Lipid Metabolism in Cultured Cells. V. Comparative Lipid Nutrition in Serum and in Lipid-Free Chemically Defined Medium.* (29026)

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It has been shown that mammalian cells in tissue culture derive their lipid requirements from serum customarily used in growth medium(1). In recent years, culture media have been developed by Evans(2), Healey(3), Waymouth(4) *et al,* in which serum nutrient is replaced by complex but completely chemically defined mixtures. Since synthetic media contain no complex or preformed lipids, it was of interest to determine lipid composition and comparative lipid nutrition of cells grown on the new media. The strain of cells chosen, L-strain mouse fibroblasts, has been grown on synthetic medium NCTC-109 over a period of 7 years(2). It still grows perfectly well, however, in 20% human serum, and can be readily transferred from one medium to the other. This paper describes experiments in which comparative lipid nutrition of L-strain cells in the 2 types of medium was studied.

Materials and methods. Chemically defined culture medium NCTC 109 and samples of calf, chicken and horse serum were supplied by Microbiological Associates, Inc., Bethesda, Md. Human serum was from Philadelphia Serum Exchange. 1-C^{14}-acetate was from from New England Nuclear Corp. Antibiotics used in culture medium (penicillin, streptomycin, achromycin and mycostatin) were from Z. D. Gilman, Washington, D. C. and Squibb Pharmaceuticals, Inc. Original culture of L-strain mouse fibroblasts adapted to growth on NCTC 109 medium was kindly supplied by laboratory of Dr. W. R. Earle, Nat. Cancer Inst. Stock cultures of L-strain mouse fibroblasts were grown in 15 ml capacity Leighton tubes or 100 ml milk dilution bottles in chemically defined NCTC 109 medium. When more rapid growth was required, medium was supplemented with 20% serum. L-strain mouse fibroblasts grow as adherent sheets on glass walls of culture vessels. Sheets were broken up into small clumps of individual cells by gentle scraping. For purposes of inoculating cultures, cell populations were approximated by counting suspensions of clumped cells in hemacytometer chambers. For purposes of calculating lipid composition, dry weight of harvested cells was determined quantitatively by colorimetric method of Bailey and Nejad(5).

Cell lipids were extracted with 1:1 ethanol ether at 45°. Extracts were dried over sodium sulfate, evaporated to dryness in nitrogen atmosphere and redissolved in boiling petroleum ether (B pt 30°-60°). Lipids in culture medium were extracted under reflux for 45 minutes with 2 × 40 volumes of 1:1 ethanol ether. Extract was evaporated and redissolved in petroleum ether, as described above. Total lipid in extracts was determined by dichromate method of Bloor(6) as modified by Bragdon(7). Total cholesterol was determined by method of Brown(8). Phospholipids were ashed by combustion with perchloric acid-hydrogen peroxide mixture, and inorganic phosphate was determined on residue by method of Allen(9). Triglycerides were determined by difference. Measurement of C^{14} in samples was made using infinite thinness plating techniques and counting in Nuclear Chicago type D47 gas flow end-window counter.

Addition of C^{14}-labeled acetate to culture media. C^{14}-labeled acetate solutions for additions to culture media were sterilized by autoclaving in saline before mixing with sterile medium constituents to give final concentrations of 14 mg/100 ml with specific activity of 0.71 μc/mg. Sterile culture conditions were maintained throughout experiments and broad spectrum antibiotic mixture (1 part to 100 parts culture medium) was also added.

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TABLE I. Balance Study on Removal of Lipids from Serum Medium by L-Strain Mouse Fibroblasts.

Time of growth	μg in 10 ml culture medium or in harvested cells			
	Cholesterol	Phospholipid	Triglyceride	Total lipid
0	70	72.9	487	630
2 days	57.3	61.2	265	384
5 "	32.4	21.9	216	270
Lipid				
Utilized (μg)	37.6	51.0	271	360
Lipid found in cells (μg)	40	97	236	383
Serum lipid utilized as % of cell lipid	94%	53%	115%	94%
% contribution to cell dry wt	4.6	11.2	27	44.8

L-strain cell culture in 100 ml capacity milk dilution bottle was harvested and medium replaced by 10.8 ml of NCTC 109 and 1.2 ml horse serum. 3 ml portions of medium were analyzed at zero time and after 2 and 5 days growth. Lipid composition of cells harvested at 5 days and lipid depletion in growth medium was measured as described in text. Triglycerides were calculated by difference of total lipid and phospholipid-cholesterol values. Lines 1,2,3 of table show progressive depletion of each lipid type with days in culture. Line 4 shows total lipid of each type removed from medium over 5 day period. Line 5 shows total lipid of each type recovered from harvested cells. Line 6 expresses depletion in serum lipid as per cent of that found in cells. Last line is per cent contribution of each lipid type to total dry weight of cell. It will be noted that 94% of cell lipid is potentially derived from serum lipid.

