorporated into the linoleic acid fraction (Table II), although all of the nonessential fatty acids were labeled. This shows that the traces of EFA present in the cells are not synthesized de novo, but represent a very efficient conservation or scavenging mechanism on the part of the cells. We have found that commercially available synthetic media contain appreciable amounts of EFA as contaminants, and EFA are also present in traces in media custom made in the laboratory using stringent precautions to avoid contamination by lipids.

TABLE IV

Fatty Acid Composition of MBIII Cells Grown on Lipid Free Medium[a]

Fatty acid	Control, 10% serum	Lipid free
14:0	4.3 ± 0.9	Trace
16:0	23.5 ± 2.7	8.9 ± 4.1
16:1	13.8 ± 2.0	4.0 ± 2.3
18:0	19.5 ± 4.2	5.2 ± 1.4
18:1	28.9 ± 2.3	5.2 ± 4.3
18:2	11.2 ± 1.9	5.2 ± 3.4
18:3	---[b]	---[b]
20:3	---[b]	29.0 ± 5.0
20:4	---[b]	Trace
22:3	---[b]	42.6 ± 6.6

[a]Cells were adapted to lipid free medium 7CLF over a period of 3-4 weeks by serial passage into decreasing serum concentration. Data was obtained from cultures which had been on lipid free medium for 7 weeks. Note that 20:3 and 22:3, not normally found in cells, comprise over 70% of the fatty acids in cells grown in synthetic lipid free medium.

[b]Did not appear on chromatogram.

The studies of EFA deficient cells show that in addition to scavenging EFA they also adapt, by accumulating large amounts of polyunsaturated fatty acids of a type not usually found in normal cells. Figure 1 shows the fatty acid composition of Ehrlich Ascites cells grown in EFA deficient mice, an experimental situation in which rapid and efficient depletion of EFA can be effected. The content of 18:2 and 20:4 drops in 2-3 days, and there is an appearance of 20:3 which increases from undetectable levels to almost 15% of the total fatty acids. The levels of 20:3 decrease when cells are transplanted back into normal mice, and the essential fatty acid composition rapidly returns to normal (Fig. 2). A similar appearance of new polyunsaturated fatty acids is observed in cells cultured in lipid free chemically-defined medium. This is illustrated by the data in Table IV, which compares the fatty acids of MBIII cells cultured in the presence of serum with those of cells grown in a medium (Gey's 7CS synthetic medium) devoid of fatty acids. Although the content of linoleate remains at ca. 5%, large quantities of 20:3 and 22:3 are synthesized. These polyunsaturates were also observed to accumulate in L cells and Chang liver cells grown in lipid free media. (More recent experiments have failed to confirm this finding for L cells.) These fatty acids presumably can replace the function of EFA at the cellular level, since the growth rate and transplantability of the EFA deficient ascites tumor cells are not significantly different from those of normal cells. The absence of these unusual fatty acids in normal cells implies that the pathways for their biosynthesis are probably repressed when sufficient EFA are present in the medium and are induced only when the

TABLE V

Per Cent of Cellular Lipid Derived From
Biosynthesis in Serum-Supplemented and in Lipid Free Medium[a]

	^{14}C Glucose		^{14}C Acetate	
Type of medium	Serum	Lipid free	Serum	Lipid free
S.A. precursor, dpm/μg	300	150	857	1000
S.A. cell lipid	23.3	136	5.82	82
Biosynthesized,[b] %	7.8	90	0.68	8.1

[a]Cells were cultivated in either Basal Medium (Eagle) containing 10% calf serum or lipid free NCTC 135. Glucose was measured enzymatically (Glucostat) and ^{14}C acetate was added with known amounts of unlabeled sodium acetate. After 7 days cells were harvested and lipid extracted. For additional details see Methods.

[b]Per cent biosynthesized calculated as ratio specific activity of lipid to that of precursor.

content of EFA is decreased.

