

# THE SOURCE OF LIPID ACCUMULATION IN L CELLS

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## ABSTRACT

Strain L cells accumulate lipid, concurrent with cessation of protein synthesis, in the stationary phase of growth from the extracellular medium and as a result of *de novo* synthesis. Cells which have been more severely damaged with an amino acid analogue also accumulate lipid from the extracellular medium, but synthesize very little lipid from labeled acetate. The possible roles which lipid accumulation may play in the cell are discussed.

## INTRODUCTION

It has been shown previously in this laboratory that strain L cells in old suspension tissue cultures accumulate lipid concurrent with cessation of growth. A similar accumulation of lipid is seen in cells in which protein synthesis has been inhibited by the amino acid analogue para-fluorophenylalanine (8). One of the major controversies regarding the appearance of fat in cells centers on the source of the lipid. Although it is well recognized that most cells are capable of synthesizing lipid, many reports support the theory that excess stainable intracellular lipid usually arises from extracellular lipoproteins. Most of these studies involve experiments *in vivo* (13, 16, 14) although the incorporation of extracellular fat by cells *in vitro* has also been demonstrated (10, 2, 4). Other investigators believe that excess amounts of lipid many accumulate intracellularly as a result of *de novo* synthesis (11, 3, 15). One worker has noted a distinct species difference in regard to the synthesis of cholesterol in aortic tissue (1). This study was initiated to evaluate the comparative role of exogenous and endogenous sources in the accumulation of lipid in strain L cells.

## MATERIALS AND METHODS

*Tissue Culture:* Strain L cells were grown in suspension tissue culture with Eagle's basal medium supplemented with 10 per cent horse serum as previously described (8). Cell counts were done using

a standard hemocytometer. Each culture was grown in a 250 ml. Erlenmeyer flask and consisted of 80 ml. of medium and free floating cells, initially inoculated in a concentration of 200,000 to 400,000 cells/ml. The flasks were agitated on a rotary shaker at 37.5°C.

*Isotopes:* Isotopes were obtained from the New England Nuclear Corporation with the following specific activities: sodium acetate 1-C<sup>14</sup>, 24.2  $\mu\text{c./mgm.}$ ; cholesterol 4-C<sup>14</sup> stearate, 10  $\mu\text{c./mgm.}$ ; and palmitic acid 1-C<sup>14</sup>, 6.8  $\mu\text{c./mgm.}$

*Preparation of Isotopes:* Cholesterol 4-C<sup>14</sup> stearate was dissolved in hot ethanol and 0.05 ml. containing 200,000 c.p.m. was pipetted directly into the culture. Acetate 1-C<sup>14</sup> was introduced directly into the culture after being dissolved in sterile distilled water. Potassium palmitate C<sup>14</sup> was prepared by the method of Fillerup (4). Labeled lipoprotein was synthesized by the cells from palmitic acid 1-C<sup>14</sup> as described below. The isotope ( $2 \times 10^6$  c.p.m.) was incubated with strain L cells in log growth for a period of 3 days. The cells were then washed 3 times, ruptured by sonic oscillation, centrifuged at 30,000 times *g* for 3 hours and the supernatant solution dialyzed against 7,000 cc. Krebs-Ringer phosphate buffer for 2 days. The labeled lipoprotein was then incubated in a flask with sterile tissue culture medium on a rotary shaker at 37.5°C. for 24 hours and the complete medium centrifuged to insure that no particulate components were present. Ethanol-ether extraction of the TCA precipitate of the labeled lipoprotein showed that over 96 per cent of the total counts were in the lipid-extractable fraction.

*Preparation of Samples:* Five ml. aliquots were removed daily from the culture flasks, the cells washed 3 times by centrifugation in Krebs-Ringer buffer, and the cell protein precipitated by the addition of 2 ml. of cold 10 per cent TCA. After being left overnight at 4°C., the precipitated protein was separated by centrifugation, and extracted twice at 50°C. for 45 minutes each in a mixed solution of ether ethanol (2:1). The resultant extract was plated on planchets as the total lipid-extractable fraction.

Cholesterol was extracted from 10 ml. aliquots of washed cells according to the method of Sperry and Webb (12), dried, and plated on planchets. The saponified acidic acetone alcohol extract was precipitated with digitonin; aliquots of the supernatant were made alkaline with a few drops of 50 per cent potassium hydroxide diluted with equal volume of water and extracted 3 times with equal volumes of petroleum ether. This extract was dried, plated on planchets, and called the non-cholesterol non-saponifiable fraction. Corrections were made for self-absorption when necessary.

