LIFE CYCLE ANALYSIS OF MAMMALIAN CELLS

II. CELLS FROM THE CHINESE HAMSTER OVARY GROWN IN SUSPENSION CULTURE

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ABSTRACT A method for life cycle analysis in mammalian cells which utilizes the collection function has been applied to the Chinese hamster ovary grown in suspension. The following durations were found for the various parts of the life cycle: S, 4.13 hours; G1, 4.71 hours; G2, 2.81 hours; mitosis, 0.81 hours.

The cell has a total generation time of 12.4 hours as opposed to 20.1 hours for the S3 HeLa cell. However, the relative lengths of each phase of the life cycle are identical within experimental uncertainty in the two cells.

INTRODUCTION

Mammalian cells grown *in vitro* offer the possibility of localizing with high resolution normal biochemical events occurring at different ponts of the life cycle, and identification of the point at which externally applied agents affect the metabolic course. In previous work, equations were developed demonstrating the pattern of accumulation of cells at any point in the life cycle, which results from addition of a blocking agent and an experimental methodology based on these equations was developed for detailed analysis of the life cycle of such cells (1, 2). The method consists in addition of an inhibitor which blocks the life cycle at a specific point and enumeration of the relative numbers of cells accumulating behind the blocking point as a function of time. An accumulation function was defined which results in a linear curve if the culture in which the inhibitor had been applied is random and in a non-linear curve which permits precise definition of the deviant population segments in non-random cultures. Experiments were described in which this method was applied to analysis of the life cycle of the S3 HeLa cell when grown as monolayers attached to glass or plastic surfaces.

In the present communication, experiments are described in which this life cycle

analysis method is applied to a Chinese hamster ovary cell (CHO) grown in suspension.

METHODS AND MATERIALS

The CHO cell used in the present paper was established in tissue culture in the Department of Biophysics at the University of Colorado in 1958 and has been described previously (3). It originated from a biopsy of an ovary of a Chinese hamster. It has been cultivated continuously since its establishment in culture, and has a chromosomal constitution similar to that which the culture displayed on original isolation; *i.e.*, a modal number of 22 with about 20 per cent of the cells deviating from this number. The cells of the Chinese hamster *in vivo* display similar inconstancies in chromosome number (4).

Cell cultivation was carried out in spinner flasks (Bellco Glass Inc., Vineland, New Jersey) of 250 ml capacity. These were mounted in a 37°C walk-in incubator, above magnetic stirrers driven by a synchronous motor at a rate of 3 revolutions per second. The medium employed consisted of F10 (5) from which the calcium had been omitted, supplemented with 5 per cent fetal calf serum (6) and occasionally 10 per cent normal calf serum as well, the latter serum usually being heated for 15 minutes at 60°C before use. For routine farming, flasks were inoculated with 100 ml of this medium containing cell inocula of 4.5×10^4 cells per ml. The pH was adjusted to the yellow-orange color of the phenol red indicator (pH of around 7.0) and placed above the magnetic spinner. Under these circumstances the cells initiated multiplication at a generation time of approximately 12 to 14 hours and maintained this rate with high regularity provided that the suspension was diluted with fresh medium sufficiently often to prevent the cell density from exceeding 3×10^5 per ml. If the cell density were maintained between 3×10^4/ml and 3×10^5/ml, logarithmic growth could be maintained for indefinite periods with no lag whatever occurring on replacement of part of the suspension with fresh medium provided that the new medium had been prewarmed to 37° and had its pH carefully adjusted before addition to the culture.

A typical life cycle analysis was carried out as follows: At a time designated zero, colcemide and H^3-thymidine were added to a logarithmic culture in concentrations of 0.25 μg/ml and 0.15 μc/ml, respectively. At various time intervals 3-ml samples were removed from the flask with a pipette and delivered into a chilled tube which was centrifuged at 1000 RPM for 3 minutes in an International refrigerated centrifuge. After removal of the supernatant, 4 ml of 0.1 M citric acid in water was added with continuous shaking. The tube was placed in a 37° water bath for 30 seconds, then centrifuged again for 3 minutes, and the supernatant discarded. Then 0.3 ml of a mixture containing 3 parts ethanol to 1 part glacial acetic acid was added dropwise with shaking and the resulting suspension allowed to stand for 4 minutes; thereafter, 0.1 ml of the suspension was pipetted onto the surface of a series of clean microscope slides and spread by means of a gentle airstream. The slides were permitted to air-dry, after which they were treated with 1 M HCl for 6 ½ minutes at 60° and stained with crystal violet. Alternatively, the previously described procedure for hypotonic expansion, fixation, mounting, and staining can be used (2). Radioautographs were prepared by standard methods, using 4 to 6 days of exposure (2). Microscopic scoring of the following quantities was performed: fraction of all cells which are in mitosis (N_M); fraction of all cells which are both H^3-labeled and in mitosis (N_M^*); and fraction of all cells which are H^3 labeled (L).