Mixture contained penicillin 1000 units, achromycin 50 μg, streptomycin 50 μg and mycostatin 50 μg per ml dissolved in saline and filtered through millipore filter (Grade HA, avg. pore size 0.45 μ) before use. Before closing cultures for incubation, pH of medium was adjusted to 7.4 by flushing culture tubes or bottles with mixture of 95% air-5% CO_2 through sterile cotton-plugged pipette.

Silicic acid column chromatography. Silicic acid (Mallinkrodt 100-200 mesh activated by heating at 110° overnight) was slurried into dry hexane and poured into column 1.8 cm diam. to give column height of 10-15 cm. Cell lipid extracts dissolved in petroleum ether were applied to top of column and C^{14}-lipids were eluted by successfully increasing concentrations of diethyl ether in hexane. Phospholipids were eluted with 100% methanol. Eluates were collected in 5 ml fractions and aliquots of each fraction were used for C^{14} determination and for chemical assay.

Results and discussion. Depletion of serum lipids by L-strain cells growing on 20% serum supplemented medium for 5 days is shown in Table I. Progressive removal of cholesterol, phospholipids and triglycerides was found, with triglyceride being most rapidly utilized. Decrease in lipid content of serum medium accounts for 94% of total lipid recovered from harvested cells. Only phospholipids of cells were apparently synthesized *de novo*. Phospholipid removed from serum corresponded to only 53% of that found in harvested cells. Decrease in serum triglycerides was 115% of that found in cells, thus suggesting possible conversion of triglycerides to phospholipid. Similar apparent conversion of triglyceride to phospholipid in the MB III strain mouse lymphoblast grown on human serum has been reported(1).

Growth rate of L-strain cells on serum-free chemically defined medium is somewhat less than in presence of serum. Total weight of cells harvested from 10 ml of serum supplemented medium is also 2-3 times that with synthetic medium alone. Despite absence of exogenous lipids in chemically defined medium, lipid composition of L-strain cells grown on synthetic medium alone was remarkably similar to that of cells grown on serum supplemented medium. The only significant difference was lower phospholipid content (Table II). It was apparent that cell lipids were now synthesized from simpler precursors in chemically defined medium. Breakdown of 68 components of synthetic medium NCTC 109 into major categories as possible carbon source is in (2). This shows 2 probable carbon sources for synthesized lipid; glucose,

TABLE II. Lipid Composition of L-Strain Cells Grown on Chemically Defined and Serum Supplemented Media.

Growth medium	% of dry weight of cell*			
	Total lipid	Phospholipid	Triglycerides	Cholesterol
NCTC 109	45 ± 3.0	6.8 ± 1.4	34 ± 3 3	3.9 ± .4
Plus 25% serum	44 ± 2.2	11.2 ± .7	27 ± 4.1	4.6 ± .4

* Mean ± S.E.M.

L-strain cells were grown for 4 days on synthetic medium NCTC 109 (10 ml) in 100 ml capacity milk dilution bottles both with and without addition of human serum (25%). pH of growth medium was maintained at 7.4 with gas phase of 95% air/5% CO_2. Cell lipids were extracted and determined as described in *Methods* section. Triglycerides are calculated by difference from total lipid and phospholipid-cholesterol values.

present at 100 mg/100 ml, and sodium acetate at 5 mg/100 ml. Since 100 ml of synthetic medium will support growth of approximately 8-10 mg of L-strain cells over a 5-day period, and L-strain contains about 40% lipid, it is apparent that these 2 components either single or in combination, are potentially a sufficient carbon source. We have also found† that L-strain cells utilize detergent Tween 80 as carbon source. This (polyoxyethylene sorbitan mono-oleate) is present in synthetic medium NCTC 109 merely as a vehicle for solubilization of fat soluble vitamins(2). In 7-year period during which L-strain has been grown on this medium, it has apparently adapted to use oleic acid portion as source of carbon for lipid synthesis.

Depression of de novo *lipid synthesis by added serum.* Most of L-strain lipid has been shown by experiments described above to be derived from serum when grown on serum medium, and from simpler precursors in medium when grown on lipid-free medium (Table II). Lipid composition of harvested cells was, however, essentially the same in both media. It was concluded that in presence of serum, synthetic pathway for lipid synthesis must be inactive. This was confirmed by experiments shown in Table III. L-strain cells were grown in NCTC 109 medium supplemented with C^{14}-acetate. Incorporation of C^{14} into total cell lipids was measured with various additions to growth medium. In synthetic medium alone about 20% of cell lipids were derived from acetate. Addition of 2.5%, 10% and 20% human serum depressed acetate incorporation into cell lipids by 69, 82 and 87%, respectively. In individual experiments up to 98% inhibition of acetate incorporation was observed in 20% serum medium. Inhibition is manifested both by L-strain cells preadapted to growth on 20% serum for 1 or 2 culture transfers, and by cells transferred directly from synthetic medium. Total acetate incorporation into cell lipids of serum adapted cells in serum-free medium was slightly less than that for cells preadapted for a number of generations to growth on synthetic medium alone.