Cell protein was determined by Oyama's modification of the Lowry method (9).

## RESULTS

The first group of experiments involved the accumulation of lipid in cultures which were in the logarithmic and the stationary period of growth. As shown in Fig. 1, cells in the stationary phase, which had ceased active growth, continued to incorporate exogenous palmitate and lipoprotein from the extracellular medium and also continued to synthesize lipid *de novo* from acetate  $1-C^{14}$ . The acetate label was found in high concentration in the cholesterol, as well as other lipid fractions, although it is recorded for comparative purposes in Fig. 1 as the total lipid-extractable fraction.

In the second group of experiments, protein synthesis was inhibited by para-fluorophenylalanine ( $8 \times 10^{-4} M$ ) as previously described (8).

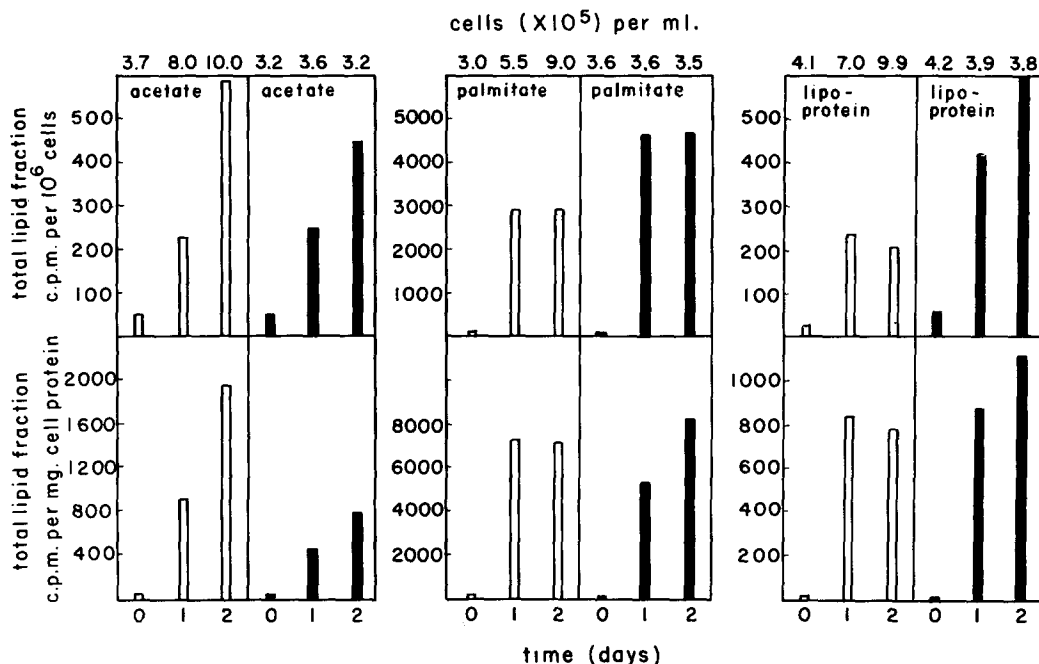


FIGURE 1

Cells from stock cultures were pooled, divided, and incubated either in fresh tissue culture medium or in exhausted medium taken from a 5-day-old culture. Labeled acetate (200,000 c.p.m.), palmitate (500,000 c.p.m.), and lipoprotein (100,000 c.p.m.) were introduced into cultures in the logarithmic (white bar) and stationary (black bar) phase of the growth cycle. Aliquots were removed daily for determination of cell count, cell protein, and radioactive assay of the total lipid-extractable fraction by the procedures described under methods. Results were recorded as c.p.m. per mg. cell protein and c.p.m./10<sup>6</sup> cells for each sample. The figures at the top of the graphs represent the cell number ( $\times 10^6$ ) per ml. in each culture. Each bar represents the average of 3 flasks.

