Lipid Metabolism in Cultured Cells VII. Linoleic Acid Content of Cells Grown on Lipid-Free Synthetic Medium.* (32024)

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Most strains of cells maintained in tissue culture are grown in media containing added serum. The relatively high lipid content of serum, coupled with the favorable proportions of polyunsaturated fatty acids means that adequate quantities of these compounds are usually available to the cells.

A limited number of cell strains however can now be grown on serum-free chemically defined media(1). In particular, the 2071 subline of L-strain mouse fibroblast has been grown for a number of years in a lipid-free medium. (NCTC 109)(2). This cell line also grows well in serum supplemented media, and when grown under these conditions derives most of the cell lipids from the serum(3). When grown in synthetic medium however the cell lipids are synthesized from simpler components of the medium, principally acetate and glucose(4). This paper describes experiments in which the fatty acid composition of L-strain cells grown both in serum and lipid-free chemically defined medium was studied. In particular, the linoleic acid content of cells after prolonged growth periods in lipid-free medium was analyzed. When considerable amounts of linoleic acid were found, the possible biosynthesis of this fatty acid from radioactive acetate added to the lipid-free growth medium was investigated.

Materials and methods. The subline of the L-strain of mouse fibroblast (L 2071) cells adapted to growth on lipid-free medium was kindly supplied by Dr. V. J. Evans of the National Cancer Institute, Bethesda, Md. Tissue culture Medium NCTC 109, serum samples and penicillin-streptomycin mixture (5,000 units of each/ml) were from Microbiological Associates, Bethesda, Md. Tween 80-free NCTC 109 medium was prepared by Microbiological Associates. Fatty acid standards were from the Hormel Institute, Austin, Minn. Azelaic acid was obtained from Eastman Organic Chemicals, Rochester, N. Y., and Caproic Acid from Nutritional Biochemicals Co., Cleveland, Ohio. Silicic Acid (100-200 mesh) was obtained from Mallinkrodt, New York, and Sodium 1-C^{14} acetate was obtained from New England Nuclear Co., Boston, Mass. Ether was rendered peroxide free by redistillation over lithium aluminum hydride. Boron trifluoride-methanol reagent was obtained from Applied Science Laboratories, State College, Pa.

Extraction and analysis of cell lipids. Cultures were grown in 100 ml capacity milk dilution bottles with 10 ml growth medium and using conventional sterile techniques. Cells from 5-6 bottles were pooled and washed twice with isotonic saline. One mg of α-tocopherol was added and the lipids were extracted with 1:1 ethanol-ether at 45°C for 45 minutes. The extract was evaporated to dryness under nitrogen and redissolved in petroleum ether. Total lipid in the extract was determined by the method of Bloor(5) as modified by Bragdon(6). Total cholesterol was determined by the method of Brown *et al*(7). Phospholipids were ashed by combustion with perchloric acid, and inorganic phosphate was determined on residue by the method of Allen (8). Lipid fractionations were carried out on silicic and columns using graded concentrations of ether in hexane and methanol in ether as described previously(9).

Analysis of fatty acids by gas-liquid chromatography. The extract was prepared for gas-liquid chromatography by the method of Hyun(10), by transmethylation with Boron trifluoride. Analyses of the methyl esters of L-strain mouse fibroblast fatty acids were carried out on a Perkin-Elmer model 154 Vapor Fractometer (with hot wire detector) and a 6-foot column packed with diethylene glycol succinate polyester. Long chain acids (C_{12} or longer) were analyzed at 190° and 28

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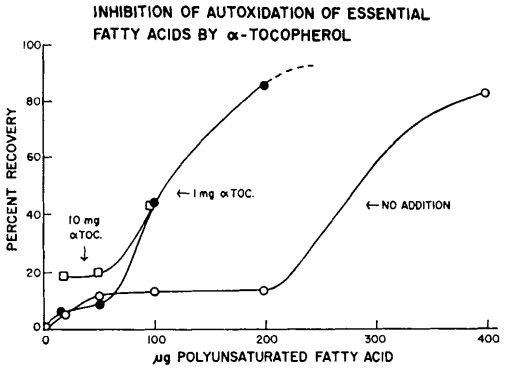

FIG. 1. A series of tubes containing quantities of methyl linoleate ranging from 10 to 400 μg dissolved in 5 ml of 1:1 ethanol ether, was submitted to the same extraction and transesterification procedures used in analysis of cell lipids (see Methods). A second and third series of tubes each contained in addition either 1 mg or 10 mg of the antioxidant α-tocopherol.

