LIFE CYCLE ANALYSIS OF MAMMALIAN CELLS

I. A METHOD FOR LOCALIZING METABOLIC EVENTS WITHIN THE LIFE CYCLE, AND ITS APPLICATION TO THE ACTION OF COLCEMIDE AND SUBLETHAL DOSES OF X-IRRADIATION

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ABSTRACT Equations are presented describing the accumulation of cells at any part of the life cycle as a result of addition of specific blocking agents. An experimental methodology using these relationships is described which makes possible analysis with relatively high resolution of the distribution of cells throughout the life cycle in normal cultures or those treated with various agents. The action of colcemide on S3 HeLa cells studied by this method revealed that colcemide has no effect on the G1, S, or G2 stages; it blocks cells quantitatively at the metaphase-anaphase region; but it accumulates mitotic figures only from the cells which have not yet entered mitosis at the time of its addition. The technique was also applied to study the efficiency of x-irradiation in delaying the entrance of G2 cells into mitosis. A definite lag was found at the lowest dose studied which was 9 rads. Only the cells confined to a central region of G2 at the time of irradiation are affected by this dose.

INTRODUCTION

While the long generation time of mammalian cells, as compared to that of microorganisms like *E. coli* or bacteriophage, constitutes a disadvantage for some kinds of studies, it affords a more favorable opportunity for delineation of the metabolic events of different parts of the cell life cycle. Howard and Pelc (1953), working with plants, and Lajtha, *et al.* (1954, 1955) who used mammalian cells *in vitro,* demonstrated that the life cycle could be divided into four distinct phases, by means of the fact that S, the period of DNA synthesis, (which can be recognized by radioautographic determination of incorporated H^3-thymidine) is separated from mitosis by an interval of several hours, which was called G2. Similarly, the period between the end of mitosis and the beginning of S was labeled G1 (Fig. 1). These observations furnished a basis for estimation of the time of these four principal divisions of

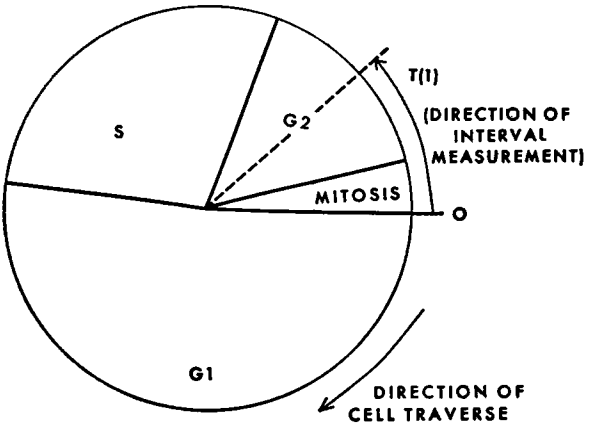


FIGURE 1 Diagrammatic representation of the 4 phases of the mammalian cell life cycle after Lajtha, *et al.* (1955). While the cells traverse this cycle in a clockwise direction, the convention adopted here counts time in a counterclockwise direction beginning from the end of mitosis which is labeled 0. Thus, the arbitrarily chosen interval $T(1)$ represents the fraction of the life cycle shown, and $N(1)$ is the fraction of the total cell population contained within this interval, which would in this case extend from the end of mitosis to the middle of G2.

the life cycle, which has been used to map actions like the mitotic lag produced by x-irradiation (Lajtha, *et al.*, 1958; Painter, *et al.*, 1959; Whitmore, *et al.*, 1961; Painter, *et al.*, 1961; Puck and Yamada, 1962; Dewey and Humphrey, 1962).

However, this method requires the scoring of labeled and unlabeled mitotic figures in random cultures, where the total fraction of mitoses does not exceed 2 to 5 per cent, so that, even with laborious microscopic analysis, the figures obtained exhibit considerable fluctuation. For this and other reasons, it is not surprising that reported values of the four stages of the life cycle shown in Fig. 1 sometimes exhibit variability of 50 to 100 per cent for the same cell.

