# EFFECTS OF TEMPERATURE ON GROWTH RATE

# OF CULTURED MAMMALIAN CELLS (L5178Y)

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## ABSTRACT

L5178Y cells were cultured in vitro at various temperatures. When the cells were in the exponential growth phase, the cells were in the "steady state of growth," i.e., the fraction of cells in the $G_1$, S, $G_2$, and M stages and the durations of each stage were constant. The life cycle analysis of the cells in the steady state of growth demonstrated that the $G_1$ stage and the S stage were affected the most by variation of temperature, and suggested that these two stages have considerable influence on the growth rate of the L5178Y cells. The calculated activation energies were positive in each stage of the life cycle, whereas the entropies of activation were negative throughout. The possible significance of these findings in our search for the regulatory mechanisms of cell growth is discussed.

Although the effect of temperature on growth rate has been studied in detail in bacteria for many years (15, 16, 19, 36), relatively little work of this nature has been carried out with mammalian cells. If combined with modern techniques of life cycle analysis, such studies in mammalian cells might shed light on the mechanisms regulating cell growth. For example, temperature change could modify cell growth in one of the following ways: (a) the rate of progress through all four stages of the life cycle ($G_1$, S, $G_2$, and M (14, 18)) could be altered to the same degree; (b) the rate of progress through only one or two stages could be affected; and (c) the rates through all four stages could be modified to different degrees resulting in a situation somewhat between (a) and (b). By pinpointing the exact stages in the life cycle where change in temperature or other environmental factors affect the growth rate, it might be possible to identify molecular events occurring simultaneously as the mechanisms involved in the regulation of growth rate.

To date, the limited observations of the effect of temperature on cultured mammalian cells are contradictory. In human amnion cells, Sisken (37,

38) reported that the main effect of temperature on growth rate is to change the rate of cell passage through the $G_1$ stage. In contrast, Rao and Engelberg (30) observed that temperature change affects the growth rate of HeLa S3 cells by modifying the rate of progress through $G_1$, S, and $G_2$ stages to a similar degree. Although Paul (24) studied the effect of temperature on the growth rate of L5178Y cells, no experimental work combining temperature effect and life cycle analysis has been reported in this cell line.

In the present paper, mouse leukemic cells (L5178Y) were incubated at various temperatures and life cycle analyses of cells growing in the exponential growth phase were carried out. The thermodynamics of the rates of progress through the four stages of the life cycle were considered to determine the "energetics" involved in cell growth. An attempt was made to relate these observations to growth regulatory mechanisms.

## MATERIALS AND METHODS

CELL LINE: The mouse leukemic cell line, L5178Y, kindly supplied by Dr. G. A. Fischer of Yale University, was used in this study. The cells were

cultured in Fischer's medium (Hyland Laboratories, Los Angeles, Calif.) supplemented with 10% horse serum, 0.02% glutamine, 0.025% penicillin, and 0.005% streptomycin (10). The cells were mass-cultured in a 250-ml flask for at least 48 hr at the temperature to be studied. The cells were then inoculated into tissue culture tubes containing fresh medium at room temperature and kept in an incubator at the temperature to be studied. From time to time, the tubes were removed in order to determine the total cell number, the percentage of eosin-stained cells, the percentage of mitotic cells, or the percentage of cells labeled with tritiated thymidine. In practice, because of the time requirements, all four measurements could not be made in one experiment. Usually, the cell count and one other parameter were determined simultaneously in each experiment.

DETERMINATION OF CELL NUMBER: The number of cells was determined by means of a Coulter counter, Type A.

MITOTIC INDEX: The cells were fixed with acetic alcohol, stained with acetoorcein and the number of mitotic cells in the total cell population (mitotic index) was determined (32, 45).

EOSIN STAINING: In order to test the possible contribution of dead cell fractions to the total population, we mixed 2 vol of the cell suspension with 1

vol of 1% eosin solution, and ascertained percentage of eosin-stained cells in the total cell population (7, 45).

PERCENTAGE OF LABELED CELLS: The cells were incubated with tritiated thymidine (2 $\mu$c/ml, specific activity, 6.7 c/mM) for 10 min and the percentage of cells taking up tritiated thymidine was estimated by radioautography (Eastman Kodak NTB emulsion) (27, 45).

## RESULTS

### Cells in the Exponential Growth Phase

Immediately after inoculation, the cell number did not increase for several hours (lag phase) (Figs. 1 and 2 a). Then, the cell count began to increase exponentially with time (exponential growth phase). After 50–100 hr in the exponential growth phase, the culture reached a final phase (stationary phase), in which there was no further increase in cell number.

The changes of the mitotic index through various phases of the growth curves were studied at various temperatures (Figs. 2 c and 3). At all temperatures studied, the mitotic index in the lag phase increased with time and reached a plateau
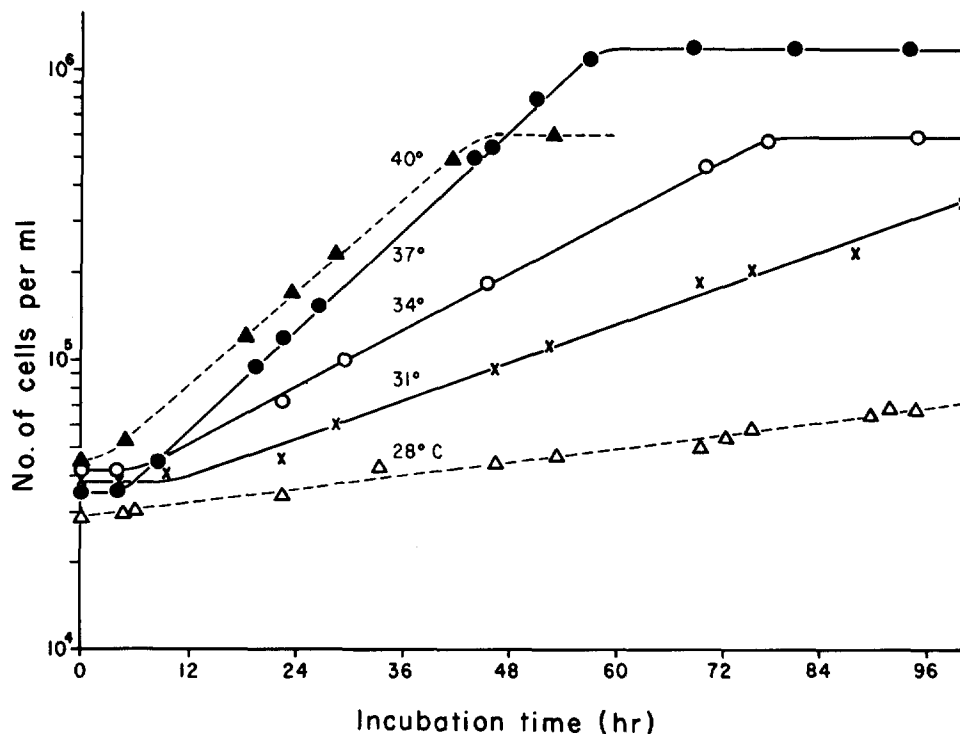


