The Relation between Protein Synthesis and Lipide Accumulation in L Strain Cells and Ehrlich Ascites Cells*

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ABSTRACT

It has long been known that fat accumulates in old injured cells both in tissue culture and in many mammalian disease states. The use of L cells grown in suspension tissue culture permitted the opportunity to study conditions in which lipide accumulation could be retarded or accelerated.

These cultures exhibit a three-phase growth curve which is similar to that previously found with bacteria and consists of a lag period, logarithmic growth period, and stationary period. Daily aliquots were removed from cultures going through these phases and protein and cholesterol content correlated with cell division.

It was found that L cells gradually accumulated lipide in the cell concurrent with retardation of cell division and protein synthesis. Conversely old lipide-laden cells, placed in fresh media and encouraged to active division with net protein synthesis progressed from a high to a low lipide/cell ratio over a period of 2 to 4 days. An amino acid analogue p-fluorophenylalanine and a mitotic inhibitor, colchicine, also markedly increased the lipide/cell ratio. Similar results were found in in vitro experiments with Ehrlich ascites cells.

INTRODUCTION

For many years pathologists and cytologists have observed the accumulation of stainable lipide in cells that are either old or injured. The old terms of fatty degeneration (meaning intracellular unmasking of lipide) and fatty infiltration (lipide deposited from exogenous sources) have been replaced by the term "fatty change."

Both at autopsy and in experimental injury the liver has been a frequent site of "fatty change." An early relationship was elucidated between dietary deficiencies and lipide deposition in the liver. Diets low in protein (16, 22, 17, 15), containing an amino acid imbalance (5), deficient in methionine (31) or tryptophane (29, 10, 1), have all produced fat in liver cells. Injury to the liver by a variety of other means apparently accentuates lipide accumulation (32).

Studies on the deposition of lipide, particularly cholesterol, on blood vessels have yielded similar results. Diets deficient in methionine produce hypercholesterolemia (14) and atherosclerosis in rats (19) and monkeys (24). Injury of some nature to the vessel also causes an increase in lipide accumulation (7, 6, 30, 33).

The literature indicates that lipides appear in injured cells. There is little indication as to why it appears or from whence it comes. The emphasis in explanations has been on a cellular degenerative process associated with faulty breakdown of fat or a cellular membrane alteration allowing increased accumulation of abnormal lipides from exogenous sources.

In our laboratory mouse L strain fibroblasts are being grown in suspension cultures. It is possible with this system to evaluate the accumulation of lipide in cells during various stages of growth and when subjected to different forms of injury.

Materials and Methods

Strain L (Earle) cells were grown in suspension tissue culture according to the procedure described by Kuchler (21). Each 250 cc. Erlenmeyer flask contained
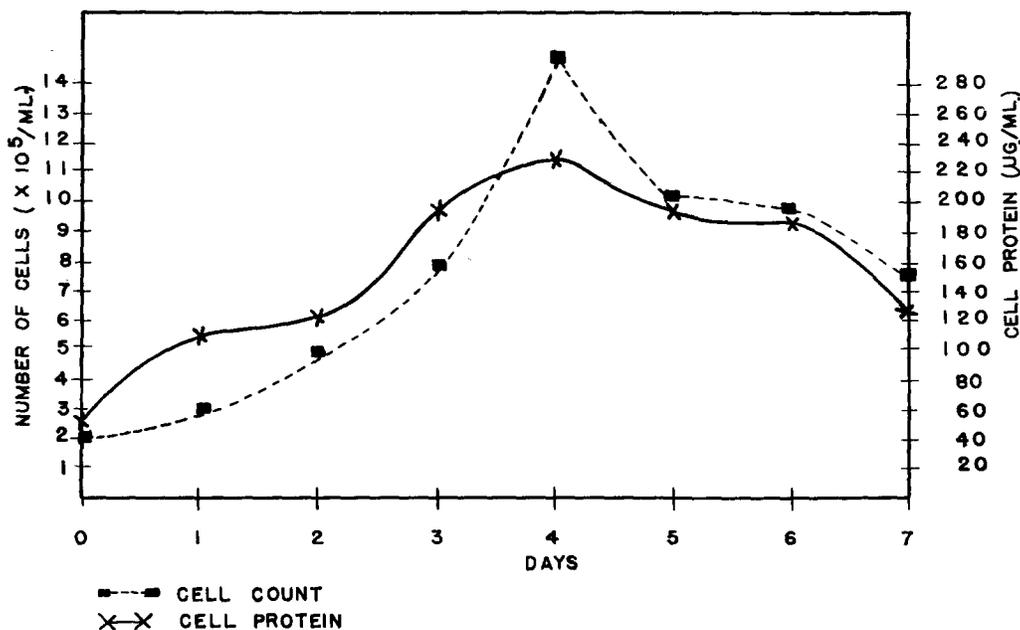
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FIG. 1. Cell growth of strain L cells.

Eagle's basal medium (72 ml.) (12), horse serum (8 ml.), penicillin (100 units/ml.), and streptomycin (100 μg/ml.). Cultures were gassed with 8 per cent CO_2 in air. The flasks were then placed on a rotary shaker in an incubator maintained at 37.5°C. Each culture initially contained 200,000 cells/ml.