In the present paper, delineation of component parts of the life cycle is described, utilizing blocking agents which halt the progress of cells around the cycle at a point which can be readily identified by microscopic examination. The accumulation of cells at the point of block is then scored at various times. The number of these blocked cells rises steadily with time, so that counting becomes rapid and less subject to statistical fluctuations. Labeled DNA precursors and other specific indicators for time-localized metabolic processes can still be used, separately or in combination. Under these circumstances, one can obtain: a direct indication of randomness of any cell population; precise measurement of the duration of each recognizable division of the life cycle; the fraction of the cell population present at any time in various parts of the life cycle for random, singly phased, or polyphased cultures; the point in the life cycle at which various normal metabolic events occur; and the points at which externally applied agents exercise their action.

The general equations for the accumulation of cells in any particular part of the life cycle, as a result of addition of a blocking agent have been developed (Puck, 1962) and are presented in the Appendix for the case where the block occurs at the end of mitosis (Appendix equation (7)), and where the block occurs at any other point (Appendix equation (14)). The use of these general relationships makes possible measurement of the time interval separating any group of cells which forms a distinguishable counting increment, from the point of block. In the sections which follow, these general equations are modified for use with particular agents, and compared with experimental observations.

METHODS

All experiments here described were performed with S3 HeLa cells, grown and treated by methods described previously (Ham and Puck, 1962). It is essential for such a procedure that the cells sampled be representative of the entire population. This requirement makes for difficulty in attempting to fix cells directly on the glass surface on which they have grown because of the tendency of mitotic cells to become spherical and detach from the glass. Hence, the cells were grown on glass, then harvested by trypsinization, expanded hypotonically, fixed, and plated on a new slide, in accordance with the following procedure:

1×10^5 S3 cells are inoculated into 30 mm plastic Petri dishes along with 1.5 ml of nutrient medium. In earlier experiments the medium was N16HHF (Ham and Puck, 1962). More recently we have used F10 (85 per cent), normal calf serum (10 per cent), and fetal calf serum (5 per cent) (Ham, 1963). The plates are incubated for 1 or 2 days, after which the medium is removed and replaced with fresh, prewarmed medium, and incubation resumed. After approximately 1 hour, the inhibitor, usually colcemide, 0.25 μg/ml, is added and this becomes zero time. H^3-thymidine (0.025 to 0.04 μc/ml) may be added simultaneously. At successive time intervals thereafter, plates are removed from the incubator and the supernatant liquid from each transferred to a specific test tube which is always identified with its particular sample. Each plate is then washed with 1.0 ml of standard trypsin (Ham and Puck, 1962), which is then added to its same test tube, and a new trypsin addition made. The plate is reincubated for 4 minutes at 37°C, at which time the cells are virtually completely released from the plastic dish. The resulting cell suspension is quantitatively transferred to the selfsame test tube, which is then centrifuged for 4 minutes at 2°C at a speed of 600 to 1000 RPM in refrigerated centrifuge (International Equipment Co., Boston). All of the supernantant is then poured off, and 2ml of a solution containing 2.8 gm NaCl per liter of 0.002 M phosphate buffer at pH 7 is added. After 5 minutes of standing in this hypotonic solution at room temperature, the cells are resuspended with gentle shaking, and the centrifugation is repeated but at a speed of 700 to 900 RPM. The supernant is again discarded, and 0.50 ml of fixative (absolute ethanol: acetic acid = 3:1) is added. After 5 minutes of standing, 1 ml of 65 per cent acetic acid is added, and the tube stored for at least 2 hours in the refrigerator. A few drops are then transferred to a clean microscope slide which is blown upon gently to disperse the droplets over the surface. The slide is dried in air, heated at 60°C in 1 M HCl for 6½ minutes, and stained by immersion for 3 minutes in a crystal violet solution. The resulting preparation permits easy scoring of mitoses as well as reasonably good chromosomal delineation. The mitotic index is determined by counting until a total of 100 or 200

mitoses, or at least 1000 interphase cells have been scored. If radioautographs are to be prepared, the slides are de-stained in acid alcohol; dried; liquid NTB3 (Eastman Kodak) emulsion applied; and an exposure period provided whose duration is adjusted in accordance with the specific activity of the radioisotope employed. The resulting slides are developed, fixed, washed, and stained by procedures which are now routine in many laboratories.

