

THE PLATEAU PHASE OF GROWTH OF THE L-M STRAIN MOUSE CELL IN A PROTEIN-FREE MEDIUM

I. PATTERNS OF PROTEIN AND NUCLEIC ACID SYNTHESIS AND TURNOVER¹

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MAINTENANCE of a metabolically active population of non-dividing mammalian cells *in vitro* for prolonged periods of time has practical application in the production of viral vaccines, hormones and enzymes [14]. Under these conditions the synthetic activities of the cell are directed both toward the preservation of cell components and specialized function, a situation which is seen in the adult animal. While this idealized situation probably is never achieved *in vitro* the restricted cell turnover which characterizes the plateau phase of the growth cycle approaches this condition.

Once rapid cell division ceases, the plateau phase ensues. In bacterial systems this period is sometimes so short as to be imperceptible whereas in mammalian cell populations, *in vitro*, the markedly longer generation time and slower metabolic rate afford an opportunity for careful scrutiny and manipulation [15] of this phase even though it is relatively short by comparison to the *in vivo* situation.

The lag and logarithmic phases of the growth cycle of various mammalian cell populations *in vitro* have been delineated in terms of increase in cell number and of protein and nucleic acid synthesis [2, 6, 7, 10, 20, 22]. Although lipid accumulation has been associated with the cessation of protein synthesis during the plateau phase [3], little attention has been devoted to this period in its entirety or to changes occurring in the culture medium.

In the studies reported here, cells were grown as a uniform suspension

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in a chemically defined medium supplemented only with peptone, methylcellulose and a polysulfonic acid. In the absence of extraneous protein and nucleic acid, quantitation of both cellular and medium components reflected changes due to the direct activity of the cells free of complicating factors such as serum protein commonly employed in culture medium.

MATERIALS AND METHODS

Serial subcultures of L-M strain mouse fibroblasts [8] were grown in 200 ml of modified synthetic mixture 199¹ supplemented with 0.5 per cent Bacto-peptone² in 500 ml screw-capped Florence flasks. The flasks were agitated on a rotary shaker, having a one inch radial stroke, at 110 rpm. The incubation temperature was 35°C. Addition of 0.1 per cent of methylcellulose³ and 0.025 per cent Darvan No. 2,⁴ a polysulfonic acid dispersing agent [12] prevented clumping of the cells throughout the entire growth cycle in both stock and experimental cultures. Initially the pH of the medium was adjusted to 7.6 by the addition of 0.4 ml of 1 N NaOH per 100 ml of medium. Sodium bicarbonate was omitted in order to facilitate release of carbon dioxide produced by the cells in radioactive tracer experiments [5]. Carbon dioxide thus released represented only that attributable to cell metabolism. Cells were harvested in late logarithmic phase (appr. every 6–7 days) and were resuspended in fresh medium at a cell density of 4×10^5 cells per ml. Only log phase cells were used to initiate stock and experimental cultures.

A Model A Coulter Counter⁵ was employed to monitor the development of both stock and experimental cultures and increase in cell number was used as the basic index of growth.

The complete tissue culture medium for each stock and experimental culture was incubated for 48 hr at 35°C prior to use. At monthly intervals stock cell suspensions were inoculated into thioglycollate and trypticase soy broth for detection of possible bacterial contaminants. In addition, at bi-monthly intervals, aliquots of cells were transferred to a modified PPLO agar [1] to detect the presence of pleuropneumonia-like organisms. Calf serum (20 per cent) was employed instead of human blood in the PPLO medium and the plates were incubated for seven days in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide at 35°C.

At various intervals throughout the growth cycle cell viability was checked employing the erythrosin B dye exclusion technique [17]. One half ml of cell suspension plus 0.2 ml of 0.4 per cent erythrosin B solution was placed in a 12 × 75 mm test tube and mixed thoroughly. After a 5 minute interval at 25°C, 100 cells were examined under the microscope. The number of unstained cells in the sample was

¹ The modified medium 199 was prepared according to the formula and directions of Salk, Youngner, and Ward [19] with the exception that the following compounds were omitted: D-ribose, D-deoxyribose, glutathione, 1-cysteine hydrochloride, ascorbic acid, vitamin A, adenosinetriphosphate, penicillin, streptomycin.

² Difco Laboratories, Detroit, Mich.

³ 15 CPS, reagent grade, Dow Chemical Co., Midland, Mich.

⁴ R. T. Vanderbilt Co., New York, N.Y.

