STATIONARY PHASE OF CULTURED

MAMMALIAN CELLS (L5178Y)

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ABSTRACT

The stationary phase of the mammalian cells L5178Y in culture can be divided into two stages: (a) an early phase characterized by the decline of mitotic index, followed by a stabilization of the cell number, and (b) a late stage, occurring several hours after the flattening of the growth curve, during which dead or dying cells appear in the cultures. The estimates of rates of cell progress showed that the rates from G_1 to S and from G_2 to M were affected in the early stationary phase. The main cause of cessation of increase in cell number in the early stationary phase is resulted from the decline in mitotic index, which is caused by prolongation of the G_2 period. The importance of the G_2 stage in regulating the cell growth is discussed in relation to other known situations in the literature.

INTRODUCTION

The growth curve of cells in culture is, in general, made up of three phases: the latent phase before growth begins, the exponential growth phase in which cell numbers increase rapidly, and the final or stationary phase in which the rapid increase in cell numbers gradually slows. In a previous paper (22) the exponential phase of L5178Y leukemic cell line was shown to be in a "steady state of growth." In this state, the fraction of cells in each of the four stages of the life cycle is constant as is the time spent in each stage. The rate of progress of cells from one stage to the next is also constant. Alteration of the generation time of the cells by temperature reduction during the exponential growth phase has been shown to be due mainly to alteration in the rate of the cells' progress from the G_1 to S stages (22).

In the present paper, the stationary phase of the growth curve was studied in the mouse L5178Y leukemic cell line. The main characteristic of the stationary phase is a cessation of the rapid rate of increase of cell numbers which is so prominent in the exponential phase. The purpose of the present study is (a) to discover how the cell number stabilizes in the stationary phase, and (b) to evaluate these results from the point of view of regulatory mechanisms of cell proliferation.

MATERIALS AND METHODS

The mouse leukemic cell line L5178Y in suspension culture was used throughout the present experiment (4, 21). The time characteristics of this cell line in the exponential growth phase are as follows: generation time, 10.8 hr; G_1 period, 1.8 hr; S period, 7.3 hr; G_2, 1.2 hr; and M period, 0.55 hr (22).

TABLE I
Estimation of the Rate of Cell Progress from One Stage to the Next

Stages	Methods*	References
M → G$_1$	Rate of disappearance of M stage cells following G$_2$ block caused by irradiation	21
	Rate of appearance of daughter cells in a cell population harvested at the M stage	17
	Rate of increase of cell numbers in synchronized cell cultures	18
G$_1$ → S and S → G$_2$	Rates of appearance and of disappearance of pulse-labeled cells (S stage cells) in synchronous cultures	17
	Difference in per cent of labeled cells after continuous labeling and pulse-labeling, with the following techniques:	
	(a) Continuous labeling and pulse-labeling of separate aliquots of same culture	21
	(b) Continuous labeling with low specific activity thymidine-^{3}H and pulse-labeling of same culture with high specific activity	10
	Difference in per cent of ^{3}H-labeled, ^{14}C-labeled, and ^{3}H-^{14}C-labeled cells after pulse-labelings with thymidine-at various time intervals	23
G$_2$ → M	Rate of accumulation of metaphase cells after treatment with Colcemid	13

* Labeling is done with thymidine-^{3}H unless otherwise specified.

The cells were grown until their number reached 8 × 10^5 cells per milliliter. They were then transferred to fresh, warm culture medium to make a final cell concentration of approximately 4 × 10^5 cells per milliliter. Aliquots of the culture were removed from time to time and the cell number in each was estimated by a Coulter counter (Model A). In each aliquot, one other parameter of the culture was obtained by the following methods: the mitotic index was estimated in acetoorcein-stained samples; the per cent of labeled cells was determined by radioautography after pulse-labeling with tritiated thymidine; the per cent of dead or dying cells was estimated in eosin-stained preparations (3, 21, 22).

The rate of progress of cells from one stage in the life cycle to the next was studied by methods considered to be suitable for each rate estimate of exponentially growing cell suspensions in our hands (see Table I). The rate of progress from G$_1$ to S and from S to G$_2$ was estimated by a combined method of pulse-labeling and continuous labeling with thymidine-^{3}H (21). The rate of progress from G$_2$ to M was estimated by a modified method of Puck and Steffen (13; also reference 22), with an optimum concentration of Colcemid for this cell line (0.75 μg/ml) and a time period of 5 hr to allow maximal accumulation of M stage cells (Fig. 1). The rate of progress from M to

G$_1$ stages was studied by the X-irradiation-induced G$_2$ block method (21).