The Source of Cellular Lipid in Cultured Cells

We are also studying the sources of the various components of the complex lipids in cells. When cells are cultured in lipid free chemically defined medium, all components of the lipids are derived by de novo biosynthesis. Table V shows results of experiments in which various ^{14}C-labeled carbon sources were added to the growth medium for cells cultured in the presence and absence of serum. The data indicate that glucose and acetate are the two main carbon sources for lipid biosynthesis for strain L2071 cells, and provide approximately 90% and 8% of the total lipid respectively. When the medium is supplemented with serum, however, lipid biosynthesis from the simple precursors is inhibited up to 95%. When the specific activity of the cell lipid is compared to that of the particular radioactive precursor, it is found that less than 1% of the lipid is derived from acetate and only about 8% from glucose, when serum is present. This indicates that under the usual culture conditions, when the medium is supplemented with serum the cells derive their lipids from the abundant serum lipoproteins. We then set out to determine whether there is a uniform uptake of all serum lipid or whether certain serum lipids can serve as preferential sources of cellular lipid. Isotopic dilution studies adding ^{14}C-labeled fatty acid or ^{14}C-triglyceride to the medium (Table VI) show that under normal conditions of growth, about 85% of the cell lipid is derived from serum free fatty acids and only 2% of the lipid is derived directly from serum triglyceride. These experiments suggest that although there is a far greater amount of triglyceride and phospholipid present in serum, the cells preferentially take up free fatty acid and use it to synthesize glycerides and phospholipids intracellularly. This is supported by experiments that determine the source of glycerol in the cell lipids (Table VII). The data indicate that the glycerol portion of the lipid is derived almost entirely from glucose. ^{14}C-glycerol added to the medium is utilized only in the case in which there is a large excess available. If serum glycerides or phospholipids were taken up intact, the specific activity of the lipid glycerol would be expected to be much lower than that

TABLE VI

Per Cent of Cellular Lipid Derived From
Serum Triglycerides vs. Free Fatty Acids Present[a]

Precursor	1-^{14}C Tripalmitin	1-^{14}C Na Palmitate
S.A. precursor, dpm/μg	65.8	22.7
S.A. cell lipid	1.49	19.4
Derived lipids,[b] %	2.3	85

[a]WI-38 cells were grown to a 10-fold increase in cell mass in 10% calf serum to which tracer amounts of radioactive lipid were added. Specific activity of precursors was assayed by extracting total lipid, and isolating free fatty acid and triglyceride by the method of Howard and Kritchevsky (18). Triglyceride was assayed by the method of van Handel and Zilversmit (19), and fatty acid by the method of Duncombe (20). For additional details see Methods.

[b]Per cent of lipids derived from given precursor calculated as ratio S.A. of lipid to that of precursor.

TABLE VII

Source of Glycerol in Cell Lipids[a]

Precursor	^{14}C Glucose	^{14}C Glycerol	^{14}C Glycerol plus excess glycerol
S.A. precursor, dpm/μg	66.7	2605	136
S.A. lipid glycerol	56.8	232	24
Derived from given precursor, %	85	8.8	17

[a]L cells were subcultivated in Minimal Medium (Eagle) containing 10% fetal calf serum and supplemented with ^{14}C glucose or glycerol. Glucose in the medium was measured enzymatically (Glucostat), and glycerol by the method of Spinella and Mayer (21). Cell lipids were saponified overnight at 60 C in 4 N ethanolic KOH. Glycerol was assayed on the remaining aqueous residue by the method of Bailey (22) after both nonsaponifiables and fatty acids were extracted.

observed experimentally. It should be noted that the derivation of the glycerol portion of the triglyceride molecule from glucose can account for the above-mentioned figure of 8% of the lipid derived from glucose when cells are grown in serum. Further evidence on the differential sources of the precursors of the complex lipids is obtained when phospholipid synthesis is studied. When ^{32}P and ^{14}C-acetate incorporation into lipids of these serum-grown cultures are compared (Fig. 3) the rate of ^{14}C-acetate incorporation is low, indicating that the fatty acid portion is not synthesized de novo. ^{32}P incorporation is much more rapid, suggesting that phospholipids are assembled intracelluarly rather than being taken up and used intact. Our calculations indicate that the rate of ^{32}P incorporation into lipids is more than adequate to account for the measured cellular content of phospholipids.