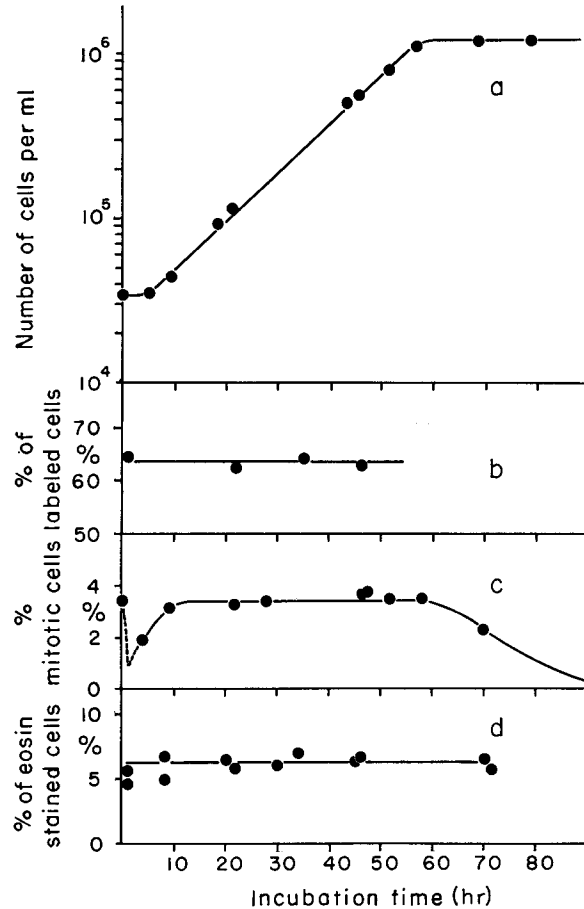FIGURE 1   The growth curves of L5178Y cells at different temperatures.

FIGURE 2 The relationship of the growth curve, the percentage of labeled cells, the percentage of mitotic cells, and the percentage of eosin-stained cells with incubation time at 37°C.

as soon as the cells entered the exponential growth phase. The constant plateau value of the mitotic index was maintained throughout the exponential growth phase. When the cells reached the final stationary phase there was a gradual decrease of the mitotic index.

Other growth characteristics of the cells, i.e. the percentage of the labeled and the eosin-stained cells in relation to the mitotic index and the growth curve, are shown in Fig. 2 for the cell cultures maintained at 37°C. In Fig. 2 *b*, it can be seen that the percentage of cells labeled with thymidine-$^3$H was constant during the exponential growth phase. In Fig. 2 *d*, one notes that the percentage of eosin-stained cells also remained constant during the exponential growth phase. In contrast to the mitotic index, these two parameters did not show any drastic change in the lag phase or in the early stationary phase.

The percentage of labeled cells represents the population of S stage cells. Similarly, the mitotic index indicates the percentage of M stage cells, and the percentage of the eosin-stained cells is proportional to the fraction of dead or dying cells.[1] The constancy of the fractions of S stage cells, M stage cells, and dead cells observed in the exponen-

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1 In the continuous labeling experiment (Fig. 5), the maximum percentage of labeled cells after 3.6 hr was 96% rather than 100%; thereafter, the percentage of labeled cells increased slowly with time. When one extrapolated this second slope to zero hour, a value of 94% was obtained. The difference of 6% between 100 and 94% is the same magnitude as the 5–6% of eosin-stained cells and, we believe, is accounted for by dead or dying cells which cannot be labeled. The second slow increase of the percentage of labeled cells is probably due to the gradual disappearance of non-labeled dead cells from the cell population.
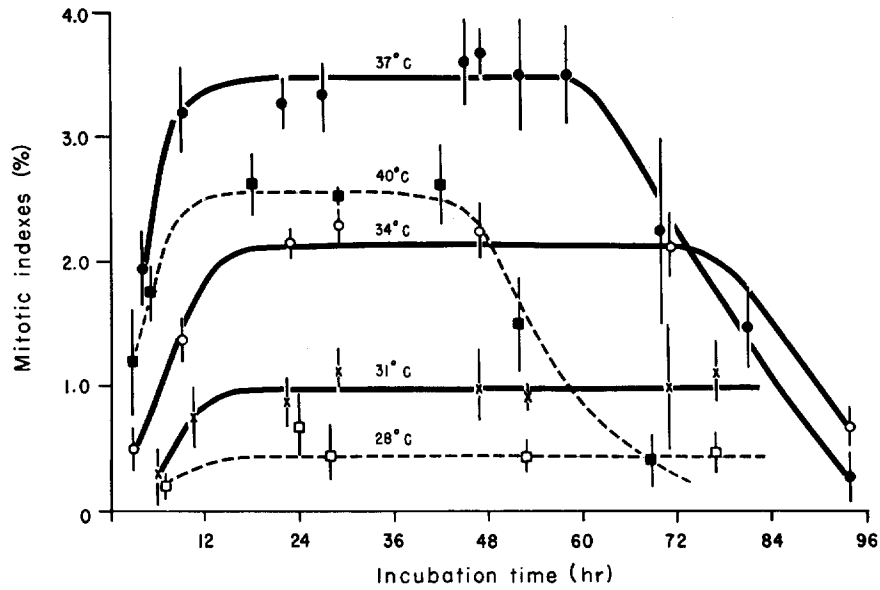
FIGURE 3 Variation of the mitotic index through various phases of the growth curves at different temperatures. The mitotic index of the exponential growth phase at 31°C in this figure is different from that given in Table II. The reason for this is that Table II uses the average mitotic indices of several experiments whereas the value in this figure represents that of one experiment.