Since only two measurements could be carried out on each aliquot, e.g. cell number and mitotic index, and since the cell number was always counted in each experiment, the results of different experiments were compared by superimposing growth curves on one another to adjust them to the same time scale relative to the stationary phase. In order to express the data on the same time scale, "zero" time was defined, for convenience, as the time when the extrapolation of the straight line of the logarithm of cell numbers in the exponential growth phase intercepts that line drawn through the constant cell number in the stationary phase. As is discussed later in the text, zero time occurs 5 hr after the mitotic index begins to decrease. The determinations of the rates of cells' progress were carried out at zero time to assure us that we were definitely dealing with the "stationary phase." All experiments on stationary phase cultures were repeated at least twice, often simultaneously with those on exponentially growing cultures. The parameters of the life cycle estimated for the exponential growth phase in this study were in good agreement with those previously determined (22).
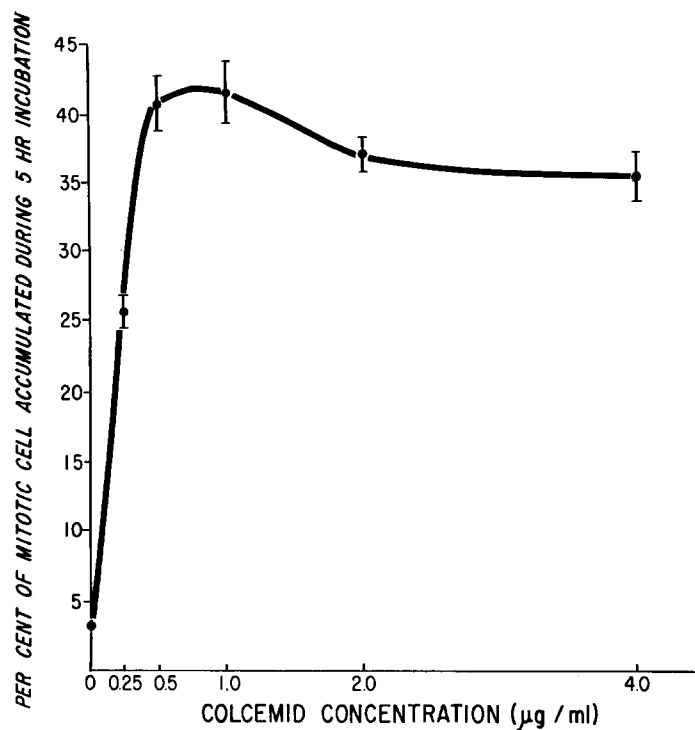
FIGURE 1 The accumulation of mitotic cells after 5 hr treatment with various concentrations of Colcemid.

RESULTS

Description of Stationary Phase

During the transition period from the exponential growth phase to the stationary phase, the per cent of eosin-stained cells, the per cent of mitotic cells, and the per cent of cells pulse-labeled with thymidine-^{3}H were estimated (Fig. 2). The first noticeable change during the transition was a decrease in the mitotic index (Fig. 2, C) (22). The gradual decline of the mitotic index continued for at least 15 hr. The beginning of the leveling off in the rate of the increase of cell number seemed to coincide in time with the onset of decrease of the mitotic index; however, the cell number did not stabilize completely until 6 or 7 hr after the decrease in mitotic index had begun (Fig. 2, A). The per cent of pulse-labeled cells (S stage cells) in the stationary phase remained at 62 ± 3% (22), essentially the same as that in the exponential growth phase (Fig. 2, B). Several hours after zero time, however, a significant decrease in the per cent of labeled cells was noted. The per cent of eosin-stained cells (assumed to be dead or dying cells)

(21, 22) remained nearly the same as that in the exponential growth phase for 6–7 hr after the zero time and then increased sharply.

From these observations, the stationary phase can be divided into two stages. The first (A) starts with the onset of decline of the mitotic index and is followed by the virtual cessation of the increase in cell numbers. The second stage (B) begins with the sudden increase in the number of eosin-stained cells.