⁵ Coulter Electronics, Hialeah, Fla.

expressed as per cent of viable cells. Experimental cultures were considered to be in the terminal stages of the stationary phase when a marked decrease in cell viability was observed since lysis did not always immediately follow loss of viability. In most instances cell number remained constant even when viability reached 0 per cent.

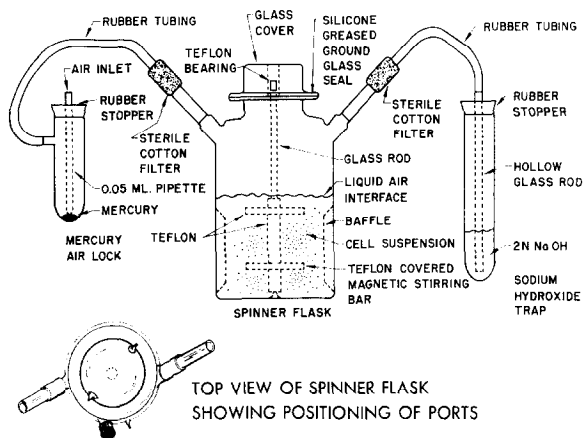


Fig. 1.—Culture apparatus for tracer study.

To initiate experimental cultures late log phase suspension cultures were transferred *in toto* by 100 ml volumetric pipettes to several 900 ml capacity modified spinner flasks (Fig. 1) mounted on a multiple spindle magnetic stirrer.¹ Three screw-cap openings were so positioned that one was used for introduction and sampling of the culture; one was attached to a mercury manometer (Fig. 1) for introduction of sterile air as needed, and one was connected to a sodium hydroxide reservoir for entrapment of carbon dioxide produced. Each flask contained 500 ml of cell suspension and was agitated at a rate of 300 rpm (without foaming or cell destruction) at $36^{\circ} \pm 0.5^{\circ}\text{C}$.

A modified Schmidt-Thannhauser procedure [21] was employed for isolation of nucleic acids and protein. Ten ml samples of the experimental culture were transferred to 16 ml screw-capped test tubes at desired intervals and centrifuged immediately. The medium was withdrawn aseptically and transferred to another screw-capped tube. Both cells and medium were stored at -60°C until the culture had completed the stationary phase of growth. At this time all samples of packed cells were individually resuspended in 2.5 ml of cold distilled water to induce lysis and 2.5 ml of cold 1 N perchloric acid was added immediately both to stop enzymatic activity and to precipitate the nucleic acid-protein complex. After one hour's extraction at 4°C the samples were centrifuged in the cold and the supernatant fluid containing the acid soluble fraction was transferred to another screw-capped tube for storage. The residual pellet was digested overnight (18 hr) at 37°C in 5 ml of 0.1 N sodium hydroxide to release the RNA and the digest was re-precipitated at 4°C by addition of 5 ml of 0.5 N perchloric acid. After centrifugation, the supernatant fluid was ana-

¹ Eberbach Corporation, Ann Arbor, Mich.

lyzed by the orcinol reaction [13] for ribonucleic acid and read at 670 $m\mu$ on a Coleman Junior spectrophotometer. Values were obtained from a standard curve established from yeast RNA. The precipitate was heated at 90°C in 10 ml of 0.5 *N* perchloric acid for 15 min, cooled and the resulting suspension centrifuged. The supernatant fluid was then assayed for deoxyribonucleic acid employing the Burton modification of the Dische diphenylamine reaction [4] and read at 600 $m\mu$ against a standard curve derived from herring sperm DNA. The protein residue was resuspended in 5 ml of 1 *N* NaOH and incubated overnight (18 hr) at 37°C. Using bovine serum albumin as a standard, protein content was determined by the Lowry method [11]. The samples of medium originally separated from the cells were treated similarly except that initially 0.5 ml of 11 *N* perchloric acid was added to 10 ml of medium yielding a final concentration of 0.55 *N* acid.