TABLE I

*Relationship of the Fraction of the Cells to the Duration of Different Stages of the Life Cycle During the Exponential Growth Phase*

$$1 = \frac{G_1}{N} + \frac{S}{N} + \frac{G_2}{N} + \frac{M}{N} \qquad (1)$$

$$T = t_{G_1} + t_S + t_{G_2} + t_M \qquad (2)$$

$$\alpha = \frac{\log 2}{T} = \frac{0.693}{T} \qquad (3)$$

| Cell stage | Fraction of cells at one stage | | Duration of this stage | |
|---|---|---|---|---|
| M | $\dfrac{M}{N} = e^{\alpha t_M} - 1$ | (4) | $t_M = \dfrac{1}{\alpha} \log\left(\dfrac{M}{N} + 1\right)$ | (8) |
| $G_2$ | $\dfrac{G_2}{N} = e^{\alpha t_M}(e^{\alpha t_{G_2}} - 1)$ | (5) | $t_{G_2} = \dfrac{1}{\alpha} \log\left(\dfrac{G_2}{M + N} + 1\right)$ | (9) |
| S | $\dfrac{S}{N} = e^{\alpha(t_M + t_{G_2})}(e^{\alpha t_S} - 1)$ | (6) | $t_s = \dfrac{1}{\alpha} \log\left(\dfrac{S}{G_2 + M + N} + 1\right)$ | (10) |
| $G_1$ | $\dfrac{G_1}{N} = 2(1 - e^{-\alpha t_{G_1}})$ | (7) | $t_{G_1} = \dfrac{1}{\alpha} \log\left(\dfrac{1}{1 - \dfrac{G_1}{2N}}\right)$ | (11) |

Log, $\log_e$ ; T, generation time; N, total number of cells; $\alpha$, a constant.

tial growth phase indicates that the fractions, the duration of all four stages ($G_1$, S, $G_2$, and M), the rate of cell progress from one stage to the next, and the rate of cell death must be constant during the exponential growth phase. In other words, the cells in the exponential growth phase are in the "steady state of growth."

## Life Cycle Analysis of Cells in the Steady State of Growth

As described in the previous section, cells in the exponential phase are in a steady state of growth in which the fraction of cells in each stage of the life cycle and the duration of the four stages are constant. Stanners and Till (39) and Puck and Steffen (28) have developed equations for cell proliferation kinetics in the steady state of growth. From the kinetics of Stanners and Till, we have derived equations which relate the fractions of cells to the duration of each different stage. These equations are summarized in Table I.

In a complete life cycle analysis, one is confronted with nine unknowns: T, $G_1/N$, $t_{G_1}$, $S/N$, $t_S$, $G_2/N$, $t_{G_2}$, $M/N$, and $t_M$. Since all these unknowns can be related in eleven equations (Table I), one has to determine values for at least four unknowns to make a complete life cycle analysis. Some of the methods available for the measurement of these unknowns are tabulated in the Appendix. Since each method has its advantages and disadvantages as well as its own inherent variations, one has to consider the methods carefully before selecting one for life cycle analysis.

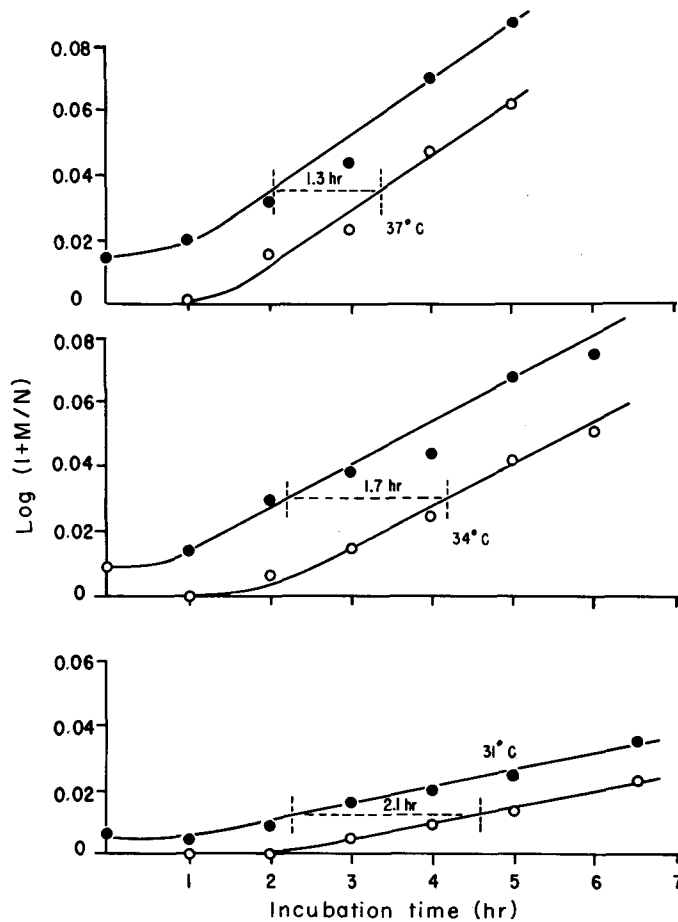In the present study, we have chosen several methods of life cycle analysis which can be adapted



Figure 4 Determination of the duration of the $G_2$ period at different temperatures. ●, log $(1 + M/N)$; ○, log $[(1 + M/N)/(1 + \overline{M - M^*}/N)]$.

to suspensions of cultured cells. In principle, T, M/N, S/N, and $t_{G_2}$ were experimentally estimated and complete life cycle analyses at three different temperatures were then made by applying these values to equations (1), (2), (3), (5), (8), and (10) (see Table I). The equations were programmed for computer analysis.