Rate of Cells' Progress from one Stage to the Next

The rate of cells' progress from one stage to the next was always estimated approximately at zero time. This time was selected because the cell population was definitely in the stationary phase, but dying or dead cells were not yet present to complicate the observations. The rate of progress of cells from G_1 to S during the 2 hr period following zero time was about 74% of that of the exponential growth phase; after this, the rate declined rapidly with increasing time (Fig. 3). The rate of progress of cells from S to G_2 in the first 2 hr after zero time
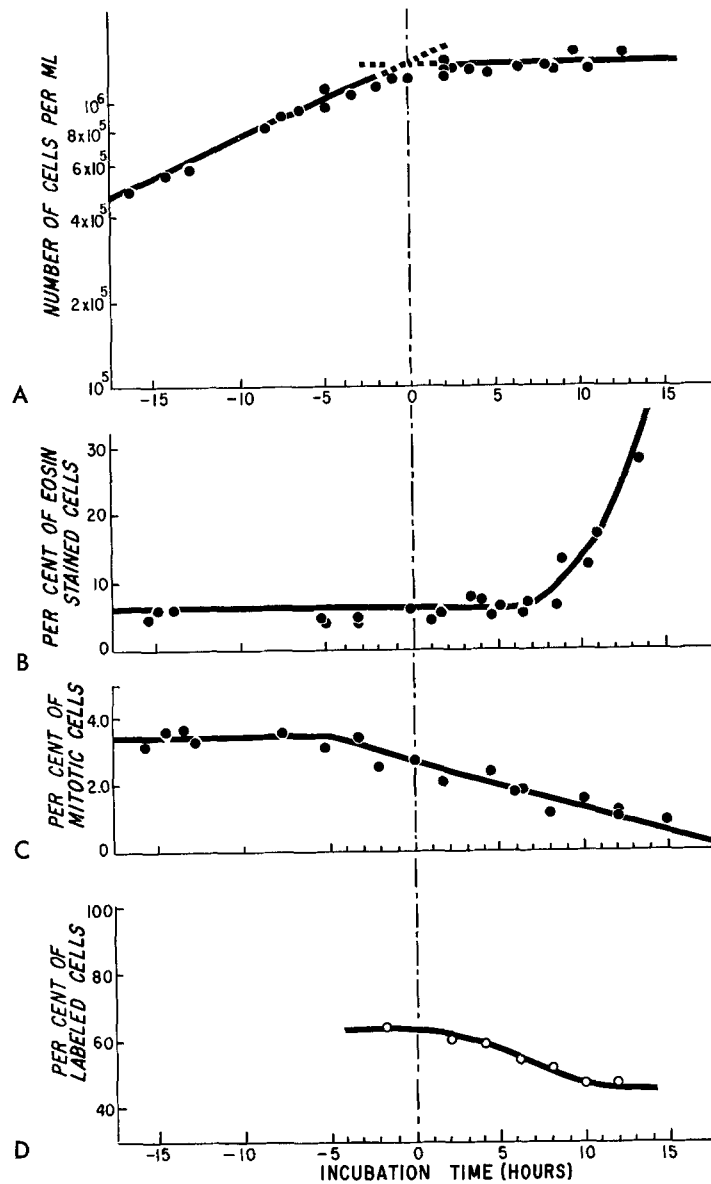
FIGURE 2 Relationship of cell number (A), per cent of eosin-stained cells (B), per cent of mitotic cells (C), and per cent of labeled cells (D) through the period extending from the end of exponential growth phase to the start of the constant cell number in the stationary phase. The intersection of the extrapolated slopes of cell numbers in the exponential and stationary phases is defined as the zero time.

was around 95% of that in the exponential growth phase; thereafter, the rate decreased rapidly with time (Fig. 3). It is interesting to point out that the rate of progress from G_1 to S was always lower than that from S to G_2, and that the decrease of the per cent of pulse-labeled cells (S stage cells) in the

stationary phase was the result of the different in these two rates (Fig. 3).