The formulae used in the treatment of the data have been derived previously (1, 2)

and are reviewed as follows: (The only case considered here is where colcemide collects only those mitoses which form after its addition, reference 2.)

The collection function, Log $(1 + Ni/k)$, for N_M, the total mitotic index, is given by:

$$\log (1 + N_M) = \frac{0.301}{T} (t) \tag{1}$$

The collection function for labeled mitoses:

$$\log (1 + N_M^*) = \frac{0.301}{T} (t - T_{G2}) \quad \text{for} \quad t \geqq T_{G2} \tag{2}$$

The collection function for total cells labeled with H^3-thymidine:

$$\log \left(1 + \frac{L}{k}\right) = \frac{0.301}{T} (T_S + t) \tag{3}$$

where: N_M = the fraction of cells which are in mitosis
T = generation time of the culture
t = elapsed time after addition of the colcemide
N_M^* = the fraction of cells which are both labeled and in mitosis
T_{G2} = the average time required to traverse the G2 interval
L = the fraction of all cells which are labeled
k = $2^{T_{G2}/T}$
T_S = the time required to traverse the S interval

In the course of these experiments it was found that flasks seeded with a cell inoculum and stored for as long as 48 hours at 2°C could be reincubated with stirring so as to initiate logarithmic growth with the standard generation time, with virtually no lag period. A flask seeded with the standard medium and cell inoculum is incubated for 3 hours at 37°C as described. It is then removed from incubation and plunged into an ice-water bath in the cold room. The rapid chilling is necessary to prevent cell attachment to the glass, which occurs if the cooling proceeds slowly in the absence of stirring. At any time up to 48 hours, the flask can be restored to the magnetic stirring device in the 37°C incubator with resumption of normal growth. This procedure is convenient to permit experiments to begin early in the morning, particularly after week-end interruptions.

EXPERIMENTAL RESULTS

1. *Difficulty Due to Mitotic Degeneration.* A difficulty was experienced in that the accumulation function for mitotic figures was linear only for the first 6 or 7 hours of each experiment, and thereafter bent toward the horizontal, instead of continuing to rise in linear fashion until almost all the cells were mitotic, as in the S3 case (2). Table I presents the data for a typical experiment, (columns 1 through 4) and Fig. 1 demonstrates the deviation of the uncorrected collection function. Analysis of these data revealed that the deviation is due to a degeneration of mitotic figures which begins on the average about 6 hours after they have been formed. This is illustrated in Fig. 2, which demonstrates the change in the fraction of un-

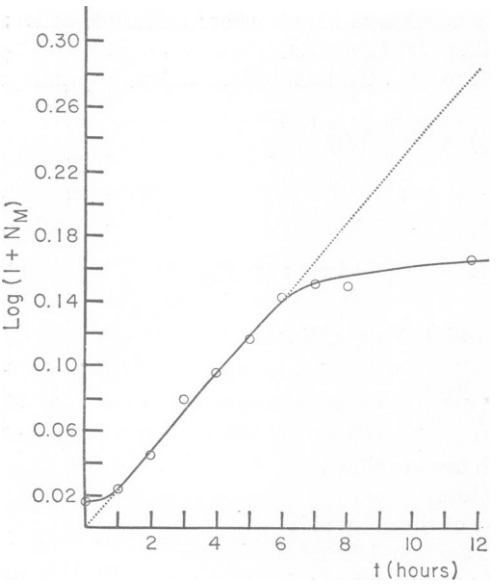

FIGURE 1 Uncorrected collection function for total mitotic figures, demonstrating that the curve bends over and becomes almost horizontal after 6 hours, when only 40 per cent of the cells are in mitosis.