Since, in reality, the cell population always contains a small percentage of dead cells, it is necessary to introduce a correction factor. An accurate correction for dead cells can be made only by knowing the fraction of dead cells, the stage in the life cycle when death occurs, and the rate of disappearance of dead cells from the cell population. Since the effort to obtain such information would be considerable, a simple way to approximate this factor was used. Assuming that the eosin-stained cells are dead or dying (45),[1] the correction factor, 1–(eosin staining)/100 was applied in the following manner:

T (*generation time*). The population doubling time was estimated from the linear portion of growth curves in Fig. 1. T was obtained by multiplying the population doubling time by the correction factor.

M/N (*fraction of M stage cells in the total living cell population*). The mitotic index was divided by the correction factor to obtain the true M/N.

S/N (*fraction of S stage cells in the total living cell*

*population*). The percentage of cells pulse-labeled with tritiated thymidine was divided by the correction factor to give S/N.

$t_{G_2}$ (duration at the $G_2$ stage). This was calculated by the following two methods: The first was slightly modified from that of Puck and Steffen (28) in that colchicine (0.6 $\mu$g/ml) was substituted for Colcemid.

Also $\log\left[\left(1 + \dfrac{M}{N}\right)\Big/\left(1 + \dfrac{M - M^*}{N}\right)\right]$, instead of $\log(1 + M/N)$, was plotted against time. In this equation, M is the number of mitotic cells, $M^*$ is the number of mitotic cells labeled with tritiated thymidine, and $N$ is the total number of living cells. Theoretically, our equation is more accurate than that of Puck and Steffen (28), but practically little difference was found in the values of $t_{G_2}$ estimated by the two methods. Fig. 4 summarizes the results obtained at the different temperatures. The second method of calculating $t_{G_2}$ utilized the equation:

$$t_{G_2} = t\,\frac{\log\left(1 + \dfrac{M}{N} - \dfrac{M^*}{N}\right)}{\log\left(1 + \dfrac{M}{N}\right)}$$

where $t$ is the time of sampling of cells after addition of tritiated thymidine and colchicine. This

TABLE II

*Percentage of Eosin-Stained Cells, Population Doubling Time, Mitotic Index, Percentage of Labeled Cells and Duration of $G_2$ Period at Different Temperatures*

| Temperature | Eosin staining | Population doubling time | Generation time* | Mitotic index | $\dfrac{M\ddagger}{N}$ | Percentage of labeled cells | $\dfrac{S\S}{N}$ | $t_{G_2}$ |
|---|---|---|---|---|---|---|---|---|
| °C | % | hr | T | % | | % | | hr |
| 40 | | 12.0 | 11.3‖ | 2.6 ± 1.4 | 2.8‖ | | | |
| 37 | 5.9 ± 0.8 | 11.5 | 10.8 | 3.4 ± 0.1 | 3.6 | 62.4 ± 3.3 | 66.3 | 1.2 ± 0.2 |
| 34 | 5.3 ± 0.5 | 20.0 | 18.9 | 2.1 ± 0.6 | 2.2 | 65.1 ± 3.4 | 68.7 | 1.8 ± 0.1 |
| 32 | | 24.0 | 22.6‖ | | | | | |
| 31 | 6.3 ± 1.1 | 28.0 | 26.2 | 1.6 ± 0.5 | 1.7 | 63.6 ± 4.2 | 67.9 | 2.1 ± 0.1 |
| 28 | | 81.0 | 76.3‖ | 0.5 ± 0.1 | 0.5‖ | | | |

* Generation time (T) = (population doubling time) × (1 − (eosin staining)/100)

‡ $\dfrac{M}{N} = \dfrac{\text{(No. of S stage cells)}}{\text{(No. of total living cells)}} = \dfrac{\text{(mitotic index)}}{100} \times \dfrac{1}{(1 - (\text{ eosin staining})/100)}$

§ $\dfrac{S}{N} = \dfrac{\text{(No. of S stage cells)}}{\text{(No. of total living cells)}} = \dfrac{\text{(percentage of labeled cells)}}{100} \times \dfrac{1}{(1 - (\text{eosin staining})/100.)}$

‖ Corrected by use of the average of eosin staining (5.8%) of three temperatures (37°, 34°, and 31°C).