The rate of progress from G_2 to M is shown by the accumulation of mitotic cells after Colcemid treatment (Fig. 4). The rate of progress from G_2 to M in the stationary phase was about 84% of that
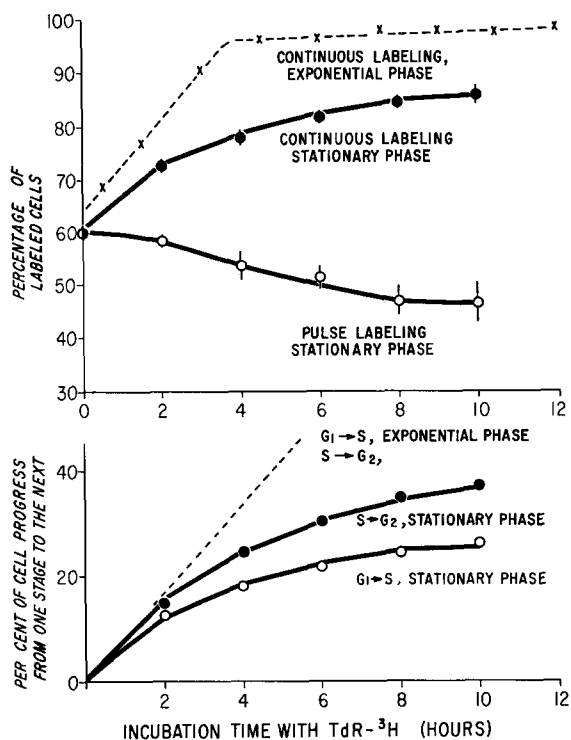
FIGURE 3 The per cent of cells labeled by the continuous and pulse-labeling techniques, and the rate of progress of cells from G_1 to S and from S to G_2. Upper figure: per cent of cells continuously labeled or pulse-labeled in the exponential and stationary phases. Labeling was started at the zero time. Lower figure: the rate of progress of cells estimated from the upper figure. The slope of the dotted line and solid lines corresponds to the rates from G_1 to S and from S to G_2.

in the exponential growth phase. This indicated a decrease in the rate of entry of cells into the M stage.

The cause of such a decreased rate of progress from G_2 to M is found by studying the slope of the curve for labeled mitotic cells, as plotted against time, compared to the slope of the curve for the total mitotic cells (Fig. 4). That in the exponential growth phase the slopes of both curves are parallel indicates that the duration of G_2 period is constant (1.21 ± 0.25 hr). That in the stationary phase, however, the slope of the curve for labeled mitotic cells, as plotted against time, is less steep than the slope of the curve for the total mitotic cells indicates that there is a gradual prolongation of G_2 period. In Fig. 4 the G_2 period was approximately 1.27 hr at zero time and 5 hr later was prolonged to 2.19 hr.

The rate of cell progress from M to G_1 is shown in Fig. 5. In this figure, the dotted line represents the mitotic index. After a G_2 block by X-irradiation was induced (see reference 21), the rate of fall of the mitotic index in the stationary phase was nearly as rapid as that in the exponential growth

phase. This indicates that the cell progress from M to G_1 in the stationary phase was not significantly altered from that observed in the exponential growth phase.

Binucleated Cells and Polyploid Cells

During the transition from the exponential to the stationary phase there was a significant increase in binucleated cells (Table II). The per cent of polyploid cells, on the other hand, did not increase (Table III).