Cells were scored microscopically for markers which consisted either of mitotic figures or the presence of silver grains revealing H^3-thymidine labeling. At least 100 of the marked cells, or 1000 unmarked ones were scored in each case. In some experiments correction was made for dead cells, which were taken to be interphase cells which failed to take up H^3-thymidine even after 24 hours, or which failed to exclude trypan blue. The per cent of such cells varied from 0 to 9 per cent. X-irradiation was performed with a constant voltage source, operating at 230 kv and 15 ma. The plates were maintained at a constant temperature of 37°C so as to prevent temperature shocks. The filtration of the beam and dose measurement has been described elsewhere (Puck and Marcus, 1956; Puck *et al.*, 1957).

RESULTS

1. *Variance Measurement.* The relationships developed in the Appendix hold strictly only for the case where the generation time, T, is constant for all cells. Otherwise the actual doubling time may be different from the mean doubling time of the cells and modifications are required in the equations.[1] The distribution of T among the cells of an S3 population was measured by cinephotomicrography.[2] Cells of a standard, growing culture were photographed once a minute for several days, and, from the resulting record, the time between mitoses was measured for all the mitoses on the film. Such measurements yielded a mean generation time of 20.5 hours and a standard deviation of 1.85 hours. This latter figure was taken as sufficiently small to permit use of the equations in the Appendix as a reasonable approximation.

2. *Mode of Action of Colcemide.* Experiments were first undertaken with colcemide as a blocking agent to prevent completion of mitosis. Since colcemide halts mitosis in the metaphase-anaphase region, it was expected that only the cells already in telophase would go on to complete mitosis. Since cells in telophase are already doubled, we can for convenience score these with G1. Thus, one would expect that, if mitoses were scored at various time intervals after addition of colcemide, they would obey the equation

$$\log [1 + N(\mathrm{M})] = \frac{0.301}{T} [T(\mathrm{M}) + t] \qquad (1)$$

[1] For example, if the generation time, T, obeyed a Poisson distribution, in which the variance is equal to the mean, the equation (1) of the Appendix would be replaced by $n = n^0 \exp^{t/T}$.

[2] The apparatus employed was originally constructed by Dr. Joseph Engelberg during his sojourn in this department.

which is derived in the Appendix as equation (7) and where $N(M)$ is the mitotic index; $T(M)$, the duration of mitosis, and t the time after addition of colcemide. Actually, however, the experimental curve exhibits an initial shoulder, after which it achieves a linear rise with the expected slope. Moreover, the extrapolation back of the linear portion of the curve to zero time meets the axis at the point (0,0), (Fig. 2).