psi of helium. Short chain acids (less than C_{12}) were analyzed at 105°C and 20 psi. Lipid extracts from tissue cultures contained only 200-400 μg of unsaturated fatty acids. Calibration of the extraction procedure for quantities of this order of magnitude was carried out with a 5-acid standard containing equimolar amounts of the methyl esters of palmitic, stearic, oleic, linoleic and linolenic acids. It was found that when less than 0.4 mg of total polyunsaturated fatty acids was present, losses due to autoxidation were considerable (Fig. 1). Recovery of linoleic acid was improved when α-tocopherol was added as an antioxidant, and was essentially 100% from samples containing 200 μg or more.

Permanganate oxidation of linoleic acid. In order to locate precisely the position of the double bonds in the linoleic acid fraction, permanganate oxidation by the method of Tinoco and Miljanich(11) was carried out. The linoleic acid fraction was recovered from the collection port of the gas chromatograph by trapping in petroleum ether. Short chain monocarboxylic acids in the products of oxidation were analyzed at a column temperature of 100°C and 20 psi of helium. Short chain dicarboxylic acids plus long chain monocarboxylic acids were analyzed on the same column at 190°C and 28 psi helium. This procedure formed a useful additional identification for linoleic acid since 2 of the expected oxidation products (caproic and azelaic acids) were themselves readily identifiable by gas chromatographic analysis.

Incorporation of C^{14}-acetate into cell lipids. Synthetic growth medium was supplemented with 1-C^{14} acetate (50 microcuries per 100 ml) and to ensure maximum incorporation, cells were grown for 6 days without replenishment of the medium. Cell lipids were extracted and radioactivity in the individual fatty acids was measured in two ways. Simultaneous analysis of mass and radioactivity was made on single samples using a Barber Coleman model 5000-S gas liquid chromatograph fitted with a stream splitter and a carbon dioxide combustion flow line coupled to a proportional counter. In the second method a Perkin-Elmer gas chromatograph was equipped with a thermal conductivity detector so that the sample was not destroyed but was collected in 1 ml fractions by passing the effluent gas into petroleum ether. The apparatus was calibrated for the 'lag' (approximately 1 minute) between the thermal conductivity detector and the effluent at the sample port, by use of a standard containing C^{14}-oleic acid.

Results. Lipid composition of L-strain cells grown on serum-supplemented medium. The L2071 subline of L-strain mouse fibroblasts grew well on a medium consisting of NCTC 109 supplemented with 10% human serum. The gross lipid composition of this strain is relatively independent of the lipid composition of the medium(3). The individual fatty acid composition of the cell lipids however was very similar to that of the serum used in the growth medium. In particular, the unsaturated fatty acid composition of the cell lipids (25.3% oleate and 16.9% linoleate) was essentially the same as that of the serum (26.4% and 17.2%, respectively). The principal difference was in the lower myristate content of the cells (2.5% *vs* 10%) and the higher content of stearic acid (22.4% in cell lipids *versus* only 10% in the serum) (Table I).

Cell lipids were fractionated by chromatography on silicic acid columns into 5 major groups (cholesterol esters, triglycerides and fatty acids, cholesterol, mono and diglycerides and phospholipids). The fatty acid composi-

TABLE I. Fatty Acid Composition of L-Strain Cell Lipids Compared to Serum Growth Medium.

Fatty acid	Percent composition	
	Cell lipids	Serum lipids
Myristate (14:0)	2.5	10.0
Palmitate (16:0)	25.3	27.3
Palmitoleate (16:1)	1.7	6.3
Stearate (18:0)	22.4	10.0
Oleate (18:1)	25.3	26.4
Linoleate (18:2)	16.9	17.2

Cells were grown for 4 days on NCTC 109 medium supplemented with 10% human serum. Lipids were extracted from harvested cells and from growth medium and fatty acid composition was analyzed as described in Methods.

tion of each class was then analyzed seprately. Most of the linoleic acid was found in the phospholipid and triglyceride fractions (Table II).

Fatty acid composition of L-strain cells grown on synthetic medium. L-strain cells maintained in NCTC 109 synthetic medium grew more slowly than in serum supplemented medium with a doubling time between transfers of about 1 week. In preliminary experiments, analysis of the fatty acid composition of cells grown on synthetic medium indicated that although the linoleic acid content was much less than that of cells grown in serum, the cell lipids still contained about 7% of this compound even after prolonged periods of growth in the lipid-free medium.