DISCUSSION

The purpose of the present paper is to show how the cell population is adjusted during the transition from the exponentially growing phase to the stationary phase; therefore, the kinetics of the altered cell proliferation have been studied by means of life-cycle analysis. As to the cause of the stationary phase, deletion of essential nutrients or accumulation of toxic metabolic wastes in the medium, or both, are probably responsible, but no conclusive studies of this aspect have been made at the present time.
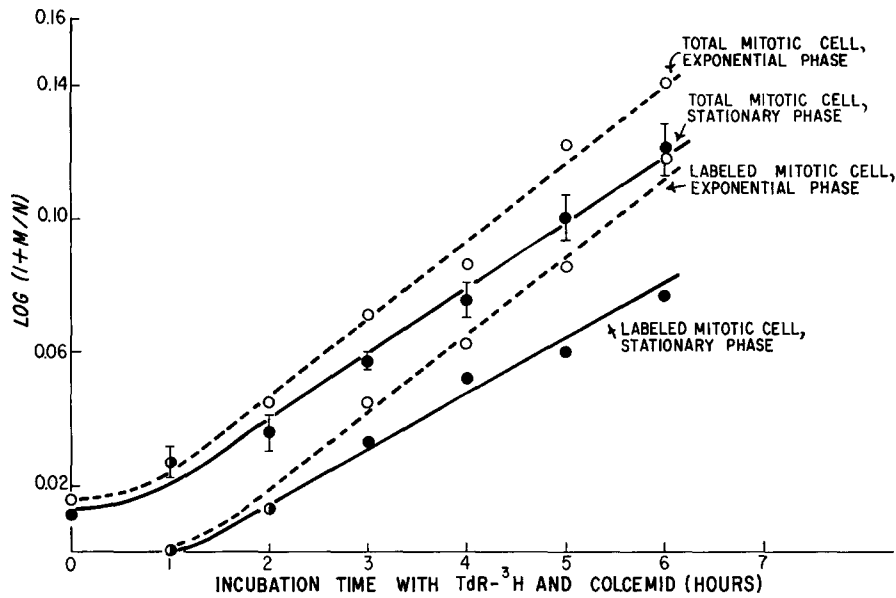
FIGURE 4 Rate of progress from G_2 to M and duration of G_2 stage in the exponential growth phase. Dotted lines with open circles represent total and labeled mitotic cells in the exponential phase. The solid lines with closed circles represent the total and labeled mitotic cells in the stationary phase. At zero time in each experiment, Colcemid (0.8 μg/ml) and thymidine-^{3}H (0.2 μc/ml) concentration and 67 mc/mM specific activity) was added. The slope of the curve for total mitotic cells is equivalent to the rate of cell progress from G_2 to M. The duration of the G_2 stage is equivalent to the horizontal distance between the slope of the total and the labeled mitotic cells. In the exponential growth phase, the two slopes are parallel and the G_2 duration is 1.21 ± 0.25 hr, which is in good agreement with the previously reported value of 1.22 ± 0.22 hr (22). In the stationary phase, the duration of the G_2 period increased gradually from 1.3 to 2.1 hr at the end of this experiment.
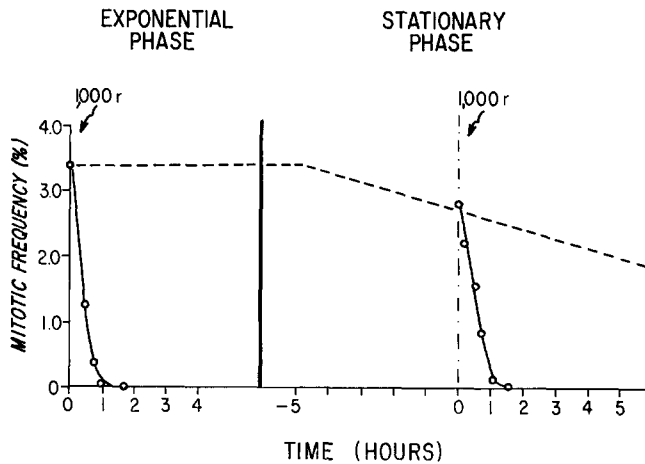

FIGURE 5 Rate of progress of the cells from the M to the G_1 stages in the exponential and stationary phases. The dotted line represents the mitotic index of the cell population as the cell progresses from exponential growth phase to stationary phase. When 1,000 r of gamma-rays were administered at zero time to different aliquots of the cultures in the exponential and stationary phases, the lines with open circles show the rate of fall in mitotic index. The slopes of these solid lines reflect rate of progress from M to G_1.

Kinetics of the Stabilization of the Growth Curve in the Stationary Phase

The stationary phase is characterized by a flattening of the growth curve. This could be explained by one of the following mechanisms: (a) the increase in cell numbers resulting from mitotic division is compensated partly by loss of dead cells; (b) certain mitotic cells develop into binucleated or polyploid cells rather than divide

TABLE II

Per Cent of Binucleated Cells in the Exponential Growth Phase and the Stationary Phase

Growth phase	Time*	Per cent of binucleated cells‡
	hr	
Exponential	−15	1.4 ± 0.6
	−10	1.4 ± 0.4
	−5	1.3 ± 0.1
Stationary	+5	2.3 ± 0.1
	+10	2.8 ± 0.2
	+15	3.6 ± 0.3

* Time in relation to the zero time.
‡ Two separate experiments were carried out. 2,000 cells were counted for each point in one experiment.