Cell lipids were fractionated on silicic acid and radioactivity in each lipid fraction was measured. Marked inhibition of C^{14}-acetate

† J. M. Bailey, unpublished data.

TABLE III. Inhibition of C^{14}-Acetate Incorporation into L-Strain Cell Lipids by Added Serum.

No. of exp	Added to NCTC 109 growth medium	Total c.p.m. in cell lipids*	% inhibition
8	None	8725 ± 650	Control
3	2.5% serum	2683 ± 610	69
3	10 % "	1563 ± 555	82
7	20 % "	1151 ± 340	87

* ± standard error of mean.

Stock L-strain cells (preadapted for a number of generations on synthetic medium alone) were grown in 10 ml portions of NCTC 109 medium supplemented with C^{14}-acetate (see text). Control cultures contained no further additions. Experimental cultures were supplemented with either 2.5%, 10% or 20% human serum and final volume of all cultures was adjusted to 12.5 ml with Gey's balanced saline. pH was adjusted to 7.4 with gas phase of 95% air, 5% CO_2 and cultures were grown for 4 days at 37° in 100 ml capacity milk dilution bottles. Cells were harvested, washed twice with saline and lipids extracted as described in *Methods* section. Dry weight of lipid extracted cell residue was determined colorimetrically(5). Radioactivity in cell lipids is expressed on basis of 1 mg dry weight cells.

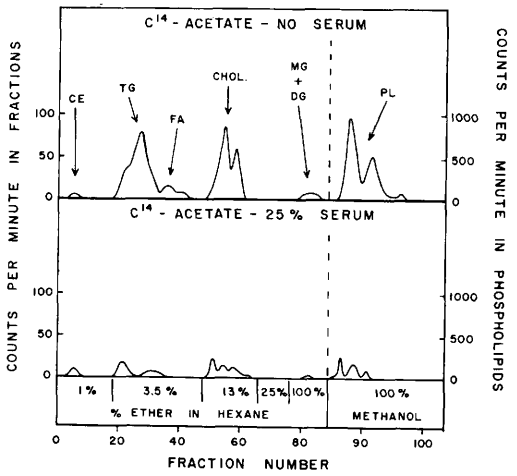

FIG. 1. Silicic acid chromatography of L-strain mouse fibroblast lipids following growth on C^{14}-acetate supplemented medium. Cells were grown on chemically defined medium alone or in same medium supplemented with 25% human serum. Medium contained C^{14}-acetate (14 mg/100 ml specific activity 0.7 μc/mg). Following growth for 4 days at 37°, cells were washed with saline and radioactive lipids extracted, as described in *Methods* section. Representative aliquots of C^{14}-labeled lipid extracts dissolved in petroleum ether were applied to top of silicic acid column (15 cm × 1.8 cm diam.) and 5 ml fractions were eluted with successively increasing concentrations of ether in hexane. Cholesterol esters (CE) were eluted by 1% ether, triglycerides (TG) and fatty acids (FA) by 3.5% ether, cholesterol (CHOL) by 13% ether, and mono- and diglycerides (MG + DG) with 25% and 100% ether, respectively. Phospholipids (PL) were removed by 100% methanol. Upper curve shows radioactive lipids from cells grown on synthetic medium alone; lower curve shows lipids from cells grown on serum supplemented medium.

incorporation by added serum affected synthesis of all lipid classes (Fig. 1). The following comparative analysis of percentage inhibition was obtained: Cholesterol esters (60%); triglycerides and fatty acids (89%); cholesterol (83%); mono- and diglycerides (82%) and phospholipids (86%). High relative incorporation into phospholipid fraction (Fig. 1) has been found to result from presence of bound amino acid in this fraction. Properties of this bound amino acid have been described(10). The location and nature of apparent "feedback" blocks produced by added serum are being further studied.

Summary. Mammalian cells in tissue culture derive most of their lipid requirements from serum customarily used in growth medium. L-strain mouse fibroblasts were grown both on serum supplemented medium and on a recently developed chemically defined lipid-free medium (NCTC 109). Lipid composition of cells was essentially independent of growth medium used. When grown in synthetic medium, cell lipids were synthesized *de novo* from C^{14}-acetate added to medium. In serum supplemented medium *de novo* synthesis from C^{14}-acetate was almost completely depressed. C^{14}-labeled cell lipids were fractionated on silicic acid columns. Serum depressed acetate incorporation approximately equally in all lipid classes. It seems possible that this represents operation of feedback type mechanism for control of cellular lipid composition.

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