Since the experiments described above were conducted using cultures having a relatively low population density (10^5 cells/ml), one obviously wonders what happens in conditions in which the free fatty acid is depleted. Figure 4 shows results of earlier experiments not utilizing radioactive precursors, which indicated that both triglyceride and phospholipid can be utilized in serum supplemented cultures. In these cultures, which were 50-100 times more dense than those of the above experiments (having 5 x 10^6 cells/ml), 100% of the triglyc-

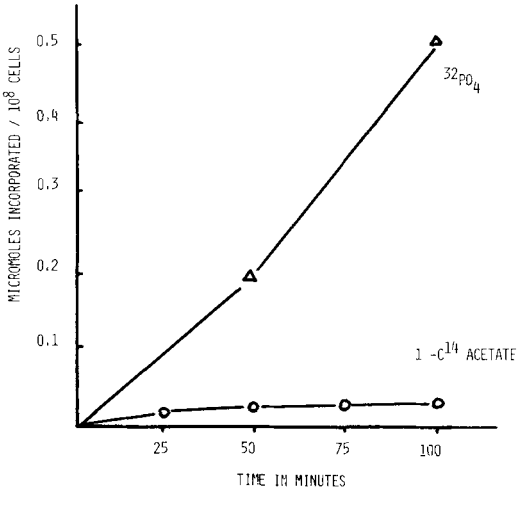

FIG. 3. Incorporation of $^{32}PO_4$ and acetate-1-^{14}C into phospholipids of WI-38 cells. WI-38 cells grown in Basal Medium Eagle supplemented with 10% calf serum were harvested and suspended in buffered balanced salt solution (10^7/ml) containing the appropriate isotope of known specific activity. The suspension was shaken in a water bath at 37 C and at intervals aliquots were removed and the cells washed in cold balanced salt solution. Phospholipids were isolated by thin layer chromatography, and radioactivity was determined without elution from the silicic acid by the method of Snyder and Stephens (23).

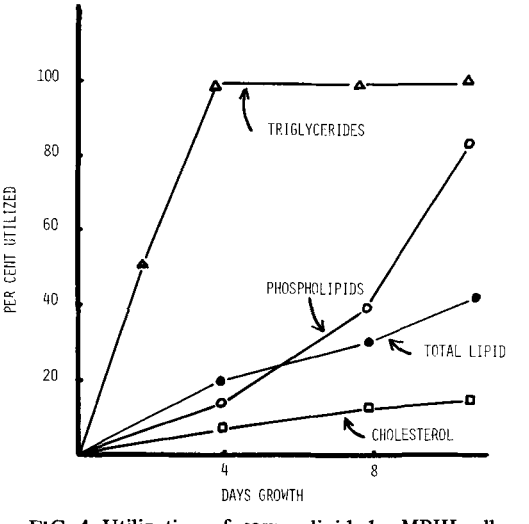

FIG. 4. Utilization of serum lipids by MBIII cells. Depletion of phospholipids and triglycerides was measured in roller tube cultures of MBIII cells in a medium consisting of human placental cord serum diluted with one part of balanced salt solution. Lipids were extracted and assayed as described in Methods. Final population density was 6x10^6 cells/ml.

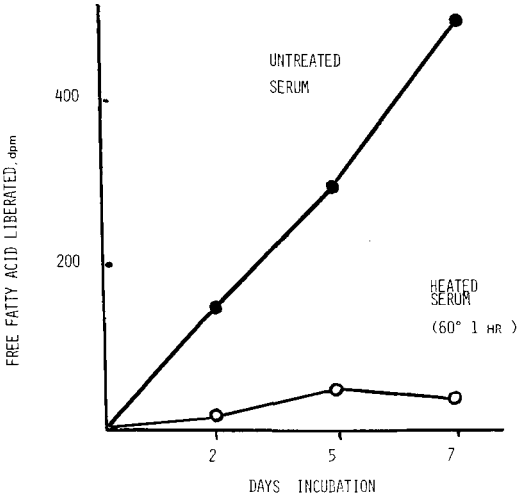

FIG. 5. Hydrolysis of ^{14}C-triglyceride in culture medium supplemented with fetal calf serum in the absence of cells. Minimal Medium (Eagle) was supplemented with 10% fetal calf serum to which had been added tracer amounts of ^{14}C carboxyl labeled tripalmitin using the procedure described in Methods. The sterile medium was incubated for 7 days at 37 C. Lipid was extracted from samples of the medium at the indicated intervals, and fatty acid was isolated and the radioactivity determined as described in the footnote to Table 6.