TABLE III

*Per Cent of Polyploid Cells**

Growth phase	Polyploid cells %
Exponential‡	4.9 ± 0.6
Stationary§	
A	4.1 ± 0.8
B	3.0 ± 0.7

* L5178Y cells are near diploid (40 ± 1 chromosomes). Polyploid cells, here, are those with more than 50 chromosomes; tetraploid cells constituted about 20–30% of the polyploid cells.
‡ Cells in the exponential growth phase (about 300,000 cells/ml) were treated with Colcemid (0.8 μg/ml) for 5 hr; 4,000 mitotic cells were examined.
§ The stationary phase cells (zero plus 13 hr) were transferred to fresh medium for 10 hr and then treated with Colcemid either for 4 hr (*A*) or for 7 hr (*B*).

into daughter cells; and (*c*) the number of mitotic cells decreases.

The first mechanism can be discarded because the cells did not stain with eosin until 11–12 hr after the decline in mitotic activity. Furthermore, the dying or dead eosin-stained cells persisted in the medium for several days and, following this, were only slowly eliminated. The second mechanism can also be ignored, since polyploid cells constituted only 4% of cells in the stationary phase and binucleated cells accounted for about 3% of the total population; neither figure is large enough to explain the flattening of the growth curve. This leaves the third mechanism, namely a decrease in the number of mitotic cells, as the one likely to be responsible for the diminution in the rate of increase in cell numbers. As has been noted, the decrease in the per cent of mitotic cells is the first observable change in the transition from exponential to stationary phase. Assuming that the rate of cells' progress from G_2 to M is the same in the exponential and stationary phases, as is indicated by Fig. 5, one can calculate the expected increment of new cells from the mitotic index. Fig. 6 shows that the early portion of the curve of the calculated increase in cell numbers can be superimposed on the actual curve in the early stationary phase. Later, however, a discrepancy between the two curves becomes apparent. We conclude, therefore, that the decline in mitotic index is sufficient in itself to account for the flattening of the growth curve in the early stationary phase.

The Cause for Decline of Mitotic Index in the Early Stationary Phase

From the 0 hr to 0 + 2 hr in the stationary phase, the rate of cell progress from G_1 to S was depressed to 74% of that in the exponential growth phase, the rate from S to G_2 was depressed to 95%, and the rate from G_2 to M was depressed to 84%. Since a slowdown of cell progress in any part of the life cycle, other than M stage, will ultimately lead to a decline in the mitotic index, the question then is which part of the life cycle is responsible for the early decline of mitotic index.

Although the rate of progress from G_1 to S is most severely affected at about zero time, this cannot be the cause of the depressed mitotic activity early in the stationary phase for the following two reasons: (*a*) it would take at least 8.4 hr (the duration of the S and G_2 periods) before such a change would affect the mitotic index, and (*b*) such a depression in the rate of progress from G_1 to S would cause a decrease in S stage cells before affecting the mitotic index. Since the mitotic index decreased in the early stationary phase before a change in the S stage cells was noted, it is apparent that a change in cells' progress from G_1 to S cannot affect the mitotic activity early in the stationary phase. This, then, means that the depressed rate of progress from G_2 to M is the cause of the decline of the mitotic index during the early stationary phase. As is illustrated in Fig. 4, the depressed rate of
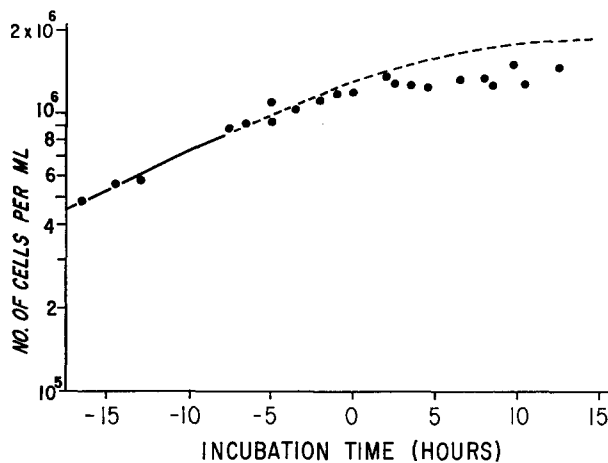
FIGURE 6   Changes of observed cell numbers and of cell numbers calculated from the mitotic index. Solid circles represent the cell numbers observed at various times; solid line refers to the cell number during exponential growth phase; and dotted line is the calculated cell number.

progress from G_2 to M is believed to be the result of prolongation of the G_2 period.