Para-fluorophenylalanine and colchicine (California Corporation for Biochemical Research) were introduced directly into the L strain cultures in a final concentration of 8×10^{-4} M for para-fluorophenylalanine and 10^{-7} M for colchicine. Control cultures from the same homogeneous stock cultures were set up in an identical manner.

The use of suspension tissue culture permitted the removal of accurate daily samples for determinations of cell number, cell protein, and lipide. The cell counts were done by counting ten squares in duplicate with a standard hemocytometer. After removal of appropriate aliquots (1 ml. for protein, 15 ml. for lipides) the cells were washed three times in 5 ml. of a modified Krebs-Ringer phosphate solution (3). Cell protein was determined by Oyama's modification of the Lowry method (26). Cells taken for lipide analysis were first extracted under a reflux condenser in a 60°C. water bath for 1 hour with 25 ml. of an alcohol-ether solution (1:1). Suitable aliquots of this extract were taken for determination of cholesterol by the Schoenheimer-Sperry method (28), phospholipides by a modified Fiske-SubbaRow method (34), and fatty acids by the method of Stoddard and Drury as modified by Man and Gildea (23).

Ehrlich ascites cells were used in a series of experiments of short term duration. The tumor was maintained in Swiss-Webster mice and harvested as described by Klein (20). The pooled cells from the non-hemorrhagic ascitic fluid of 10 to 12 mice were washed three times in 50 ml. of cold Krebs-Ringer phosphate solution (pH 7.4). Usually the cells were then resuspended in Eagle's basal medium with horse serum, as previously described for strain L cells, although some cells were resuspended only in fresh Krebs-Ringer solution. In all instances a cell concentration of approximately 2×10^7 cells/ml. was obtained. Samples of 10 ml. volume were incubated in 50 ml. stoppered Erlenmeyer flasks and placed on a rotary shaker in the 37.5°C. incubator for periods of 3 to 4 hours. Para-flurophenylalanine was added to the non-control flasks in a final concentration of 10^{-2} M and 10^{-3} M and colchicine was added to others in a final concentration of 10^{-5} M and 10^{-6} M. Aliquots for protein and lipide determinations were taken initially and at the end of the incubation period. After washing, the cells were analyzed in the manner described for L strain cells. The cells were counted in a standard blood counting chamber before and after incubation. No change in cell number was noted and less than 5 per cent of the cells permitted the entrance of trypan blue into their nuclei.

OBSERVATIONS

Other workers have previously shown a characteristic growth curve when strain L cells are grown in suspension culture as shown in Fig. 1 (21). There is usually a 1 day lag period before division commences. The cells only divide when
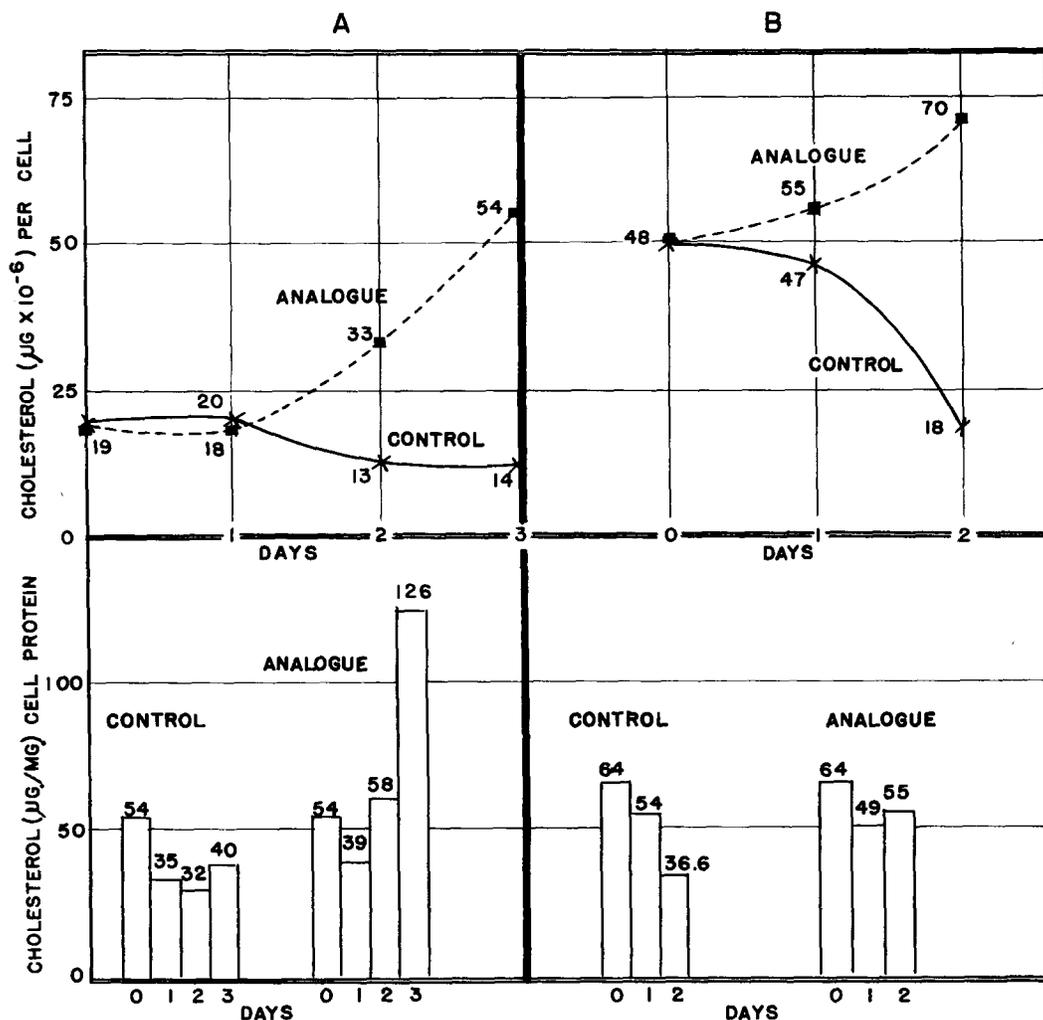
Fig. 2 A. Effects of p-fluorophenylalanine 8 × 10⁻⁴ M on cholesterol in young strain L cells.