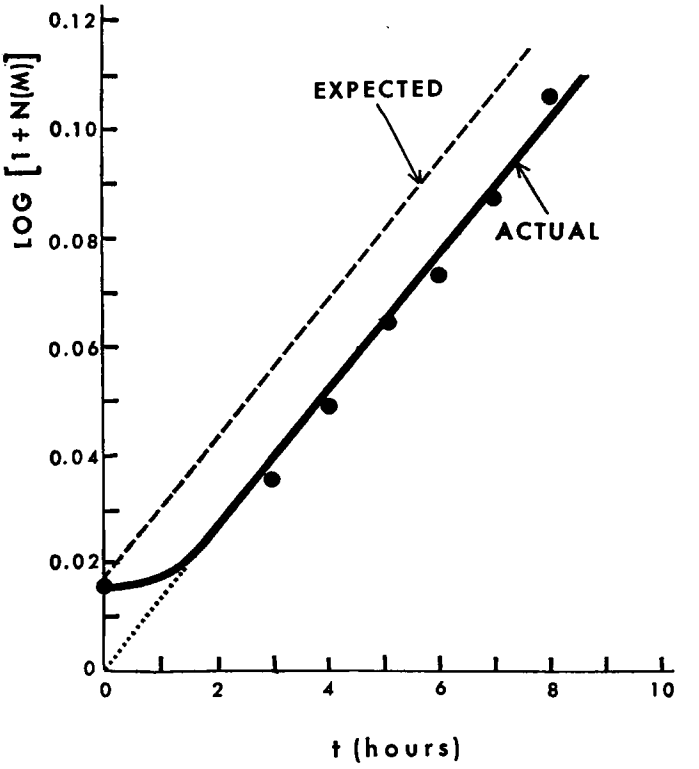


FIGURE 2 Comparison of the expected and actual curves obtained when colcemide was added to an S3 culture and periodic sampling carried out for determination of the mitotic index, $N(M)$. The total generation time, as determined from the slope of the experimental curve was 23.7 hours. If the cells already in mitosis at the time of colcemide addition had been retained at that point by the drug, the curve should have started up linearly from the Y axis intercept. Instead, there is an initial lag of slightly over an hour in the collection of mitoses, and the final curve is a straight line which extrapolates back to the origin. This behavior indicates that only the cells entering mitosis after the addition of colcemide are blocked by the drug.

This lag in the action of colcemide might be due to a delay in penetration of the compound to its active site. In that case, if the rate-limiting process involves colcemide diffusion, the lag should be decreased by increase in the colcemide concentration of the medium. However, a 20-fold increase in the colcemide concentration had no effect whatever on the curve. This result, plus the fact that the linear portion extrapolates back to

the point (0,0), implies that the colcemide is specifically prevented from acting on all cells which have already entered mitosis, even though they have not yet reached the point at which the blocking action is exerted. Hence, only the cells that are exposed to colcemide before entering mitosis are accumulated on reaching the metaphase-anaphase region. It appears possible that colcemide is prevented from entering such cells. This proposed explanation is consistent with the known striking change that overtakes the cell membrane when prophase begins, which is evident in the sudden transformation of flattened, glass-attached cells into spherical bodies. The change in structure of the cell membrane accompanying this transformation could conceivably decrease strongly the penetration of colcemide.

The data of Fig. 2 indicate that text equation (1) or Appendix equation (7) must be modified for use with this agent, so as to take into account the initial lag. Under these circumstances, the cell population continues to multiply during the entire period of mitosis, $T(\mathrm{M})$, after addition of the colcemide. Hence, the total cell number, n, is given by:

$$\begin{aligned} n &= n^0 2^{t/T} \quad \text{for} \quad T(\mathrm{M}) \geq t \geq 0 \\ n &= n^0 2^{T(\mathrm{M})/T} \quad \text{for} \quad t \geq T(\mathrm{M}) \end{aligned} \tag{2}$$

where

n^0 is the total number of cells at $t = 0$
n is the total number of cells at any time, t
$T(\mathrm{M})$ is the time of mitosis
T is the total generation time;

and

t is elapsed time after colcemide addition.

At $t = 0$,

$n(\mathrm{M})/n^0 = 2^{T(\mathrm{M})/T} - 1$ where $n(\mathrm{M})$ is the number of cells in mitosis. This follows from equation (4) of the Appendix.

If the cells already in mitosis at $t = 0$ were indeed accumulated with the rest, the observed mitotic index at any time, t, would be obtained from equation (6) of the Appendix as the quantity

$$2^{[T(\mathrm{M})+t]/T} - 1.$$

Since the cells already in mitosis are not accumulated, but pass on through another complete cycle, the total accumulation of mitoses at any time t, must be decreased by subtracting those cells which were in mitosis at $t = 0$.