eride was depleted and more than 50% of the phospholipid was also taken up. Recent experiments suggest that the triglyceride and presumably also phospholipid are not taken up intact, however, but are first hydrolyzed to free fatty acids. Fatty acid utilization was assayed suing triglyceride labeled with ^{14}C fatty acids in cultures having a population density of about 10^6 cells/ml. Under these conditions 33% of the cell lipid was derived from triglyceride; however triglyceride radioactivity also appears in the free fatty acids of the medium. When the specific activity of the various fractions is measured, it is found that the specific activity of the cell lipids more closely resembles that of the free fatty acids in the medium rather than the triglycerides. Moreover under the same culture conditions, when radioactive free fatty acid was added, a rapid decrease in radioactivity in the medium was observed (Table VIII). Almost all of the original fatty acid as measured by radioactivity was utilized, but the actual free fatty acid content of the medium as measured by chemical analysis remained relatively constant during the 7 day culture period. This implies that the fatty acid used is being replaced from some endogenous source. The source of the nonradioactive free fatty acid is most likely serum triglycerides, although there is evidence suggesting that sterol esters may also

TABLE VIII

Utilization of Serum ^{14}C Fatty Acids in Dense Cultures[a]

	Day 0	Day 7
dpm	49,900	992
μg	220	306
Specific activity	442	3.24

[a]L strain mouse fibroblasts were cultivated in Basal Medium (Eagle) supplemented with 5% fetal calf serum. ^{14}C-Palmitic acid was added to the medium in tracer amounts. Specific activity of the fatty acids in the medium was measured at zero time and after growth for 7 days as described in Table VI. Note the marked decrease in radioactivity without a corresponding decrease in the total fatty acid content of the medium.

be a significant source of free fatty acid (see below). The results suggest that when triglyceride is hydrolyzed, the products enter the free fatty acid pool of the medium and are taken up and utilized by the cells in that form.

The mechanism of the hydrolysis of triglyceride is currently being investigated. Preparations of calf and fetal calf sera appear to contain a triglyceride lipase (Fig. 5) which can be detected by addition of ^{14}C-triglyceride to culture medium in the absence of cells. This hydrolysis of triglyceride is eliminated by preheating the serum at 60 C. The observed rate of hydrolysis is not large enough, however, to account for the total cell free fatty acid requirements in dense cultures. It seems that under these conditions hydrolysis is also mediated by a cellular enzyme. The possible secretion of such a lipase into the medium and conditions which may regulate this are at present under investigation.

Uptake and Excretion of Sterol Esters by Cultured Cells

Earlier experiments indicated that when cultured in lipid free medium, cells are able to synthesize cholesterol from labeled acetate or glucose added to the medium. This biosynthesis is inhibited up to 95%, however, when serum or cholesterol itself is added to the medium. The studies indicated that when grown in serum, most of the cell cholesterol is derived from the medium. The proportion of ester to free cholesterol in serum, however, is usually ca. 2:1, whereas in cells the free form of cholesterol generally predominates. Work from both our laboratory and that of Rothblat and Kritchevsky (7) indicated that there was a steady flux of both free and esterified cholesterol into and out of the cell, with sterol levels being controlled by the rates of uptake and excretion, plus any

TABLE IX

Uptake of Labeled Cholesterol, Cholesterol Esters and a Synthetic Nonhydrolyzable Ester Analog by MBIII Cells[a]

	Radioactive test compound added		
	Cholesterol	Cholesterol oleate	Cholesterol methyl ethyl caproate
Total in medium, mg[b]	13.4	19.3	3.7
Total dpm in medium[b]	923,200	137,000	24,870
Total dpm in cells[b]	225,000	33,620	7,120
Uptake of isotope, %	24.4	24.6	28.6
In cell particulates, %[c]	82	36	36
In cell sap, %[c]	18	64	64

[a]Each value represents pooled sample of five tissue culture bottles grown for 6 days in medium of 45% calf serum prelabeled with isotope by roller tube procedure. Note that cholesterol methyl ethyl caproate distributes in cell in same manner as normal esters. MBIII cells were cultured in Basal Medium Eagle containing 45% calf serum prelabeled with isotope. After 6 days cells were harvested, homogenized and centrifuged at 150,000 x g. Sterol was isolated from particulate and supernatant fractions by digitonin precipitation and radioactivity was assayed as described in Methods.

[b]Based on 100 ml medium.