B. Effects of p-fluorophenylalanine 8 × 10⁻⁴ M on cholesterol in old strain L cells.

the pH of the medium has dropped to 7.1, but during this initial lag period there is a doubling of the cell protein. Later figures will show that during this same period the cholesterol content of the cell begins to fall. The lag period is followed by a logarithmic growth period in which the cells divide every 24 to 26 hours until a cell concentration of approximately 1.2×10^6 cells/ml. is reached. During this period the lipide content of the cells is minimal. Finally the cells enter a phase where they remain unchanged in cell number for 2 or 3 days. During this stationary phase the protein content/cell may vary and the lipide content of the cell increases greatly. In several initial experiments it was found that the phospho-

lipides, neutral fat, and cholesterol all increased in the older cells which had ceased division. Cells in log growth require a residual volume of 50 ml. of medium when using a 250 ml. Erlenmeyer flask. It is thus impossible to do multiple complete lipide analyses over a period of several days. In order to follow the cells over a longer period of time and in order to obtain larger and more accurate samples, the cholesterol content was taken as an indicator of the lipide content of the cells.

Since it was determined that the cholesterol increased in old cells in the stationary phase which had ceased active protein synthesis, it was decided to accentuate this process with an amino
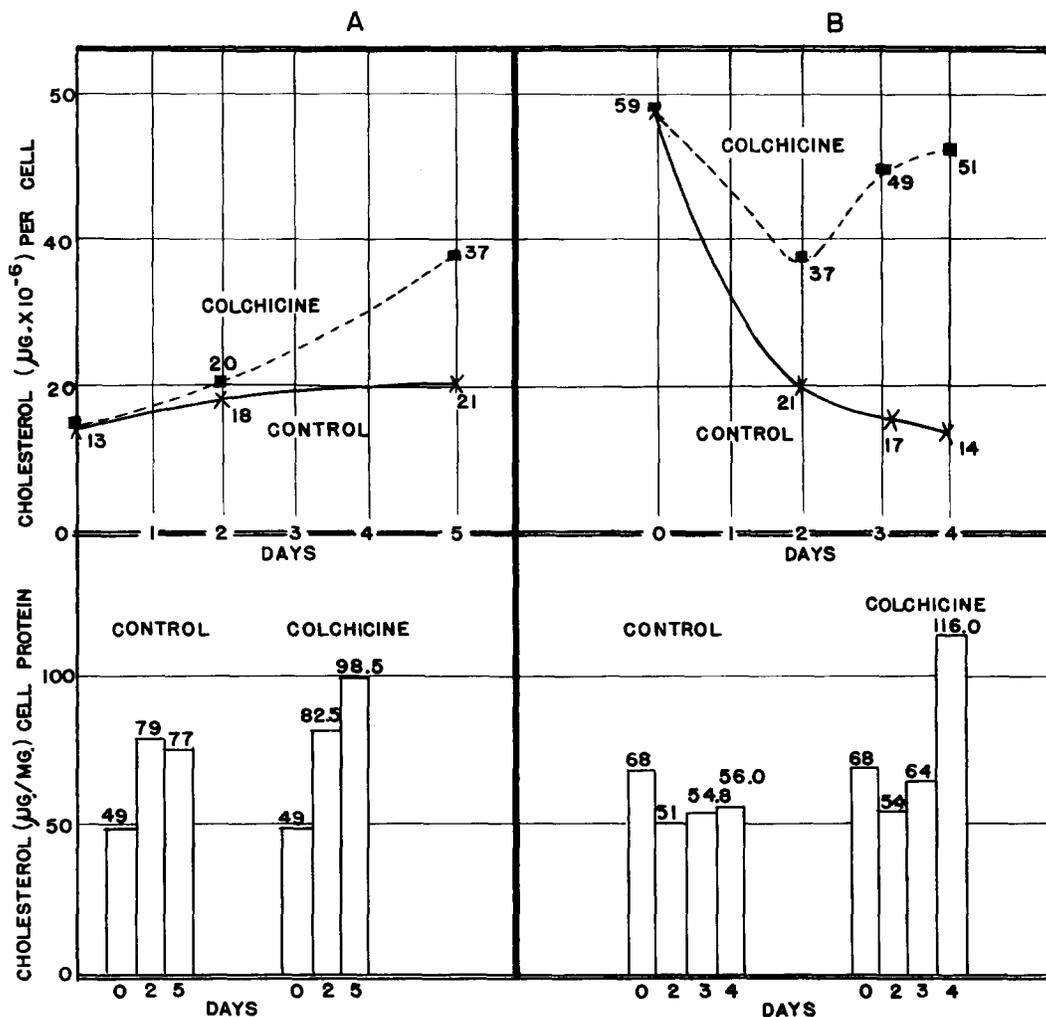
FIG. 3 A. Effect of colchicine 10^{-7} M on cholesterol in young strain L cells.