RESULTS

Fig. 2 shows the pattern of nucleic acid and protein synthesis in an L-M cell population during the plateau phase. In this experiment five replicate cultures were analyzed and the range of cell number, nucleic acid and protein values obtained is designated by the vertical lines at various points on

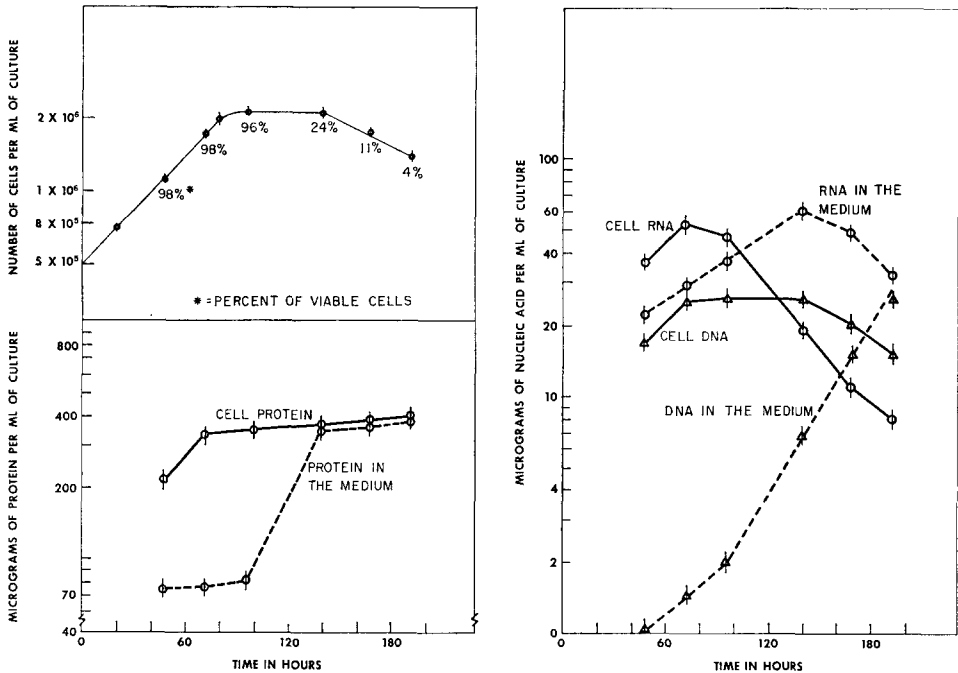


Fig. 2.—Protein and nucleic acid synthesis of L-M cells during plateau phase.

each curve. As previously observed, cell synthesis of nucleic acid and protein paralleled increase in cell number during logarithmic growth. At the onset of the plateau phase (about 80 hr on the graph) cellular RNA started to decrease and dropped precipitously once plateau phase was established. Concomitantly acid precipitable RNA was detectable in the medium. The subsequent loss of precipitable RNA in the medium during the period of decline probably was attributable to the action of liberated RNase from disrupted cells. This pattern of RNA loss was observed consistently in similar experiments. The cellular RNA content decreased apprx. 60 per cent during the plateau phase. However, DNA values remained relatively constant and paralleled the growth curve during the log, plateau and decline periods. It is interesting that the loss of cell viability occurred simultaneously with the rapid decrease in RNA.

Cellular protein increased slightly throughout plateau phase and a significant increase in extracellular protein also occurred shortly after the onset of the stationary phase in every series of experiments. The qualitative nature of this material was not analyzed, but it either was a product secreted by cells directly or accumulated due to cell lysis. The latter is not likely in that cell number remained constant and appreciable DNA was not detected in

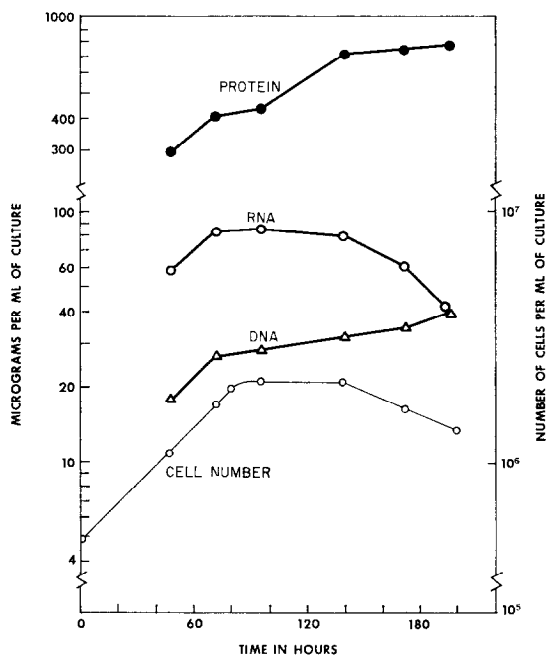


Fig. 3.—Total protein and nucleic acid production during plateau phase.

the medium. It is possible that some form of extracellular synthesis was operative in the presence of enzymes leaking from the cells. Merchant and Kahn [16] and Pumper [18] have noted the synthesis of collagen-like protein under analogous conditions. As seen in Fig. 3 the total amount of RNA detected in the system (cellular and medium) never exceeded the highest level attained early in the plateau phase. On the other hand the total amount of DNA increased slightly as the plateau phase progressed and well into the period of decline. Total protein production increased markedly upon the establishment of the plateau phase despite the rapid loss of RNA from the cells.