For the sake of simplicity, we will consider only observations of the mitotic index taken at a time $t \geq T(\mathrm{M})$. This is not a severe restriction because of the relatively short duration of mitosis in mammalian cells. However, where one wishes also to consider shorter time intervals, the appropriate equations are readily derivable.

$$\frac{n(\mathrm{M})}{n^0} = 2^{[T(\mathrm{M})+t]/T} - 1 - (2^{T(\mathrm{M})/T} - 1)$$

$$= 2^{[T(\mathrm{M})+t]/T} - 2^{T(\mathrm{M})/T}$$

$$= 2^{T(\mathrm{M})/T}(2^{t/T} - 1) \tag{3}$$

In addition, the observed mitotic index, $N(\mathrm{M})$, at any time, t, will be smaller than $n(\mathrm{M})/n^0$ because of the cell division which occurred during the period of doubling of the cells initially in mitosis. The final number of cells is then $n^0\, 2^{T(\mathrm{M})/T}$.
Hence,

$$N(\mathrm{M}) = \frac{n(\mathrm{M})}{n}$$

$$= \frac{n(\mathrm{M})}{n^0 2^{T(\mathrm{M})/T}}$$

$$\therefore\ N(\mathrm{M}) = 2^{t/T} - 1$$

or

$$\log\,[1 + N(\mathrm{M})] = \frac{0.301t}{T} \quad \text{for} \quad t \geq T(\mathrm{M}) \tag{4}$$

This equation expresses the experimental fact noted in Fig. 2 that after the first hour the mitotic index accumulation function is a straight line extrapolating to the origin.

From the slopes of curves like that of Fig. 2, in which the accumulation function for mitoses is plotted against time, values for generation time for S3 HeLa cells under the experimental conditions described, of about 20 hours are obtained. This value agrees with that obtained from analysis of colony sizes of single cells plated in the absence of inhibitors (Puck, *et al.*, 1956), and with the value obtained by direct measurement of the intermitotic time as described in section 1. Hence, the conclusion may be drawn that colcemide affects no part of the life cycle except the metaphase-anaphase region.

3. *Life Cycle Analysis of a Random Culture.* We now proceed to a complete analysis. In this experiment, colcemide and H^3-thymidine were simultaneously added at $t = 0$ to a series of plates which were sampled at progressive times.

In Table I are presented the experimental data on the observed total mitotic index (total mitoses/total cells); the radioautographically labeled mitotic index (labeled mitoses/total cells), and the fraction of all cells (mitotic and interphase) which are labeled with H^3-thymidine. Fig. 3 presents the data for mitosis accumulation of labeled and total cells, respectively. The two curves conform well with the theoretical expectation. Both bend toward the horizontal as t approaches T, presumably because the variance in T is not zero. Thus, at the moment of colcemide addition, any interval in the life cycle is gaining faster moving cells from the preceding interval at a roughly similar rate as it loses such cells to the following region.

TABLE I

EXPERIMENTAL APPLICATION OF THE LIFE CYCLE ANALYSIS

Data from a typical experiment in which the fractions of the cell population (*a*) in mitosis (column 2), (*b*) labeled with H^3-thymidine (column 7), and (*c*) both labeled and in mitosis (column 5) were determined at various times after the simultaneous addition of colcemide and H^3-thymidine.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
t	Mitotic index $N(M)$	Log $[1 + N(M)]$	F = Fraction of mitoses labeled with H^3-thymidine	$N^*(M)$ = Labeled mitotic index = $N(M) \times F$	Log $[1 + N^*(M)]$	$N(L)$ = Fraction of all cells labeled with H^3-thymidine
	hrs.					
0	0.0350	0.0150	0	0	0	—
1.5	0.049	0.0208	0.0024	0.0024	0.009	0.330
2.5	0.0735	0.0310	0.0134	0.0134	0.0044	0.360
3.5	0.115	0.0473	0.0230	0.0230	0.0099	0.459
4.5	0.141	0.0573	0.0395	0.0395	0.0166	0.495
5.5	0.174	0.0697	0.0644	0.0644	0.0269	0.515
8.0	0.244	0.0948	0.110	0.110	0.0413	0.685
10.0	0.364	0.135	0.218	0.218	0.0860	—
22.0	0.840	0.265	0.634	0.634	0.213	0.694
24.0	0.920	0.283	0.745	0.745	0.242	0.775

However, once the blocking agent has been added and new cells are prevented from reentering the cycle, the cells most recently emerged from mitosis gradually lose their faster moving members without replenishment, so that ultimately only the very slowest part of the population is left. The curve for labeled mitoses exhibits an initial shoulder for similar reasons. (Another effect which could contribute to the bending of the curve as t approaches T would be gradual disintegration of mitotic figures after 16 or more hours, if these should be unstable.)