B. Effect of colchicine 10^{-7} M on cholesterol in old strain L cells.

acid analogue, para-fluorophenylalanine. After a wide range of different doses, a concentration of 8×10^{-4} M was found to cause no apparent increase or decrease in cell number, although a moderate increase in cell protein was noted over a period of 3 to 4 days. Concentrations of 10^{-5} M had no effect on logarithmic growth, while concentrations of 10^{-2} M resulted in death and dissolution of the majority of cells in a 24 hour period. The normal concentration of phenylalanine in Eagle's basal medium is 1×10^{-4} M. Fig. 2 shows the effect of p-fluorophenylalanine on the cholesterol content of L strain cells. In Fig. 2 A, cells were taken from a culture in logarithmic growth,

broken into two identical cultures, and introduced into fresh medium. One of the cultures contained p-fluorophenylalanine (8×10^{-4} M) the the other remained as a control. The initial cholesterol content per cell and per mg. protein is very low and remains so in the control cultures. In the analogue-treated culture, the cholesterol content rises rapidly, both on an individual cell basis and in relation to the cell protein. In the experiment shown in Fig. 2 B, cells were obtained from an old culture of cells in the stationary phase of the growth curve. Sudan-stained sections showed the lipide content in these old cells to be very high when compared with young cells
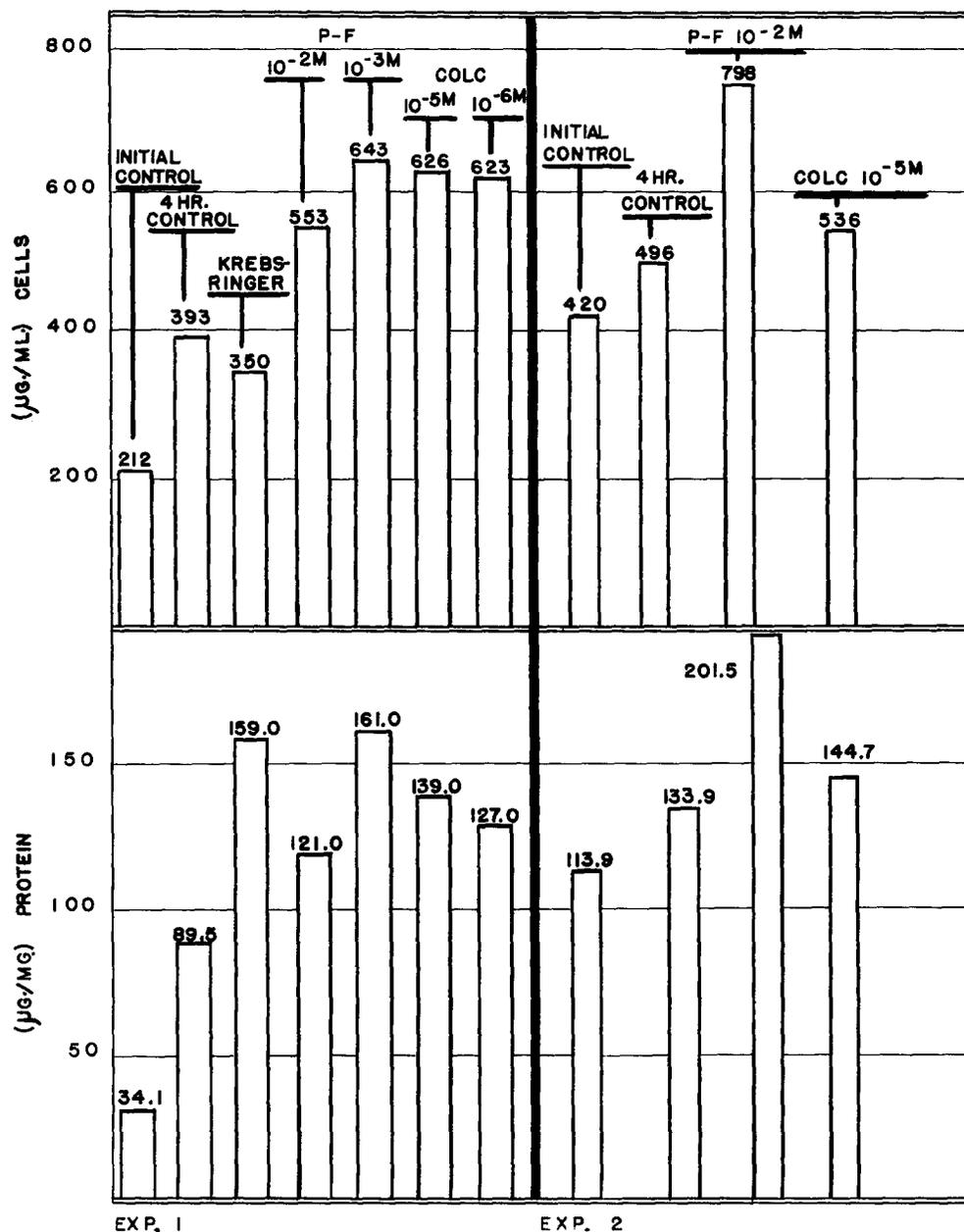
FIG. 4. Total lipides in Ehrlich ascites cells given *p*-fluorophenylalanine and colchicine.