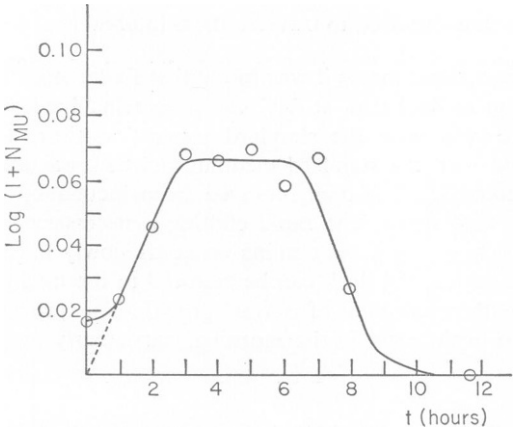

FIGURE 2 Demonstration of the instability of the mitotic figures obtained by the procedure described. N_{MU} is the fraction Unlabeled mitoses/Total cells. If the mitoses were completely stable, this fraction should increase until it reaches the value of N_{G_2}, the fraction of the cells which were initially in the G2 interval, and remain there indefinitely, because the subsequent mitoses would all be labeled. While the curve does flatten at the expected point, the final value is not constant, but begins to fall at around 6 hours, and eventually reaches zero, indicating complete disappearance of these particular mitotic figures.

labeled mitotic cells with time. These represent the cells which had already completed the S period (*i.e.*, DNA synthesis) at zero time. Hence, this fraction of

the population should rise steadily at first then reach a plateau which remains constant. Instead, however, the fraction of the cells which are unlabeled and in mitosis begins to drop sharply between 6 and 7 hours, indicating that the lifetime of a mitotic figure is only of this magnitude under the prescribed conditions.

This analysis of the limited lifetime of mitotic figures makes possible simple correction for this mitotic degeneration. The total fraction of unlabeled mitotic figures is calculated at each sampling period, and by means of a plot like that of Fig. 2 the limiting value can be determined. For all observations beyond 7 hours, the extent of mitotic loss through degeneration of these unlabeled mitotic figures can then be determined and this value added to the mitotic index, N_M, to yield a corrected value of N_M. This procedure serves only to correct for degeneration which has taken place among the unlabeled cells. Since no correction at all is required for the first 6 to 7 hours, this measure effectively extends the period of accurate determination of the mitotic index by 2 to 3 hours, so that the great majority of the life cycle period is covered without any further correction. It was not feasible to use a constant correction factor for all experiments since the amount of mitotic degeneration shows appreciable variation in different experiments. In the next paper of this series an alternative measure applicable to monolayer cultures, which eliminates mitotic degeneration experimentally, is described (7).

2. *Complete Life Cycle Analysis.* In Table II is presented the corrected values of N_M, and in Fig. 3 is shown the accumulation functions for the corrected

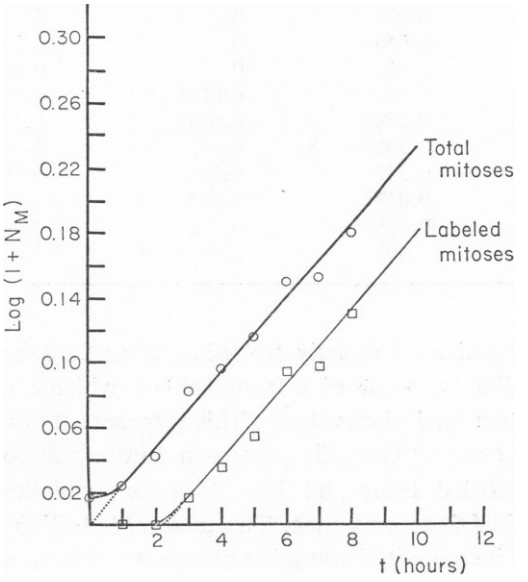

FIGURE 3 The collection functions for total mitoses, Log $(1 + N_M)$, and for labeled mitoses, Log $(1 + N_M^*)$ after correction for mitotic degeneration. The point at which the "labeled mitoses" curve intersects the X axis represents T_{G_2}, and is 2.3 hours. (2)

total mitotic index and for the labeled mitotic index respectively. The following conclusions may be drawn:

(*a*) It is evident that the collection function for total mitoses is a reasonably straight line and extrapolates back to the origin. Hence, this culture appears to be satisfactorily randomized and behaves like S3 monolayer cultures (2) in that it does not permit colcemide to accumulate the cells already in mitosis at the time of its addition but colcemide does collect all the new mitoses which form after its addition.