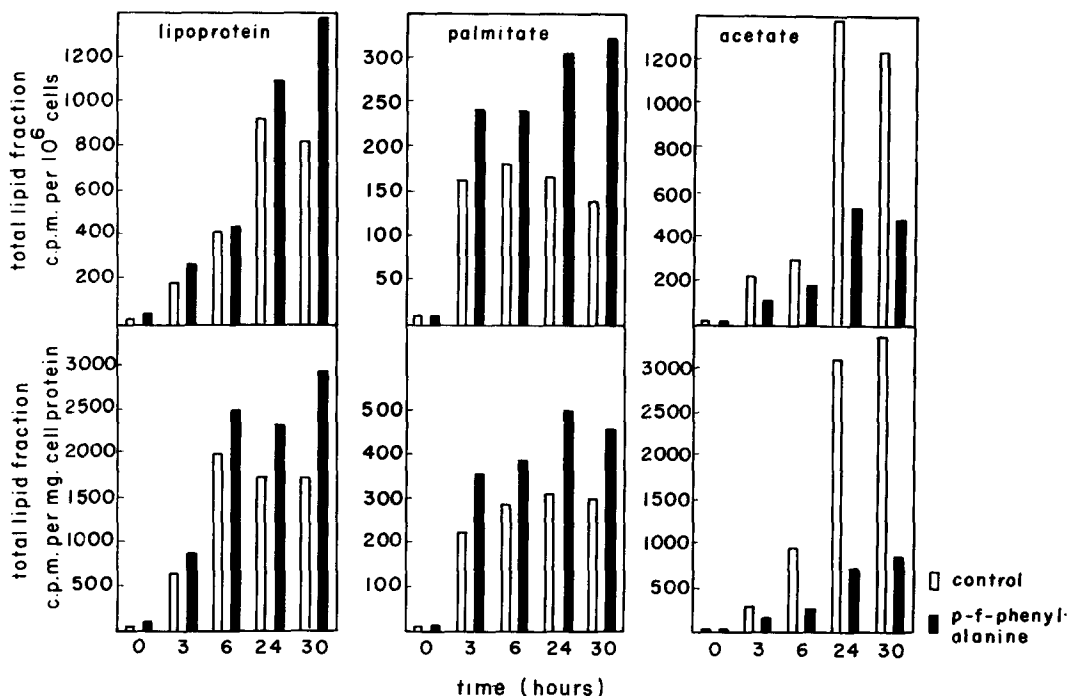


FIGURE 2

Cells from stock cultures were pooled, divided, and incubated in control flasks without analogue or in experimental flasks containing para-fluorophenylalanine ( $8 \times 10^{-4}$  M). Labeled acetate (100,000 c.p.m.), palmitate (100,000 c.p.m.), and lipoprotein (100,000 c.p.m.) were introduced into both control and experimental flasks and aliquots removed at the stated period for determination of cell count, cell protein, and radioactive assay of the total lipid-extractable fraction by the procedures described under methods. Results were recorded as c.p.m./mg. cell protein or c.p.m./ $10^6$  cells for each sample. Each bar represents the average of 3 flasks.

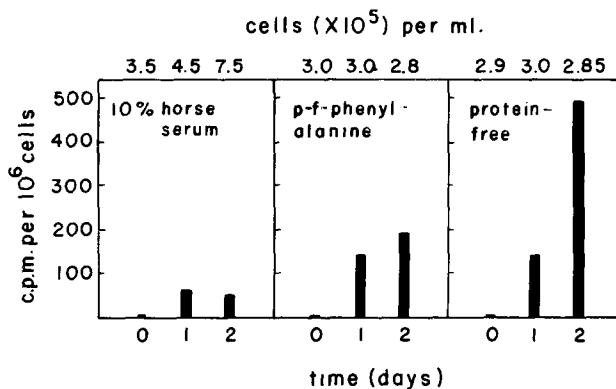


FIGURE 3

Cells from stock cultures were pooled, divided, and incubated with labeled cholesterol stearate (200,000 c.p.m.) in one of 3 different media: basal medium and 10 per cent horse serum, basal medium and 10 per cent horse serum and para fluorophenylalanine ( $8 \times 10^{-4}$  M), or basal medium without serum protein. Aliquots were taken daily and extracted for total lipids as described under methods.