taken from a culture in rapid growth. As shown in the graph, the initial cholesterol content of the cells was very high also. The culture was broken into two identical fractions and introduced into fresh medium. The control culture gradually showed a reduction in cholesterol content coincident with the beginning of growth and rapid protein synthesis. The culture treated with the analogue retained and appeared to show a slight increase in its cholesterol content.

Another series of experiments was made in which colchicine was used instead of an amino acid analogue (Fig. 3). Colchicine apparently only interfered with protein synthesis indirectly in
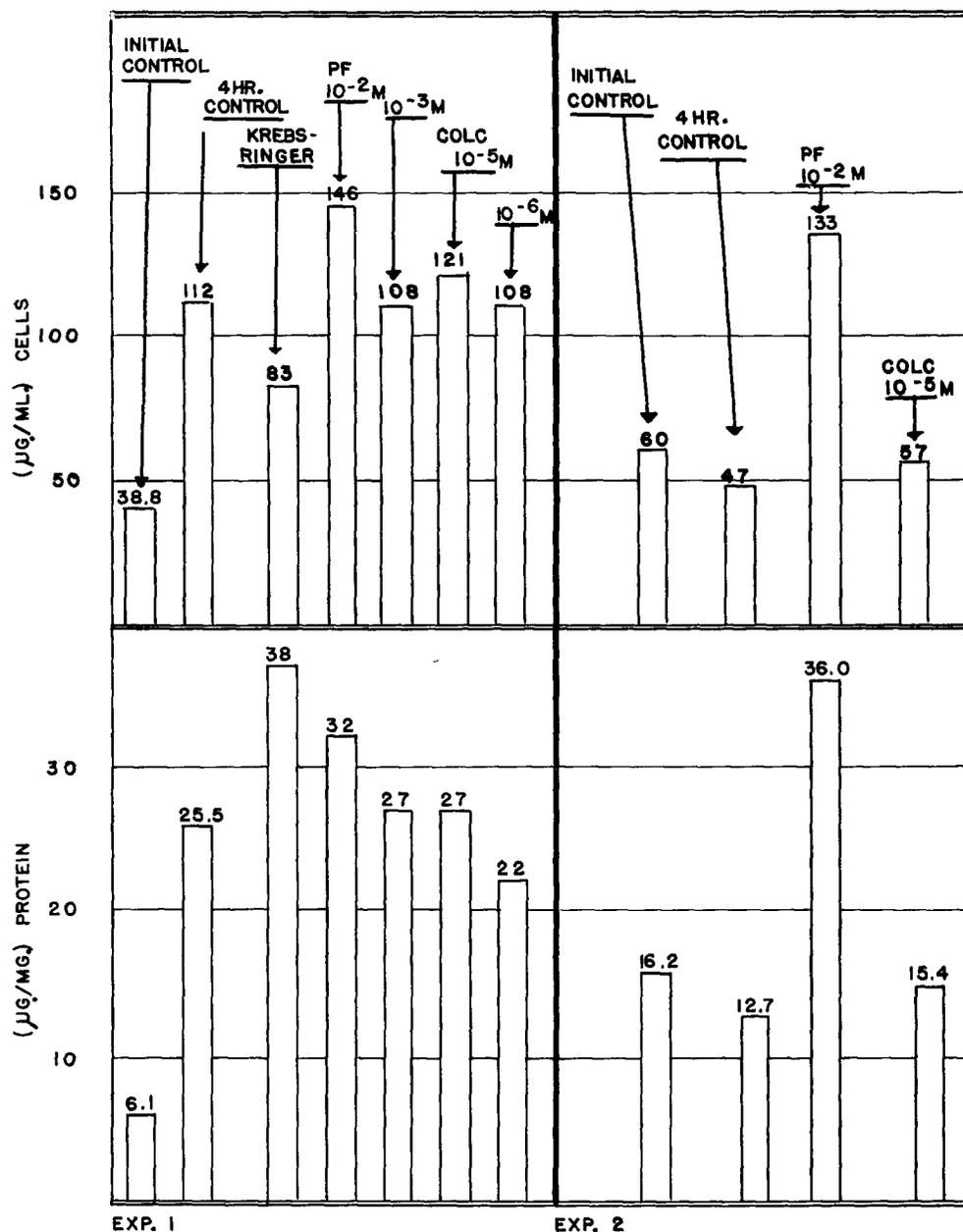
FIG. 5. Cholesterol in Ehrlich ascites cells given p-fluorophenylalanine and colchicine.

these cells when a concentration of 10^{-7} M was used. Although there was no increase or decrease in cell number noted over a period of several days, there was an initial doubling of cell protein. Concentrations of this order on these cells presumably interfered with some other essential process beside cell division. Although the effects with colchicine do not appear as marked as those with para-fluorophenylalanine, the results are nevertheless very similar. Interruption of cell division, growth, and protein synthesis by any one of several methods is associated with an increase in cholesterol.