The possibility of contamination of the medium or glassware with extraneous linoleic acid was therefore examined. A 50 ml portion of the medium was lyophilized and the residue extracted with lipid solvents and analyzed. The principal fatty acid found was oleic acid but trace amounts (of the order of 1% of the total fatty acid recovered) of linoleic acid were also present. It was found that oleic acid was contributed to the medium by Tween 80 (Tween oleate) which is added at a level of 1.5 mg% during preparation of the medium in order to solubilize the fat soluble vitamins(2). Analysis of the fatty acid composition of Tween oleate showed it to contain about 70% oleic acid with varying proportions of other acids including linoleic acid (2.7%) as impurity (Table III). A modified NCTC 109 medium from which Tween 80 was omitted and which showed no detectable traces of linoleic acid was therefore used in subsequent experiments. At this time also the fatty acid composition of the "Microsolve" detergent used in cleaning tissue culture glassware was determined. Palmitate, stearate and oleate were the principal constituents and only 1.9% linoleic acid was found (Table III). Since glassware after boiling in dilute microsolve solution was rinsed 10 times with tap water and then twice in distilled water before use it was concluded that no significant amounts of linoleic acid were supplied in the cultures *via* the detergent used.

TABLE II. Fatty Acid Composition of Lipid Classes of L-Strain Mouse Fibroblasts Grown in Serum Medium.

	Percent composition of lipid class			
	TG&FA	P. lipid	C.E.	MG&DG
Myristate (14:0)	5.1	3.1	7.3	4.4
Myristoleate (14:1)	—	—	1.5	0.9
Palmitate (16:0)	24.0	21.1	32.6	43.5
Palmitoleate (16:1)	4.8	4.1	1.2	5.2
Stearate (18:0)	23.7	29.2	27.7	21.8
Oleate (18:1)	27.0	29.2	30.8	26.1
Linoleate (18:2)	6.3	10.2	—	—

Key: TG&FA—Triglyceride plus fatty acids; P. lipid—phospholipids; C.E.—cholesterol esters; MG&DG—mono- and diglycerides.

Cells were grown for 4 days on NCTC 109 medium supplemented with 10% human serum. Lipids were harvested and separated into five major fractions by chromatography on silicic acid columns. Fatty acid composition of each fraction was analyzed separately.

TABLE III. Fatty Acid Analysis of "Microsolve"-Tissue Culture Detergent and "Tween 80".

	Percent composition	
	"Microsolve"	"Tween 80"
Laurate (12:0)	3.8	—
Myristate (14:0)	6.7	2.5
Myristoleate (14:1)	3.6	—
Palmitate (16:0)	26.7	9.7
Palmitoleate (16:1)	3.8	9.1
Stearate (18:0)	20.5	4.0
Oleate (18:1)	31.9	69.9
Linoleate (18:2)	1.9	2.7

Incorporation of C^{14}-acetate into lipids of cells grown in Tween 80-free synthetic medium. Cells adapted to NCTC 109 synthetic medium were grown for a number of generations in Tween 80-free NCTC 109, and the composition of the cell lipids was

TABLE IV. Lipid Analysis of L-Strain Mouse Fibroblasts Grown in Lipid-Free Medium.

Fatty acid	Percent composition of cell lipids			
	Exp 1	Exp 2	Exp 3	Mean
Myristate (14:0)	2.8	2.9	—	2.8
Palmitate (16:0)	25.7	28.8	26.0	26.8
Palmitoleate (16:1)	3.2	—	—	3.2
Stearate (18:0)	27.4	28.7	53.4*	28.0
Oleate (18:1)	32.9	38.7		35.8
Linoleate (18:2)	5.2	5.8	7.5	6.2

* Peaks were not well resolved.

A subline of L-strain mouse fibroblasts adapted to growth in chemically defined medium NCTC 109 was grown for a number of generations in the same medium from which Tween 80 was omitted. Cell lipids were extracted and analyzed as described in Methods.

analyzed periodically (Table IV). The fatty acid composition of the cells remained essentially constant, and in particular the linoleic acid content varied only between 5.2% and 7.5%.