(*b*) From the slope of the curves of Fig. 3, the generation time, T, is calculated to be 12.9 hours. This value is in accord with that obtained by measurement of the cell numbers in unblocked cultures as a function of time.

(*c*) From the value of N_M in Table I at $t = 0$, the time of mitosis, T_M, is found to be 0.71 hours from the equation, $N_M = 2^{T_M/T} - 1$.

TABLE I

EXPERIMENTAL DATA IN A TYPICAL LIFE CYCLE ANALYSIS OF CHO CELLS IN A SUSPENSION CULTURE (LA 17)

Time	N_M = Total mitoses ÷ Total cells	N_M^* = Labeled mitoses ÷ Total cells	L = Total labeled cells ÷ Total cells
hrs			
0	0.039	0	0
1	0.056	0	0.396
2	0.110	0	0.481
3	0.208	0.0386	0.554
4	0.248	0.0835	0.593
5	0.306	0.132	0.661
6	0.387	0.244	0.695
7	0.417	0.252	0.742
8	0.408	0.348	0.849
11.8	0.463	0.463	0.838

(*d*) T_{G2} can be evaluated directly from Fig. 3 as 2.30 hours. It can also be estimated from the limiting value of L from Table I, which can be taken as 0.844, the average of the two final observations. This represents the value of $(1 + N_M - N_{G2})/(1 + N_M)$ at $t = 0$. (The cells already in mitosis will complete division and eventually become labeled. Hence, all the cells in the population will be labeled except those present in G2 at zero time. The factor $(1 + N_M)$ in the denominator takes account of the increase in the number of cells.) Since N_M at zero time is 0.039, N_{G2} is 0.162, and T_{G2} becomes 2.70 hours (2). Averaging these two values yields 2.50 hours for T_{G2}, which was also used to obtain the figures in the last column in Table II.

TABLE II

CALCULATED QUANTITIES FROM DATA OF TABLE I

Time	N_{M_u} = Unlabeled mitoses ÷ Total cells	ΔN_M = Correction for mitotic degeneration = $0.169 - N_{M_u}$	$N_M + \Delta N_M$ = Corrected N_M	Log $(1 + N_M)$ Corrected	Log $(1 + N_M^*)$	Log $(1 + L/k)$‡
hrs						
0	0.039	—	0.039	0.0166	0	—
1	0.056	—	0.056	0.0237	0	0.1290
2	0.110	—	0.110	0.0418	0	0.1523
3	0.169	—	0.208	0.0821	0.0166	0.1711
4	0.165	—	0.248	0.0962	0.0348	0.1813
5	0.174	—	0.306	0.1159	0.0542	0.1981
6	0.143	0.026	0.413	0.1501	0.0948	0.2058
7	0.165	0.004	0.421	0.1526	0.0976	0.2172
8	0.060	0.109	0.517	0.1810	0.1297	0.2408
11.75	0.000	(0.413)§	(0.876)§	(0.2732)	—	(0.2388)

‡ $k = 2^{T_{G2}/T}$

§ These values cannot be obtained by use of the simple correction formula here proposed, but can be approximated from the fact evident in Fig. 2, that at 9.75 hours, all of the mitoses present at 3 hours have disappeared. Therefore, at 11.75 hours, all of the mitoses present at 5.0 hours would also have disappeared. Hence, the value at the 5 hour period can serve as the correction for the 11.75 hour period.

(*e*) T_S and T_{G1} can be evaluated from Fig. 4 in which values of the collection function for all cells labeled with H^3-thymidine have been plotted against the time. This curve begins as a straight line, but tends to flatten with time because the slowest moving cells are left behind, as has been discussed earlier (2). The straight portion of the curve has been drawn as the line with slope equal to $0.301/T$ and with the mean intercept determined by the three experimental points occurring during the first 2 hours of the experiment. T_S is found to be 4.46 hours and T_{G1} 5.08 hours.

Table III indicates the results of repeated analyses of the life cycle of this cell strain under the same culture conditions. The durations of the various parts of the life cycle are reasonably reproducible.

DISCUSSION

The present data make possible comparison of the life cycle of the CHO cell with that of S3 (2) which has a much longer generation time (20 hours as opposed to 12.3).