Ehrlich ascites tumor cells were used for another series of experiments. The cells were removed from
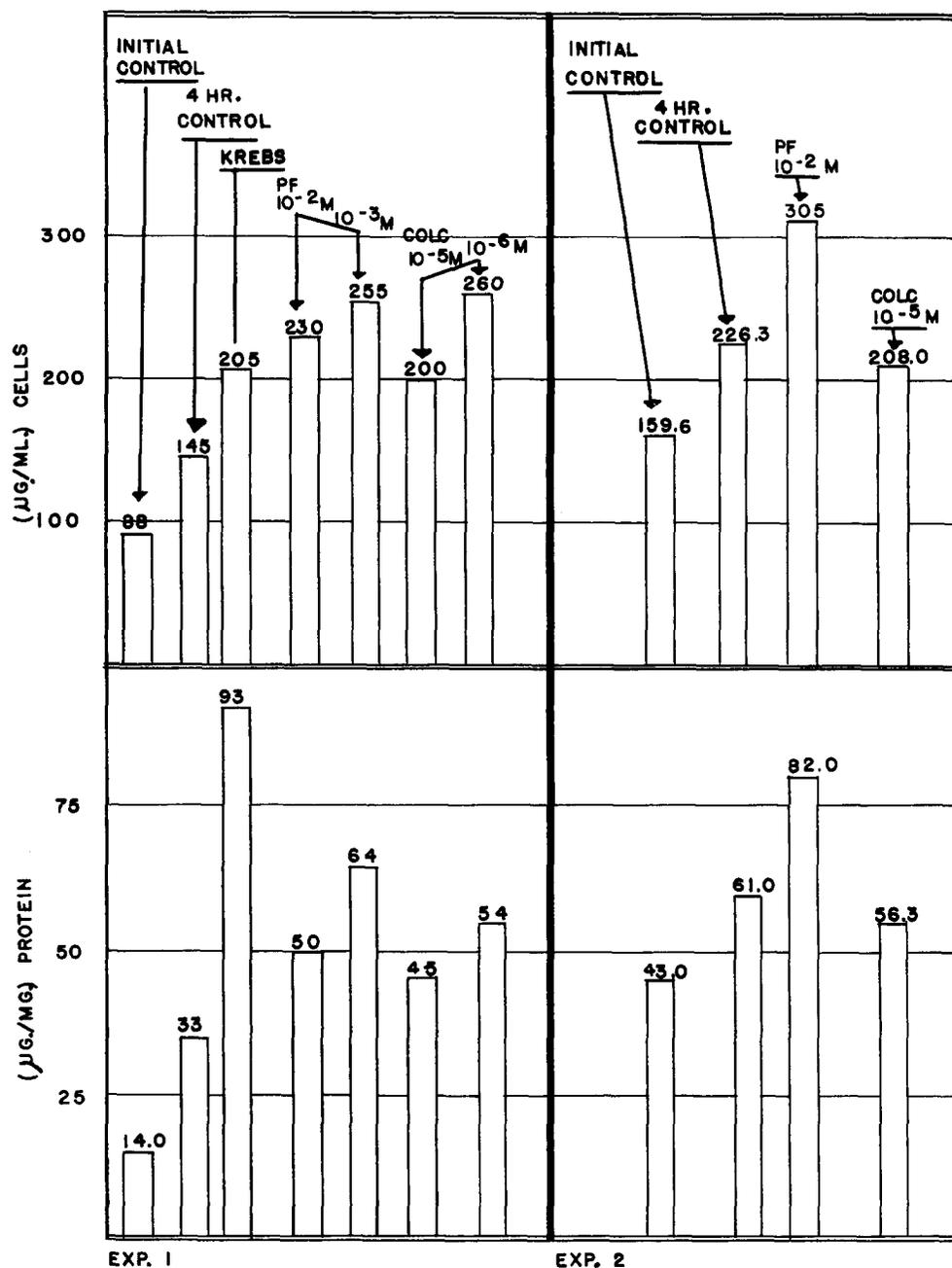
Fig. 6. Phospholipides in Ehrlich ascites cells given *p*-fluorophenylalanine and colchicine.