Acetate 1-C^{14} added to the culture medium was readily incorporated into cell lipids. Radioactive cell lipids (supplemented with non-radioactive linoleic acid as a carrier to minimize loss of radioactive material by oxidation) were converted to the fatty acid methyl esters and chromatographed. Those fractions having the same retention time as linoleic acid were treated with potassium permanganate, and the oxidation products analyzed. Two major peaks having the same retention time as the expected products of linoleic acid oxidation (azelaic and caproic acids) were observed. No significant amount of radioactivity was found in either peak. A second portion of the original lipid extract was analyzed for simultaneous determination of mass and radioactivity in a Barber Coleman model 5000-S gas liquid chromatograph. Palmitic, stearic and oleic acids were heavily labeled with C^{14} but no significant quantities of radioactivity were associated with the linoleic acid peak (Fig. 5).

Discussion. Previous work on the essentiality of polyunsaturated fatty acids in tissue culture is limited. There have been some observations which imply essentiality and others which appear to indicate that these compounds are not required by many strains of cells. For example it was shown by Ham(12) that the albumin requirement for good plating efficiency of HeLa cells in defined medium could be largely replaced by linoleic acid. Harahy *et al* have similarly reported that added linoleic acid is essential for the continued beating of rat heart cells in culture(13). On the other hand, some of the earlier defined media of the NCTC series contained added lipids including linoleic acid(14). It was subsequently reported that there was no effect on growth rate when these were removed and in later media of this series, including NCTC 109, the lipids were therefore omitted(2). Over a dozen cell lines have since been adapted to this lipid-free medium NCTC 109 which does however as noted above, have a minor source of linoleic acid in the form of Tween 80. Evans *et al*(1) have recently reported however that at least 4 of the strains which grow well on NCTC 109 (including L-2071 mouse fibroblasts) also grow well on NCTC 126 medium which contains most of the components of NCTC 109 but omits Tween 80.

Our confirmation of this observation for the L-strain fibroblast together with our finding of about 6% linoleic acid in cells grown for prolonged periods in the lipid-free medium, raised the possibility that the cells may synthesize this component *de novo*

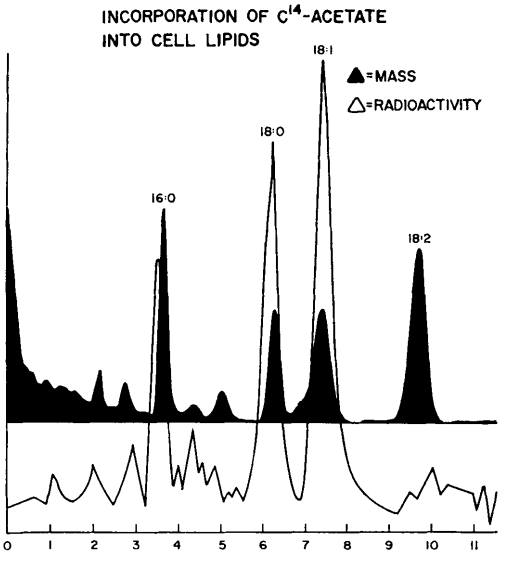

FIG. 2. Separation of fatty acid methyl esters from L-strain cells grown on C^{14}-acetate supplemented lipid-free synthetic medium.

from simpler constituents in the medium. In experiments designed to test this possibility, an active incorporation of C^{14}-acetate into palmitate, stearate and oleate was observed but no significant amounts of radioactivity were found in linoleic acid.

There are a number of possible explanations for these results. Cells may conserve linoleic acid with high efficiency under deficiency conditions and thus accumulate trace amounts present in the culture environment and which are below the present levels of detection. Assuming 100% efficiency of conservation by cells, it can be calculated for a doubling time of 1 week and a thrice weekly feeding schedule, that introduction of as little as 1 μg of linoleic acid each treatment as a contaminant of medium or glassware would suffice to maintain the observed linoleic acid content of the cells. It is also possible (although less likely) that linoleic acid differs from the other fatty acids in being synthesized from a precursor other than acetate. It will be noted that the results obtained here eliminate the possibility that deficient cells synthesize significant amounts of positional isomers of linoleic acid similar to those which have been reported in liver during polyunsaturated fatty acid deficiency(15).

Summary. The fatty acid composition of L-strain mouse fibroblasts growing both in serum and in lipid-free synthetic medium was measured. Linoleic acid comprised about 17% of the total fatty acids in cells grown on serum-supplemented medium and about 6% in cells grown for prolonged periods in lipid-free chemically defined medium. In contrast to other fatty acids however linoleic acid was not synthesized from C^{14}-acetate added to the growth medium. The most probable explanation of these anomalous findings is that cells under deficiency conditions can conserve traces of linoleic acid which are present below detectable levels in the culture environment. No synthesis of positional isomers of linoleic acid similar to that which may occur in linoleic acid deficiency *in vivo* was observed.

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