It is interesting to point out that this conclusion is in agreement with that of Macieira-Coelho et al. (9). Their conclusion was based on an entirely different type of observation, namely that human fibroblasts in the stationary growth phase pulse-labeled with thymidine-^{3}H did not show a 50% reduction in grains per cell during the subsequent incubation for 24 hr in the absence of thymidine-^{3}H. They interpreted these observations as indicating that the cells remained in G_2 stage without dividing.

Late Stationary Phase and Cell Death

In the late stationary phase, there was a rapid decrease in the rate of progress from G_1 to S as well as from S to G_2. This was followed by the sudden appearance of significant numbers of eosin-stained cells. The mitotic index, in contrast, declined continuously in the late stationary phase without any precipitous change. These results may be interpreted as indicating that cells died in G_1 and/or early S stage rather than in G_2 or M stage. Such death in the G_1 and/or S stage is analogous to that (21) occurring after exposure of this cell line to lethal doses of X-rays.

The appearance of dead cells in the late stationary phase may be partly responsible for the discrepancy between the calculated and the observed cell numbers (Fig. 6).

Consideration of the Role of the G_2 Stage in the Regulation of Cell Proliferation

Defendi and Manson (2) and many others (22) found the G_1 stage to be important in regulating

the growth of mammalian cells in vitro as well as in vivo. Other studies along this line of investigation, however, have indicated that G_2 stage may also play an important role in regulating growth of certain lines. Gelfant (5, 6) found that, in the process of wound healing of the rat's ear, some epidermal cells entered the M stage immediately while others entered the S stage. He interpreted this as indicating that in normal epithelium some cells were in a prolonged G_2 stage while others were in a prolonged G_1 stage. The presence of a population of cells remaining in the G_2 stage for a long time was also observed in osteogenic tissues (12) and in enamel tissues (14). In the gastrointestinal tract of chicken, Cameron and Cleffmann (1) made the following observations: when fasted chickens were fed, some epidermal cells of the esophagus entered the M stage promptly and others entered the S stage. This suggests that both G_2 and G_1 stages play an important role in stopping or slowing down cell growth during fasting. It is interesting to point out that farther down in the gastrointestinal tract the epidermal cells of the duodenum were only in the G_1 stage. In a cultured mammalian cell line (W1-38), repeated passage is known to bring about ultimate death of all cells. In terminal cell cultures, the prolongation of the generation time has been attributed mainly to prolongation of the G_1 and G_2 stages (8). Evidence for growth regulation in the G_2 stage is also found in the phenomenon known as "mitotic delay" induced by exposure of cells to radiation and other chemicals (7, 10, 13, 15, 21). Puck and Steffen have shown that the cause of mitotic delay after X-irradiation is a transient block in G_2 stage (13).

The present experiments and the work of

Macieira-Coelho et al. (9) constitute additional evidence showing the role of the G_2 stage in regulating cell growth in cultured mammalian cells. In contrast to the regulatory role of G_1 stage in cells in the exponential growth phase (2, 22), the regulatory role of G_2 stage seems to be evident only when the cells are facing an environmental adversity of some sort, such as injury by cold temperature (1, 5), poor nutrition (9, 12, 14, and probably the present paper), damage by X-rays (7, 13, 15, 21) or by chemicals (1, 11, 15), and aging (8). It should be noted that control of the growth rate in stationary phase is essentially different from contact inhibition which is regulated in the G_1 stage (20).

Some of the molecular events known to occur in the G_2 stage are mRNA synthesis (18, 19) and synthesis of proteins (16, 18, 19) necessary for mitosis. Other events believed to occur during the transition of G_2 to M include energy production, SH and S-S transition, breakdown of nuclear membranes, spindle formations, coiling of chromosomes, etc. (11, 15). At present, we do not have sufficient knowledge to decide whether any of these events might be responsible for the prolongation of G_2 stage.

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