the mice, pooled, washed, and incubated *in vitro* in various media as described previously. As with the L strain fibroblasts much depends on the age and stage of growth of the tumor cells when used. Young cells from mice injected 2 to 3 days previously are in the period of rapid growth. The

lipide per cell and per mg. protein shows very low values and the effect of interfering with protein synthesis is very evident. On the other hand, cells removed from mice 8 to 11 days after injection, and which have ceased active division, have very high initial lipide values per cell and per mg. pro-
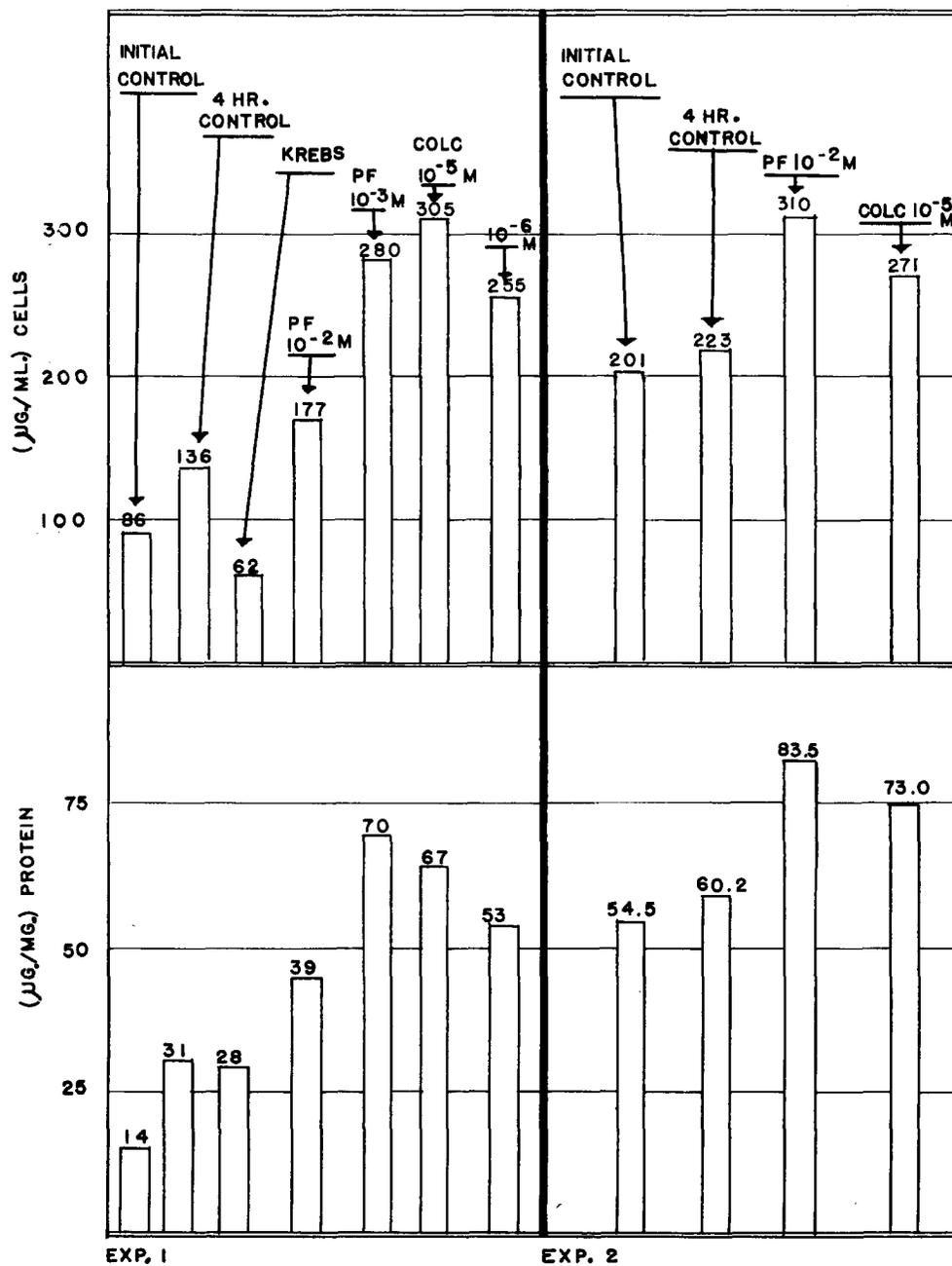
FIG. 7. Fatty acids in Ehrlich ascites cells given p-fluorophenylalanine and colchicine.

tein. With decreased mitotic indices and protein synthesis, the further inhibition of protein synthesis with analogues and colchicine produce less significant changes in the lipide values.

The age of the cell is also of great importance in regard to the amount of protein lost from the cell during the 4 hour incubation. Contrary to other reports we sometimes find a very significant decrease in protein after incubation with analogues *in vitro*. When this occurs the lipide accumulation is appreciable even when the cells are placed in Krebs-Ringer phosphate solution without serum.

This is clearly shown in Fig. 4, Experiment 1 where the total amount of lipide is several times higher than the initial values, most pronounced in those cells treated either with p-fluorophenylalanine or colchicine. Experiment 2 of Fig. 4 is a representative experiment where there was little decrease in total cell protein. Only the flask containing the amino acid analogue showed a significant increase in net lipide accumulation. The concentration of analogue 10^{-2} M has been shown to prevent almost completely the incorporation of valine-1C^{14} into these cells in control experiments.

Figs. 5 to 7 show the results when the lipides are broken into three fractions: cholesterol, phospholipides, and fatty acids. Fig. 5 depicts a marked net cholesterol accumulation in the cells regardless of whether the cells are merely placed in a new environment, as in the Eagle's basal medium control flasks and the Krebs-Ringer solution flasks, or whether the cells are actively injured by an analogue and colchicine. Figure 5, experiment 2 represents results with older cells; the increase is less marked except for the flasks containing the analogue.