DISCUSSION

The significance of the foregoing experiments rests in the fact that a model system has been developed for growth and maintenance of mammalian cells in an open system. Baseline data have been obtained for a particular cell line under carefully controlled conditions. Analysis of both cellular and medium components has revealed that the L-M strain mouse cell grown in a protein-free medium continues to synthesize protein despite the decline of RNA synthesis. While this may be a phenomenon peculiar to the L-M cell in a serum-free medium, it demonstrates that definition of growth cycles in terms of protein and nucleic acid synthesis must be restricted to the particular cell strain and cultural conditions under investigation [7, 20].

The progressive increase of protein in the cells during the plateau phase is at variance with the findings of Kuchler and Merchant [10] and Salzman [20] who reported that the content of protein per cell decreased progressively from the early logarithmic through the early plateau phase. However, both of these studies employed medium containing 5 per cent or more of serum protein. Under such conditions it is almost certain that a significant portion of the protein measured as cellular protein actually represented protein adsorbed on the cell surface from the medium. In the present studies, as the cultures developed, proteins leaking from the cells or secreted by them accumulated in the medium. Adsorption of these protein components could account for a portion of the increase noted. In a high serum containing medium adsorption of any homologous cell associated material or secretion thereof is masked in the presence of extraneous protein [9]. Therefore, it is very likely that adsorption of heterologous protein was a complicating factor in both Salzman's and Kuchler and Merchant's experiments, and the loss of "cell" protein, which they observed, may have been due to enzymatic degradation and utilization of adsorbed material as the medium was depleted. Moreover,

the production of protein characteristic of a fibroblastic cell as demonstrated by Merchant and Kahn [16] and confirmed by Pumper [18] does not occur, or is minimized, in a medium containing serum. Therefore, in its absence the conditions may be optimal for synthesis of a special type of protein, which could be reflected by increased cellular content or extracellular adsorption.

Population increase or decrease as determined by nucleic acid and protein content is not a reliable index of cell number. Kuchler and Merchant [10], Salzman [20] and others [22] have shown a decrease in RNA content per cell during the logarithmic and plateau phases of growth. The present study reveals that although protein content increases during the plateau phase and cell number remains fairly constant, RNA content decreases. Therefore, although nucleic acid and protein content are useful parameters of cell growth they must be used in conjunction with cell counts to reflect the true state of development of a culture.

The increases of total DNA from a level of approximately 28 $\mu\text{g}/\text{ml}$ at the onset of plateau to 40 $\mu\text{g}/\text{ml}$ at the termination of the experiments suggests that cell turnover and population renewal was occurring. Total DNA increased by 42.8 per cent during this period. However, the fact that total protein increased by 100 per cent indicates that more than population renewal was operative. An interplay of both new cell synthesis and specialized function appears to be the more plausible explanation of these findings. The decrease of total RNA in the presence of increased protein synthesis also indicates a divergence of pathways.

The foregoing results are in partial agreement with the observations of some investigators and are at variance with others. In this type of study, apart from the possibility of experimental error, perhaps the most important variables are the cell strain, culture medium and physical conditions of culture. In an open system where oxygen depletion and carbon dioxide accumulation are not limiting factors, all of the available metabolic pathways of the cell are unimpaired. Under these conditions the cell exerts more control over the environment than vice versa. However, it is worthy of note that all of the foregoing observations in the present study were made on a randomized population of cells. Consequently, the patterns of nucleic acid and protein synthesis represent mean trends in a population and not necessarily those of individual cells during the plateau phase. Very frequent sampling and analysis of a synchronously dividing population throughout the entire growth cycle would provide data, which might convey a more meaningful interpretation of protein and nucleic acid synthesis on a per cell basis during the plateau phase.

SUMMARY

The patterns of protein and nucleic acid synthesis during the plateau phase of growth of the L-M mouse fibroblast in suspension culture have been established. In a protein free medium analysis of both cellular and medium components has shown that cellular RNA decreases rapidly, DNA at a much slower rate and protein continues to increase. In addition protein and DNA accumulate in the medium, whereas RNA in the medium increases initially and then decreases as the plateau phase progresses.

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