[c]Based on chemical identification of isotope by digitonin fractionation.

endogenous synthesis. The uptake of esterified cholesterol by cells could not be easily studied, however, because esters readily undergo both extracellular and intracellular hydrolysis and transesterification. We have therefore utilized a synthetic cholesterol ester, cholesterol 4-^{14}C-α, α-methyl ethyl caproate (CMEC), which because of the steric hindrance of the α-Carbon atom is completely resistant to hydrolysis by esterases. The radioactive CMEC was introduced into serum by depositing the compound as a thin film inside roller tubes, and then allowing it to exchange with serum cholesterol esters by incubating the serum in the roller tube for 3 days. Cultures of MBIII mouse lymphoblast cells were then grown on medium containing the labeled serum (Table IX). The data showed that the labeled CMEC was taken up over the 6 day growth period to approximately the same extent as labeled cholesterol and cholesterol esters. Furthermore the radioactive CMEC in cell homogenates distributed between the particulate and supernatant cell fractions to the same proportions as normal cholesterol esters. Excretion was then measured in stationary cultures of prelabeled cells (Table X). In the presence of serum there was a progressive release of radioactivity from all three components—cholesterol, cholesterol ester and CMEC, into the medium; and the rates of excretion did not differ significantly for the three types of compounds. These results showed that esters are not necessarily hydrolyzed to the free form, but may also be taken up and excreted intact.

TABLE X

Excretion of Labeled Cholesterol, Cholesterol Esters and Ester Analog From Cultured MBIII Cells[a]

Time of incubation	Cell population, millions/ml	Per cent isotope excreted		
		Cholesterol	Cholesterol oleate	Cholesterol methyl ethyl caproate
2 hr	4.45	9.6	10.0	3.5
2 days	4.60	25.8	26.4	34.4
5 days	5.23	72.8	69.7	53.8
8 days	4.44	73.1	70.8	56.2

[a]Each value is the average of two tissue culture bottles harvested at the indicated times. Population density was chosen so as to maintain cells in steady state. Medium was 45% calf serum in Basal Medium Eagle. Stationary phase cultures of MBIII cells which had accumulated isotope from prelabeled serum were transferred to medium containing fresh unlabeled serum. At various times bottles were harvested and total lipid was extracted. Cholesterol and cholesterol esters were isolated by the digitonin procedure, and radioactivity was determined as described in Methods.

These experiments indicate that cell levels of cholesterol and cholesterol esters thus depend on the rates of uptake and excretion of both forms of the sterol independently, in addition to the previously observed intracellular hydrolysis of the esters (8).

DISCUSSION

Our studies of EFA deficient cultured cells can be correlated with studies on EFA deficient animals by Mead (9) and Klenk (10). These investigators reported the presence of an elongation and desaturation pathway beginning with either oleic or palmitoleic acids, and resulting in polyunsaturated fatty acids having the structures ω-7 or ω-9. It is most likely that the fatty acids identified as 20:3 and 22:3 observed in our cell cultures under EFA deficient conditions represents induction of these biosynthetic pathways, since there is independent evidence that these pathways are probably not active in cells growing in serum. These enzymes may be the same or different from those normally involved in conversion of linoleic acid to arachidonic. EFA deficient cultured cells afford an opportunity to study the role of EFAs in cell metabolism, and further experiments are in progress to investigate the mechanism of induction of synthesis of unusual polyunsaturates and their role in replacing the essential fatty acids in normal cell functions.

Earlier studies (11) indicated that there is efficient regulation of cellular lipid biosynthesis. The results so far do not indicate unequivocally which enzymes are involved, and whether the regulation is at the level of enzyme activity or if induction and repression of enzyme synthesis is involved. Preliminary studies indicate that significant increases in an acetate-activating enzyme system occur when cells are transferred to serum free medium containing acetate. The changes in rates of lipid biosynthesis in the presence and absence of serum should provide an excellent model system for the study of mechanisms of enzyme regulation in mammalian cells.