In general, the pattern with phospholipide analysis in Figs. 6, Experiments 1 and 2 parallels that shown with cholesterol. The loss of protein in the experiments shown in Fig. 6, Experiment 1 makes the phospholipide/protein ratio greatly elevated.

Fig. 7, Experiments 1 and 2 showing fatty acid content of the cells, describe results which were in general similar with the exception that those cells maintained in Krebs-Ringer solution appeared to lose large amounts of fatty acids along with the loss of protein. Perhaps this was associated with increased energy requirements of the cells resulting from the marked change in the cells' environment. Again, the increase in lipide in the cells was highly dependent on the initial lipide content and age of the cells.

DISCUSSION

We have previously mentioned the many reports in the literature in which nutritional deficiencies contributed to lipide accumulation in various cells of the intact organism. The experiments reported here with strain L cells originally isolated from a mouse fibroblast (27) may have little relation to the specialized cells of the liver, kidney, and aorta. It is, however, interesting that these three sites which readily show fatty changes in the intact organism are in fairly direct contact with the plasma and present a somewhat analogous situation to cells cultivated in media containing serum. Also the basic interconversions of fat, carbohydrate, and protein have proven to be qualitatively similar in most all cells studied.

Whether our studies have any bearing upon other cells has little relevance at present. These experiments document the well known picture of lipide accumulation in old or injured cells frequently seen by workers in tissue culture. The accumulation of cholesterol in old strain L cells is presumably associated with a deficiency of some sort, or the accumulation of an inhibitor, as this process can be reversed when the cells are placed in fresh medium. If some essential substance is missing, it is probably not a simple deficiency of amino acids. In addition to substantial amounts of amino acids in the medium, workers in tissue culture have shown utilization of serum protein for cell growth (2, 13) and large excesses of protein are thus presumably present.

Cailleau et al. have previously reported an increase in lipide content in strain L cells inhibited by other metabolic inhibitors, principally those affecting the energy-producing systems of the cell (8). It is known that p-fluorophenylalanine may be degraded to fluoroacetate and fluorocitrate. The latter compound is an inhibitor of aconitase, and inhibition of this enzyme could possibly prevent protein synthesis by interfering with the production of ATP (9). However, a concentration of p-fluorophenylalanine of 8×10^{-4} M had little effect on respiration, and we presume the drug acted as a competitive inhibitor of phenylalanine. It has also been reported that amino acid analogues are incorporated into the proteins of growing cells (11). As previously mentioned, we were able to get logarithmic growth when concentrations of 10^{-5} M para-fluorophenylalanine were used. It would be of interest to determine how much of this analogue was incorporated into the proteins of strain L cells.

The increase in lipide in Ehrlich ascites cells is most interesting since the incubations were of such short durations. The mere removal of the young cells from their normal environment constitutes an injury of some sort and results in a net increase in lipide, but this was markedly increased with the analogue. Belkin and Wodinsky (4) have previously shown that old, non-dividing

Yoshida ascites tumor cells have large amounts of stainable lipide that is immediately lost when old tumor cells are transferred into new animals. This lipide was retained in the cells, however, if small amounts of podophyllin and colchicine were injected intraperitoneally along with the cells.

There appears to be little question that experimentally *in vitro* and *in vivo* it is possible to produce an accumulation of lipide in cells by interfering with growth and protein synthesis. In some instances, as in deficient media cultures, this phenomenon is completely reversible. In other instances in which the drug or metabolic inhibitor cannot be easily removed, it appears irreversible. We believe the reversible cases may be analogous to the experiments done with nitrogen deficiency in *E. coli* by Holme and Palmstierna (18). In their experiments the cells accumulated large quantities of glycogen instead of lipide. When NH_4Cl was given to the cells, the glycogen was degraded both for energy and directly for amino acid synthesis. It does not appear unreasonable to suggest that an amino acid deficiency, the use of analogues, energy inhibitors, or the presence of other conditions incompatible with the normal protein metabolism of the particular cell being studied, accentuate or stimulate lipide accumulation by less complex or perhaps less vulnerable pathways. Of special interest in this regard is the observation of Orsi *et al.* (25) that infection of Ehrlich cells with viruses resulted in a great increase in stainable lipide in the cell. The virus presumably interfered with both nucleic acid and protein metabolism. When cells are incubated in serum it is, of course, probable that much of the lipide accumulates in the cell as a result of transport from extracellular sources.

CONCLUSIONS

1. L strain fibroblasts undergoing division and active synthesis of protein have low concentrations of cholesterol/cell and per mg. protein. Interruption of this active protein synthesis as a result of exhausted media, or drugs such as *p*-fluorophenylalanine and colchicine, causes a marked increase in the amount of cholesterol/cell and increase in cholesterol/protein ratio.

2. Ehrlich tumor cells also have a low concentration of lipide during active growth. Older cells that have ceased division have much higher levels/cell. Short term injury by several methods including a new environment, colchicine, and para-fluorophenylalanine will produce net accumulation of lipide. This may or may not be associated with increased degradation of protein.

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