The steps in the analysis of the life cycle are as follows:

(*a*) The mitotic index at 0 time is 0.035 (excluding telophase). Hence, $T(M)/T$, the relative fraction of the life cycle taken by mitosis, is 0.052 (from Appendix equation (4)).

(*b*) In Fig. 3 the accumulation function, log $[1 + N(M)]$ is plotted against the time, both for total and labeled mitoses. The slope of the linear portion is equal to $0.301/T$, from which one obtains the value, $T = 21.8$ hours for the total life cycle time.

(*c*) The separation between the two curves gives a value for $T(G2)$ directly, which is found to be 4.40 hours. From this figure, one can also determine $N(G2)$, the per cent of the original population that was in G2 at $t = 0$, by use of Appendix equation (8) where $T(1)$ is the mitotic time and $T(2)$ is the sum of the mitotic and G2 times. However, there is another way of determining $N(G2)$ and $T(G2)$: The

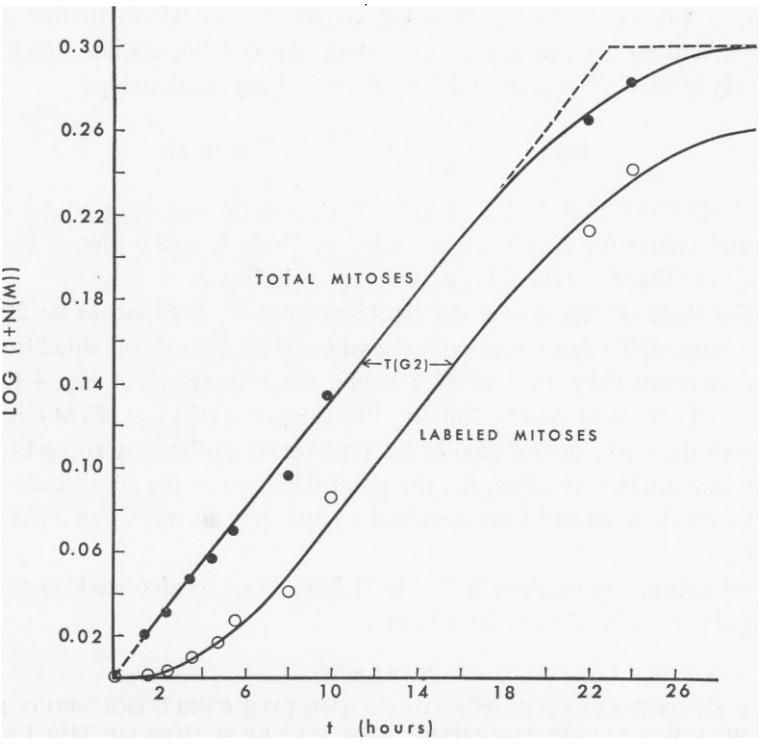


FIGURE 3 A plot of the collection function, log $(1 + N)$ for total mitotic index, $N(M)$, and for the fraction of the cell population which is both labeled with H^3-thymidine, and in mitosis for an S3 culture to which colcemide has been added. Both curves obey the expected relationships. The analysis of the resulting data is described in the text.

cells which were in G2 originally will accumulate in mitosis as unlabeled cells. All the other cells arriving in mitosis will be labeled. Hence, after the elapse of a time interval great enough so that all the cells originally in G2 are in mitosis, the difference in mitotic index for total and labeled cells, respectively, represents the original G2 population. Selecting the last four points of Table I as being sufficiently far in time for all of the original G2 cells to have entered mitosis, one finds an average difference between total and labeled mitotic indices of 0.165 ± 0.025. This figure needs to be corrected for cell multiplication by the factor, $2^{0.052}$, which yields a value of 0.171 as $N(G2)$, the fraction of the original population in G2 when colcemide was first added. By use of Appendix equation (8) one finds the corresponding G2 time, $T(G2)$, is 4.86 hours. The agreement with the previous value is reasonable, and the averages of the two sets of values may be taken as $T(G2) = 4.63$ hours; $N(G2) = 0.162$.