TABLE III

*Life Cycle of L5178Y Cells at 37°, 34°, and 31°C*

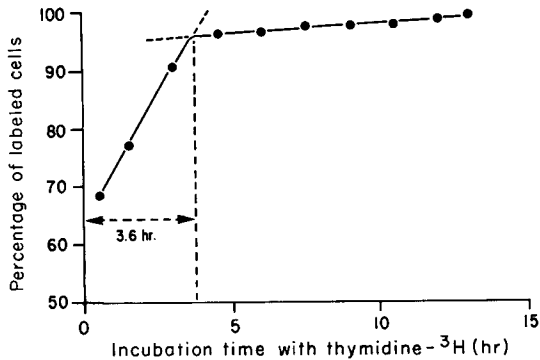| Temperature | Generation time | G₁ | | S | | G₂ | | M | |
|---|---|---|---|---|---|---|---|---|---|
| | | % | Time | % | Time | % | Time | % | Time |
| °C | hr | | | | | | | | |
| 37 | 10.8 | 21.8 | 1.8 | 66.3 | 7.3 | 8.3 | 1.2 | 3.6 | 0.55 |
| 34 | 18.9 | 22.1 | 3.2 | 68.7 | 13.3 | 7.0 | 1.8 | 2.2 | 0.59 |
| 31 | 26.2 | 24.6 | 5.0 | 67.9 | 18.5 | 5.8 | 2.1 | 1.7 | 0.63 |



FIGURE 5 The percentage of labeled cells in the continuous labeling experiment at 37°C. (Determination of the duration of G₂ plus M plus G₁ stages.)

method is somewhat more convenient because it enables us to calculate $t_{G_2}$ at different times.

The data required for a complete life cycle analysis are summarized in Table II. With these data, complete life cycle analyses of L5178Y cells at three different temperatures (31°, 34°, and 37° C) were made and are tabulated in Table III.

To check the accuracy of the values obtained by this method of life cycle analysis, we carried out a separate, entirely independent, experiment to obtain data which could be compared with calculated parameters. In this experiment, the cells were incubated continuously at 37°C with tritiated thymidine (2 μc/ml, specific activity, 6.7 c/mμ). The time required for the percentage of labeled cells to reach a maximum (Fig. 5)[1] is equal to ($t_{G_1}$ + $t_M$ + $t_{G_2}$) or 3.6 hr (see Appendix). Subtracting this from T, one obtains $t_S$ (7.2 hr). The calculated value of 7.3 hr (Table III) is in good agreement with the observed value.

## Effect of Temperature on the Life Cycle of L5178Y Cells

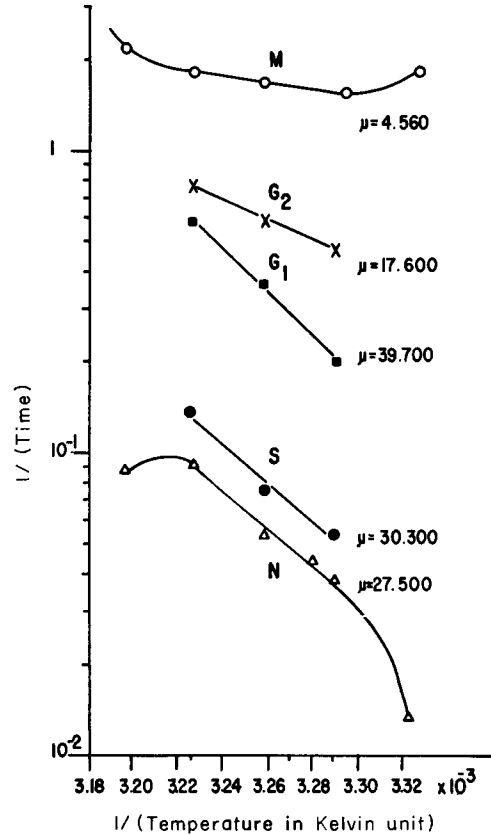In the present study, the population doubling time was estimated at six different temperatures



FIGURE 6 Arrehenius' plots of the generation time and the duration of different stages against temperature. μ is the activation energy calculated from the slope of the linear part of the plots.

(28°, 31°, 32°, 34°, 37°, and 40°C) (Fig. 1 and Table II). The optimal growth of the cells occurred at 37°C. As will be seen in Fig. 6 and in the following section, the logarithm of 1/(generation time) was related to 1/(temperature in Kelvin units) in a linear fashion at four temperatures (31°, 32°, 34°, and 37°C) (Table II).

Complete life cycle analyses were made at three

Activation Energies ($\mu$, $\Delta H\ddagger$, and $\Delta S\ddagger$) of L5178Y Cells at Various Stages of Life Cycle*

| | $\mu\dagger$ | $\Delta H\ddagger\S$ | $\Delta S\ddagger\S$ | $\left(\frac{t}{T}\right)^{\parallel}$ at 37°C | $\Delta H\ddagger \times \left(\frac{t}{T}\right)$ at 37°C | $\Delta S\ddagger \times \left(\frac{t}{T}\right)$ at 37°C |
|---|---|---|---|---|---|---|
| | | cal/mole | e.u. | | cal/mole | e.u. |
| N | 27,500 | 27,500 | −118 | 1.00 | 27,500 | −118 |
| G$_1$ | 39,700 | 32,200 | −137 | 0.16 | 5,200 | −22 |
| S | 30,300 | 29,100 | −124 | 0.67 | 19,500 | −83 |
| G$_2$ | 17,600 | 14,500 | −80 | 0.12 | 1,700 | −10 |
| M | 4,560 | 4,410 | −49 | 0.05 | 200 | −3 |

* Rates of various stages of the cells were estimated from the following equation:

$$\text{Rate} = \frac{6 \times 10^{23}}{(\text{time}) \times 60 \times 60} \left(\frac{\text{mole}}{\text{second}}\right)$$

$$\text{Time} = T, t_{G1}, t_S, t_{G2} \text{ and } t_M$$

† Using these rates and the Arrehenius' equation (1), values for $\mu$ were estimated.

$$(\text{Rate}) = Ae^{-\mu/RK}$$

(where $A$ is a constant and $R$, gas constant and $K$, temperature at Kelvin unit.)

§ Using these rates and the equation of absolute rate process, $\Delta H\ddagger$ and $\Delta S\ddagger$ were estimated.

$$(\text{Rate}) = \frac{h}{kK} e^{(-\Delta H\ddagger/RK)} e^{(\Delta S\ddagger/R)}$$

(where $h$ is Planck's constant and $k$ is Boltzmann's constant.)