Data for the latter cell, grown in the same medium but with a normal amount of Ca^{++}, so that monolayer cultures resulted, are shown in Table IV. The data per-

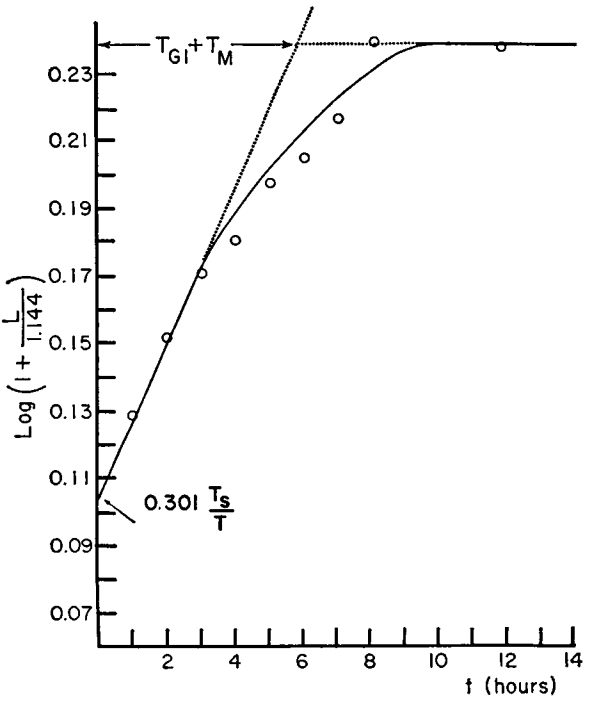

FIGURE 4 Collection function for L, the fraction of all cells labeled with H^3-thymidine. The value 1.144 is $2^{T_{G2}/T}$. (2)

TABLE III

RESULTS OBTAINED IN A SERIES OF 4 EXPERIMENTS MEASURING THE TIME REQUIRED BY THE VARIOUS PHASES OF THE LIFE CYCLE OF THE CHO CELL

The average time values and the standard deviations are presented in the first column. The third column represents the relative number of cells in each phase of the life cycle, in a random culture, calculated as in the previous paper (2). The subscript i represents the individual phase under consideration.

Phase	T_i	T_i/T	N_i
	hrs		
Mitosis	0.81 ± 0.12	0.065	0.046
G2	2.81 ± 0.33	0.226	0.177
S	4.13 ± 0.34	0.332	0.317
G1	4.71 ± 0.18	0.380	0.460
T = total generation time	12.41 ± 0.32	1.003	1.000

TABLE IV

COMPARISON OF LIFE CYCLE DISTRIBUTION OF CHO CELLS WITH THAT OF S3 HeLa CELL GROWN AS MONOLAYERS

The same medium was used in both cases except that a normal amount of Ca was employed for the S3 cell (2).

Phase	S3 (Total generation time 20.10 hrs) T_i/T	CHO (Total generation time 12.4 hrs) T_i/T
Mitosis	0.055	0.065
G2	0.22	0.23
S	0.29	0.33
G1	0.41	0.38

mits determination of whether the difference in the two generation times is due to prolongation of one or two specific phases of the life cycle in the case of the S3 HeLa cell, or whether all phases are prolonged proportionately. Table IV indicates that to a close approximation, all of the phases of the S3 cell are equally prolonged as compared to the CHO cell.

Both of these cells have been maintained in cultivation *in vitro* for many years. One arose from malignant and the other from normal tissue, but neither exhibits *in vitro* any gross behavior characteristic of a specific differentiation process. The constancy of the various parts of the life cycle relative to the whole of these two cells may be an indication that, in the main, their growth patterns reflect only a primitive reproductive cycle in which specific differentiation processes play little or no role. This particular pattern may be representative of a large variety of undifferentiated cells. Experiments comparing life cycle patterns of a variety of cell types are now in progress.

Full or partial life cycle analyses of Chinese hamster cells cultivated *in vitro* have been published by other workers (8). The values obtained generally depart from those described here, usually for reasons which may be connected with any of the following features: differences in nutrient media and growth conditions employed; differences in cell strain utilized; failure to achieve complete randomness (or complete synchronization, depending on the method employed) of the cell culture; and use of the approximation that in random culture the relative cell population of any phase of the life cycle is equal to the proportion of the life cycle time required by this phase.

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