Cultured cells also provide an experimental system for the study of cellular lipid uptake and excretion. The observation that free fatty acid is the primary source of cell lipid is consistent with experiments of Mackenzie et al. (12) and Geyer (13) who linked accumulation of intracellular fat droplets with the free fatty acid content of sera. The results from cell cultures also correlate with studies in vivo. Shapiro and others have demonstrated that in the intact animal, plasma free fatty acid, although only a small proportion of the total serum lipid, has a very short half life (14). In addition recent in vivo experiments with radioactive precursors suggest that even liver cells are unable to utilize intact triglycerides (15). We are currently extending our studies on lipid utilization to investigate conditions in which free fatty acid is depleted or absent. It seems that the utilization of serum glycerides and phospholipids under these conditions may be mediated by lipolytic enzymes, similar to those observed in vivo.

In summary, results have been presented which indicate that cultured cells control their lipid composition in a number of different ways. One is by regulation of biosynthesis. Cells were observed to synthesize lipid in lipid free medium, and this synthesis was inhibited in the presence of an external lipid supply. Similarly under conditions of EFA deficiency, quantities of polyunsaturated fatty acids of a type not normally found in large proportions are biosynthesized. This synthesis is repressed during normal EFA supply. However an additional type of regulation, involving transport into and out of the cell seems to be equally important in cell cultures. Cholesterol and cholesterol ester content is a result of both passive uptake and selective excretion. The cells preferentially utilize free fatty acid as a source of nonsterol lipid and esterify it to make glycerides and phospholipids. In addition permeability of essential fatty acids may increase under conditions in which they are depleted. Under favorable conditions all of these mechanisms act in concert toward the end result of a lipid composition which is optimal for the cellular requirements.

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REFERENCES

1. Folch, J., M. Lees and G.H.S. Stanley, J. Biol. Chem. 226:497 (1957).
2. Stahl, E., "Thin Layer Chromatography," Springer-Verlag, New York, 1969.
3. Bailey, J.M., and J. Menter, Proc. Soc. Exp. Biol. Med. 125:101 (1967).
4. Hyun, S.A., G.V. Vahouny and C.R. Treadwell, Anal. Biochem. 10:193 (1965).
5. Swell, L., and C.R. Treadwell, J. Biol. Chem. 212:141 (1955).
6. Vahouny, G.V., C.R. Boya, R.M. Mayer and C.R. Treadwell, Anal. Biochem 1:371 (1960).
7. Rothblat, G., and D. Kritchevsky, Exp. and Molec. Pathol. 8:314 (1968).
8. Bailey, J.M., in "Lipid Metabolism in Tissue Culture Cells," Edited by G.H. Rothblat and D. Kritchevsky, Wistar Inst. Symp. Monogr. No. 6, Wistar Press, Philadelphia, p. 63, 1967.
9. Mead, J.F., in "Drugs Affecting Lipid Metabo-

lism," Elsevier Press, New York, p. 16, 1961.
10. Klenk, E., in "Drugs Affecting Lipid Metabolism," Edited by S. Garattini and R. Paoletti, Elsevier Press, New York, p. 21, 1961.
11. Bailey, J.M., Biochem. Biophys. Acta 125:226 (1966).
12. Mackenzie, C.G., J.G. Mackenzie and O.K. Reiss, in "Lipid Metabolism in Tissue Culture Cells," Edited by G.H. Rothblat and D. Kirtchevsky, Wistar Inst. Symp. Monogr. No. 6, Wistar Press, Philadelphia, p. 63, 1967.
13. Geyer, R.P., Ibid. p. 33.
14. Shapiro, B., in "Lipids and Lipidoses," Edited by Schettler, Springer-Verlag, New York, p. 40, 1967.
15. Felts, J.M., and M.N. Berry, Biochem. Biophys. Acta 231:1 (1971).
16. James, A.T., Meth. Biochem. Anal. 8:1 (1960).
17. Ling, C.T., G.O. Gey and V. Richters, Exp. Cell Res. 52:469 (1968).
18. Howard, B.V., and D. Kritchevsky, Lipids 5:49 (1970).
19. Van Handel, S., and D.B. Zilversmit, J. Lab. Clin. Med. 50:152 (1957).
20. Duncombe, W.G., Biochem. J. 88:7 (1963).
21. Spinella, C.J., and M. Mayer, J. Lipid Res. 7:167 (1966).
22. Bailey, J.M., J. Lab. Clin. Med. 54:158 (1959).
23. Snyder, F., and N. Stephens, Anal. Biochem. 4:128 (1962).
24. Spitzer, J.J., and M. Gold, Proc. Soc. Exp. Biol. Med. 110:645 (1962).

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