∥ Weighing Factor.

temperatures (31°, 34°, and 37°C) to determine temperature effect at each stage of cell growth. Table III shows that the most temperature-sensitive stage was the G$_1$, followed by the S, the G$_2$, and the M stages, in that order. To illustrate, when the temperature was reduced from 37° to 31°C, the generation time was prolonged 2.43 times. The increase in each stage was: G$_1$ stage, 2.78 times; S stage, 2.53 times; G$_2$ stage, 1.75 times; and M stage, 1.15 times.

One of the original purposes of this study was to determine whether or not temperature reduction could be used to synchronize the cell population. Although the duration of the G$_1$ stage was increased 2.78 times by lowering the temperature from 37° to 31°C, the actual increase of cell number in this fraction was only 13%. This means that the temperature reduction of this magnitude does not effect good synchronization in this cell line.

### Thermodynamic Considerations of Progress of Cells through Their Life Cycle

The rate of progress of a cell through its life cycle is inversely proportional to the time required for the cell to complete its life cycle. If one

can determine quantitatively how temperature affects the rate of growth, one could calculate the "activation energies" needed for the cell to complete its life cycle by applying either Arrehenius' equation or theory of absolute rate processes (15, 16, 19).

Fig. 6 shows that when log (1/T) was plotted against 1/(temperature in Kelvin units), a linear relationship was obtained between 37° and 31°C. Using this slope, we calculated that an activation energy of 27,500 calories/mole was required for a cell to complete its life cycle. Activation energies, $\mu$, $\Delta H\ddagger$, and $\Delta S\ddagger$, similarly calculated either from Fig. 6 or from the equations given in Table IV, are tabulated in Table IV. The activation energies, $\mu$ and $\Delta H\ddagger$, were greater for the G$_1$ stage than for S, G$_2$, and M stages, in decreasing order. $\mu$ and $\Delta H\ddagger$ were smallest in the M stage. Furthermore, it was found that the entropy unit, $\Delta S\ddagger$, was negative throughout the entire life cycle.

To assess the contribution of activation energy at each stage of the life cycle, we used a weighing factor ((time of each stage)/T) (Table IV). A general idea of the energetics involved in cell passage through a complete life cycle at 37°C was shown in Fig. 7. The summation of $\Delta H\ddagger$ for
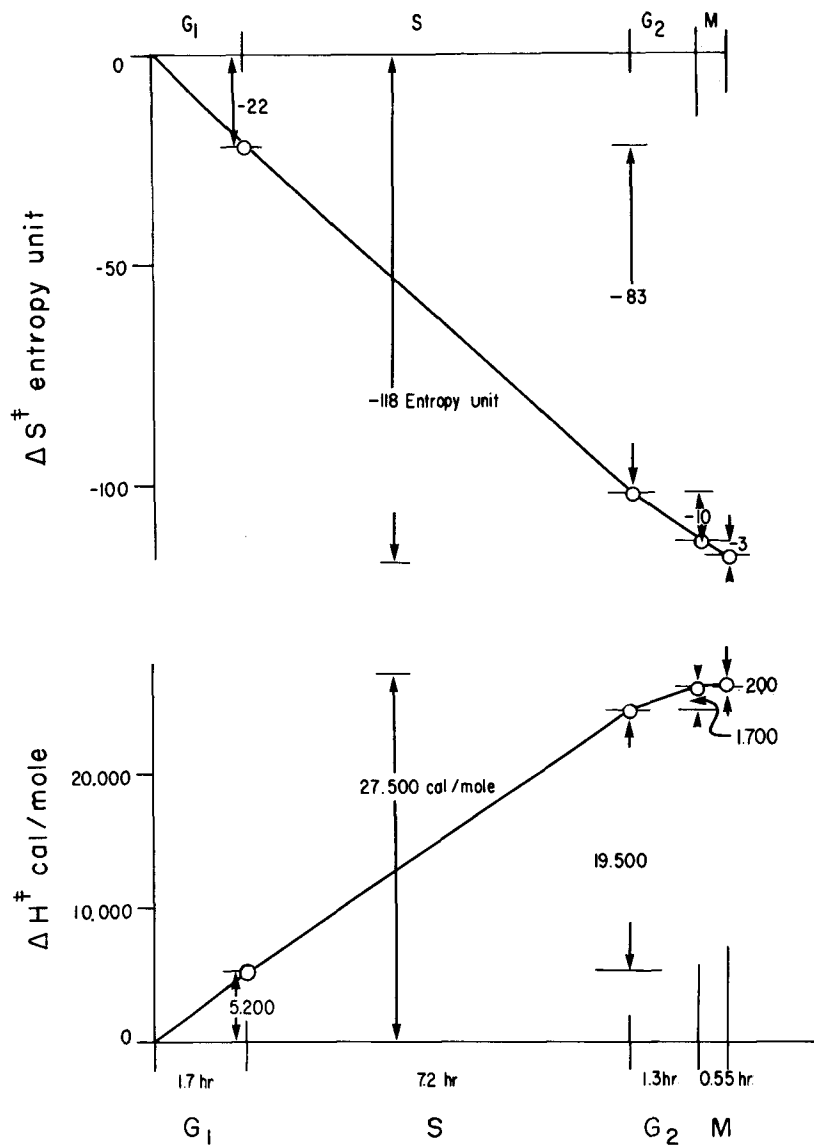
FIGURE 7 Relative changes of energy of activation and entropy of activation at the various stages of the life cycle of L5178Y cells growing at 37°C.

all four stages was 26,000 calories/mole, whereas the $\Delta H\ddagger$ for a complete life cycle estimated from the generation time was 27,500 calories/mole. The difference between these two values is regarded as negligible because of the rather large experimental error involved in the estimation of the various parameters. The activation energies estimated at 31° and 34°C are of the same magnitude as those estimated at 37°C.