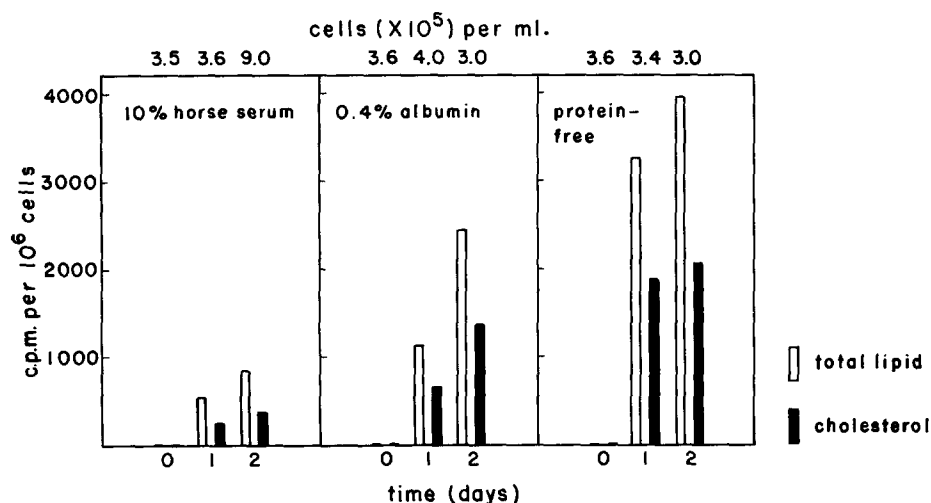


FIGURE 4

Cells from stock cultures were pooled, divided, and incubated with labeled cholesterol stearate (200,000 c.p.m.) in one of 3 different media: basal medium and 10 per cent horse serum, basal medium and 0.4 per cent albumin, and basal medium without serum protein. Aliquots were taken daily and extracted for cholesterol and total lipids as described under methods.

The cells were again incubated with labeled acetate as a source of endogenous lipid and labeled palmitate and lipoprotein as a source of exogenous lipid. As shown in Fig. 2, the lipid which accumulated within the cells came principally from the exogenous sources and comparatively little was synthesized from labeled acetate.

Cholesterol stearate was also used as a source of exogenous lipid as shown in Figs. 3 and 4. It was incorporated to a much greater degree in the cells of those cultures in which growth had been inhibited, either by para-fluorophenylalanine or serum free medium, than in the cells of control cultures containing no analogue and supplemented with 10 per cent horse serum.

#### DISCUSSION

We have presented evidence in these experiments to show that old cells in the stationary phase of the growth cycle accumulated lipid both as a result of *de novo* synthesis from labeled acetate and also from extracellular lipoproteins. It is not known whether the lack of growth in the stationary phase is the result of a deficient medium or the accumulation of toxic metabolites. As a result of inhibition of protein synthesis the cells may be able to utilize a larger portion of the acetate pool for lipid synthesis. The active acetate could be

derived not only from glycolysis but also from the ketogenic amino acids. These lipid products may later, once the conditions again become favorable for protein synthesis, be degraded both for the synthesis of high energy molecules and for non-essential amino acids. A somewhat analogous situation has been shown in *E. coli*. These cells, in a nitrogen-deficient medium, synthesized large quantities of glycogen which was later degraded when protein synthesis was initiated (7). It is, of course, possible that some of the acetate incorporated into the cells represented "exchange" or reversible metabolic processes and not true *de novo* synthesis.

Those cultures, in which protein synthesis was more drastically inhibited by the amino acid analogue, accumulated the major portion of their intracellular lipid from extracellular sources. This lipid was not reversible and the cells eventually died. It is interesting that an amino acid analogue appears to inhibit lipid synthesis from labeled acetate along with protein synthesis. It has been suggested that lipids might be involved in protein synthesis (5) and present evidence indicates that the microsomes are the site of lipid as well as protein synthesis.

There will always be some controversy concerning the question of whether the labeled lipoprotein was really incorporated into the cellular lipid

fraction or merely adsorbed on the membrane. We have tried to take all possible precautions to avoid the latter possibility. The labeled lipoprotein was dialysed for 2 days to remove all small loosely attached free acids. The soluble lipoprotein was preincubated at 37.5°C. on the shaker with complete medium and later centrifuged to insure that particulate lipoprotein was not removed with the cell aliquots. After removal of the daily samples, the cells were washed 3 times in buffered saline until the final wash was completely free of radioactivity. Cells stained with Sudan IV showed significant increases of intracellular lipid droplets. Up to 50 per cent of the label in the cholesterol experiments appeared in the non-digitonin precipitate, indicating that the cholesterol was actively metabolized to a further degradation product and not merely adsorbed to the cell membrane. Finally, the incorporation of labeled lipoprotein in this system was temperature-dependent and less than 10 per cent incorporation occurred at 4°C.

In the experience of our laboratory there exists a definite difference in membrane permeability of cells during different phases of growth. We have previously reported the greater incorporation of acridine orange in L cells during the stationary period (6). We would now speculate in regard to the present experiments that during the stationary phase cells take up more lipoprotein and other metabolites in an attempt to compensate for cellular nutritional deficiencies. We believe a somewhat similar process takes place in the injured cell, perhaps by pinocytosis (2), again in an attempt to gain essential materials to repair cellular damage. This compensatory action, of course, would be of little value to those cells in which the synthetic mechanism had been irreparably injured.

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