DISCUSSION

*Thermodynamic Considerations of the Life Cycle of L5178Y Cells*

The activation energy of 27,500 calories/mole calculated for the life cycle is a little higher than that of 15,000 calories/mole obtained for bacteria, *Escherichia coli* (16), but is of the same order of

magnitude. In the early days, activation energies for biological functions were attributed to one rate-limiting reaction in biological systems (15, 36). In L5178Y cells, however, the summation of activation energies of all four stages was found to be equal to the activation energy of a whole life cycle. This can be regarded as evidence that the activation energy of the entire life cycle (cell growth) represents the summation of activation energies of at least four rate-limiting reactions in the four stages. In reality, the activation energy of the life cycle could represent the summation of activation energies of many reactions occurring in all stages of the life cycle (15, 36).

The $\Delta H \ddagger$'s of the four stages of the life cycle were always positive, suggesting that the cells require energy to pass through the life cycle. The negative $\Delta S \ddagger$'s of the four stages indicate that ordered structures are being formed in disordered systems, such as the synthesis of macromolecules

from small molecules, assembly of subcellular structures, etc.

The $\Delta H \ddagger$ and $\Delta S \ddagger$ were the highest in the $G_1$ stage and decreased in S, $G_2$, and M stages, in that order. This indicates that the cells in the $G_1$ stage are carrying out reactions needed in preparation for a new life cycle. There are many situations where the $G_1$ stage has been shown to play an important role in regulating the growth rate of the cell (see the next section). For example, in L5178Y cells exposed to a lethal dose of gamma radiation, it was found that the cells went through one or more life cycles and died at the $G_1$ stage (45). If the radiation destroys genetic or other vital molecules in the cell, we might expect that the cell would fail to meet the high activation energy requirement of the $G_1$ stage.

The smallest activation energy is found in the M stage. This is compatible with the observation of Taylor (40) that most of the protein synthesis
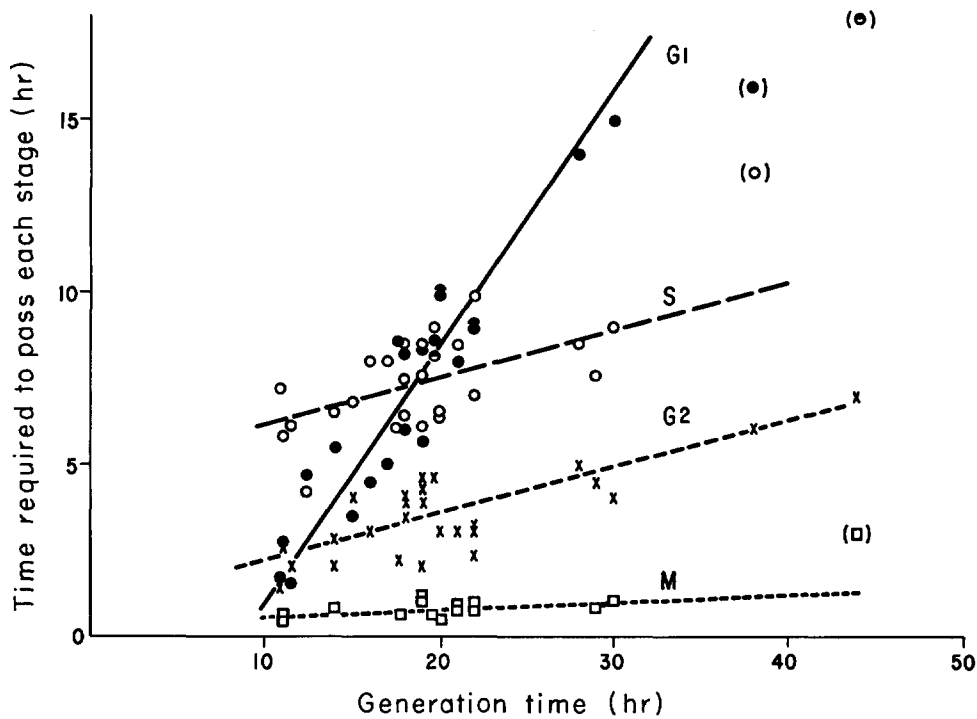


FIGURE 8 Relationship of the duration of various stages to the generation time of different cell lines. This figure is taken mainly from data given by Defendi and Manson (6), but some of the points are taken from recent work of others. Closed circle and solid line: relationship of the $G_1$ stage to the generation time. Open circle and dotted line: relationship of the duration of the $G_1$ stage to the generation time. Cross and dotted line: relationship of the duration of the $G_2$ stage to the generation time. Open square and dotted line: relationship of the duration of the M stage to the generation time.

required for the M stage is likely to be completed before the M stage. If this is so, the cells entering the M stage should complete this stage with little difficulty.In L5178Y and many other cell lines, exposure to ionizing radiation has little or no apparent effect on the cell's progress through the M stage (e.g., reference 45).

At the present time, the exact meaning of the activation energy is not clear. In the future, this could be explored by studying the relationship of activation energy to ATP production, oxygen consumption, heat production, etc.

## Factors which Affect the Life Cycle of Mammalian Cells

In the present study, only one environmental factor, temperature, was altered. It was observed that the $G_1$ stage was the most severely affected by temperature variation and that the alteration of the total generation time by temperature was attributed mainly to changes in the duration of the $G_1$ and S stages. In human amnion cells, Sisken also found that the $G_1$ stage is probably the most severely affected by temperature variation and that such changes are largely responsible for alteration of the over-all generation time (37, 38). In HeLa cells, Rao and Engelberg made the contrasting observation that the M stage is the most temperature sensitive (30). However, because of the short duration of the M stage, the change in generation time caused by temperature variation resulted mainly from the changes of the duration of $G_1$, S, and $G_2$ stages. Why the response of HeLa cells differs from that of L5178Y and human amnion cells is not known at the present time (38).

The effects of other environmental factors on the generation time of cultured mammalian cell lines have been reported. For example, the alteration of the generation time of human amnion cells and pig kidney cells by pH change was found to be the result of alteration of the duration of the $G_1$ stage (37). The second example is found in the mouse fibroblast cells released from contact inhibition. Upon release, the cells entered DNA synthesis before division, a fact which suggests that contact-inhibited cells (probably with an infinite generation time) ceased growth at the $G_1$ stage (42). Similarly, phytohemagglutinin-stimulated leukocytes were found to enter the S stage before division, indicating that circulating leukocytes are in the $G_1$ stage (1, 33).

The third example is found in the comparative studies of various cell lines in which different genetic characteristics may be considered to be analogous to a factor which altered the life cycle. Defendi and Manson (6) have attributed the variations of the generation time of different cell lines to difference in the durations of the $G_1$ stage (Fig. 8). An observation contrary to this was made by Puck, Sanders, and Petersen (29). Their comparative study of HeLa and Chinese hamster cells showed that the variation of the generation time of these two cell lines is not attributable to the variation of one specific stage, but to the variation in the duration of all stages in the same proportion.

In mammalian cells growing in vivo, the crucial role of $G_1$ stage in determining the generation time has been demonstrated with some exceptions (11, 20). They are the study of rapidly and slowly dividing cells in the same tissues (4, 46), the study of the cells of the same tissue of young and old animals (25), the study of normal and malignant cells (26, 31), and the study of normal and regenerating livers (5, 13, 17).

The search for special molecular events associated with the $G_1$ stage has been carried out in the regenerating liver (2, 3, 5, 9, 13, 43, 23), kidney cortex explant (21), phytohemagglutinin-stimulated lymphocytes (33), and cells released from contact inhibition (42). Some of the events are enhancement of RNA synthesis (21, 22, 33, 42, 43), changes of nucleoproteins (8, 44), and synthesis of enzymes necessary for DNA synthesis (2, 9, 23). However, it should be pointed out that all these studies were carried out with cells from non-growing cell populations which were stimulated to divide by some artificial means, and that in the $G_1$ stage there appear to be differences between rapidly growing cell populations and stimulated cell populations. For example, there is neither a sudden enhancement of RNA synthesis (34, 41) nor sudden burst of DNA polymerase activity (12) in the $G_1$ stage of rapidly growing mammalian cells. Other difficulties involved in relating molecular events in the regenerating livers to rapidly growing cultured mammalian cells have been also discussed recently by Seed (35). Thus, at the present time, further exploration with rapidly growing cell populations is definitely needed to find the special molecular events which are involved in regulating cell growth.

APPENDIX

*Summary of Methods of Estimation of Life Cycle*

| Cell stage | Methods of estimation of the duration of cells in a specific stage | Methods of estimation of the fraction of cells in a specific stage |
|---|---|---|
| Total cell population | 1. Counting cell number by a cell counter (a Coulter counter or a hemocytometer).[1]<br>2. Time intervals between the first and second cell divisions from film of time-lapse cinematography[2]<br>3. Time intervals between the first and second peaks of the labeled mitotic cells after pulse-labeling[3]<br>4. Rate of accumulation of mitotic cells after a complete block of progress of cells at metaphase by Colcemid treatment[4]<br>5. Rate of decrease of specific activity of DNA in the cell population with time after pulse-labeling[5] | |
| $G_1$ stage | 1. Time required for the percentage of labeled cells to reach the first plateau after X-irradiation in the continuously labeled cell population[6]<br>2. Time between cell division and appearance of labeled cells by a combined method of time lapse cinematography and radioautography.[2]<br>3. Time required for the percentage of labeled cells to reach a plateau after treatment with Colcemid in the continuously labeled cell population[4] | 1. The increment in the percentage of the labeled cells after X-irradiation of the continuously labeled cell population[6]<br>2. The increase in the percentage of the labeled cells in the Colcemid-treated and continuous labeling cell population[4]<br>3. The fraction of nonlabeled cells with low DNA content by the use of radioautography and the use of a microspectrophotometer[11] |
| S stage | 1. Time interval between the appearance and the disappearance of the labeled mitotic cells after pulse-labeling[3]<br>2. Time required for labeled metaphase cells to reach a plateau for grains per cell.[7]<br>3. Difference in the single and doubly labeled cells after $C^{14}$-labeling and $H^3$-labeling at different times[8]<br>4. Time required for the percentage of labeled cells to reach the maximum in the continuously labeled cell population[6]<br>5. Rate of accumulation of the labeled cells after Colcemid treatment in the continuously labeled cell population[4] | 1. Percentage of the labeled cells after pulse-labeling[7] |

Table cont'd

| Cell stage | Methods of estimation of the duration of cells in a specific stage | Methods of estimation of the fraction of cells in a specific stage |
|---|---|---|
| G₂ stage | 1. Time interval between the time of pulse-labeling and the time of appearance of labeled mitotic cells[9] | 1. Difference between the percentage of the labeled cells at the plateau of nonirradiated cells and the percentage at the plateau of the X-irradiated cells in the continuously labeled population[6] |
| | 2. Time interval between accumulation of mitotic cells and accumulation of labeled mitotic cells after Colcemid treatment in the continuously labeled cell population[4] | 2. Differences between the total mitotic cells and the labeled mitotic cells after Colcemid treatment[4] |
| | | 3. The fraction of nonlabeled cells with high DNA content[11] |
| M stage | 1. Time period of cell division from time-lapse cinemicrography[2] | 1. Estimation of mitotic index[1] |
| | 2. Time for the mitotic index to decrease to zero after X-irradiation[10] | |

Since there are so many papers available dealing with in vivo as well as in vitro experiments and since the authors only intend to show the general principles involved in the life cycle analysis of cultured mammalian cells, the authors took the liberty of citing only a handful of papers which are concerned with cultured mammalian cells. Labeling procedures referred to in the table have been carried out mostly by thymidine-H³ and labeled cells have been studied by radioautography.

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