(*d*) By considerations like those employed in preceding paragraphs and in the

Appendix, it can be shown[3] that, when colcemide and H^3-thymidine are simultaneously added to a culture, the fraction of the cell population which becomes radioactively labeled, $N(L)$, should obey the following relationship:

$$\log\left(1 + \frac{N(L)}{k}\right) = \frac{0.301}{T}\,[T(\mathrm{S}) + t] \tag{6}$$

where $k \equiv 2^{T(\mathrm{G2})/T}$ and $N(L)$ includes both mitotic and intermitotic cells. The experimental values for $N(L)$ are recorded in Table I, and a plot of the function (6) is shown in Fig. 4 for the same experiment as in Fig. 3.

From the slope of this curve, the doubling time, T, is found to be 20.4 hours, which is in reasonable agreement with the value of 21.8 hours obtained from Fig. 3. Using the averaged value of $T = 21.1$ hours, the Y intercept of Fig. 4 furnishes a value for $T(\mathrm{S}) = 6.04$ hours. Finally, the sum of $T(\mathrm{G1}) + T(\mathrm{M})$ is obtained directly from the curve as the time of intersection of the linearly rising branch with the asymptotic horizontal value, *i.e.* the point when all of the cells destined to pass through S have done so and have acquired a label. By subtraction of $T(\mathrm{M})$, $T(\mathrm{G1})$ is obtained.

The final values summarized in Table II have been recalculated on the basis of the averaged generation time of 21.1 hours.

TABLE II

SUMMARY OF THE RELATIVE PERIOD OF THE LIFE CYCLE OCCUPIED BY EACH OF THE FOUR MAJOR DIVISIONS, AND THE FRACTION OF THE ENTIRE POPULATION DISTRIBUTED IN EACH IN A RANDOM CULTURE

	$T(i)/T$	$N(i)$	$T(i)$
			hours
G2	0.216	0.167	4.56
S	0.286	0.260	6.04
G1	0.408	0.479	8.40
Mitosis	0.052	0.035	1.10
Totals	0.962	0.941	20.10

[3] At $t = 0$, $N(\mathrm{S}) = 2^{[T(\mathrm{M}) + T(\mathrm{G2})]/T}\ (2^{T(\mathrm{S})/T} - 1)$, from equation (8) of the Appendix, where $T(1) = T(\mathrm{M}) + T(\mathrm{G2})$ and $T(2) = T(\mathrm{M}) + T(\mathrm{G2}) + T(\mathrm{S})$. This quantity is equal to $n(\mathrm{S})/n^0$. If cell multiplication actually stopped as soon as colcemide is added, $N(L)$, at any subsequent time, would be equal to

$$2^{[T(\mathrm{M})+T(\mathrm{G2})]/T}(2^{[T(\mathrm{S})+t]/T} - 1).$$

However, in the presence of colcemide, the cells multiply for the first $T(\mathrm{M})$ hours. Therefore, the observed fraction of labeled cells,

$$N(L) = \frac{n(L)}{n^0} \times \frac{1}{2^{T\mathrm{M}/T}} = 2^{T(\mathrm{G2})/T}(2^{[T(\mathrm{S})+t]/T} - 1), \tag{5}$$

from which equation (6) follows.

It should be noted that the value for mitosis excludes telophase, which is added to G1. The totals of all the partial values agree reasonably well (within 6 per cent) with the averaged value obtained directly from the curves of Figs. 3 and 4, as do the summations for the fractional population and the fractional generation times.

4. *Life Cycle Analysis in Cells Irradiated with Sublethal Doses of X-Ray.* In previous reports from this laboratory (Puck and Yamada, 1962), the reversible lag produced by x-irradiation of S3 cells was studied. It was found that cells in G2 when irradiated with doses less than 100 rads underwent a delay in reaching mitosis of approximately 0.1 hour per rad. Cells in S and G1 were not immediately affected by these low doses of irradiation, but displayed a lag when they also reached G2.

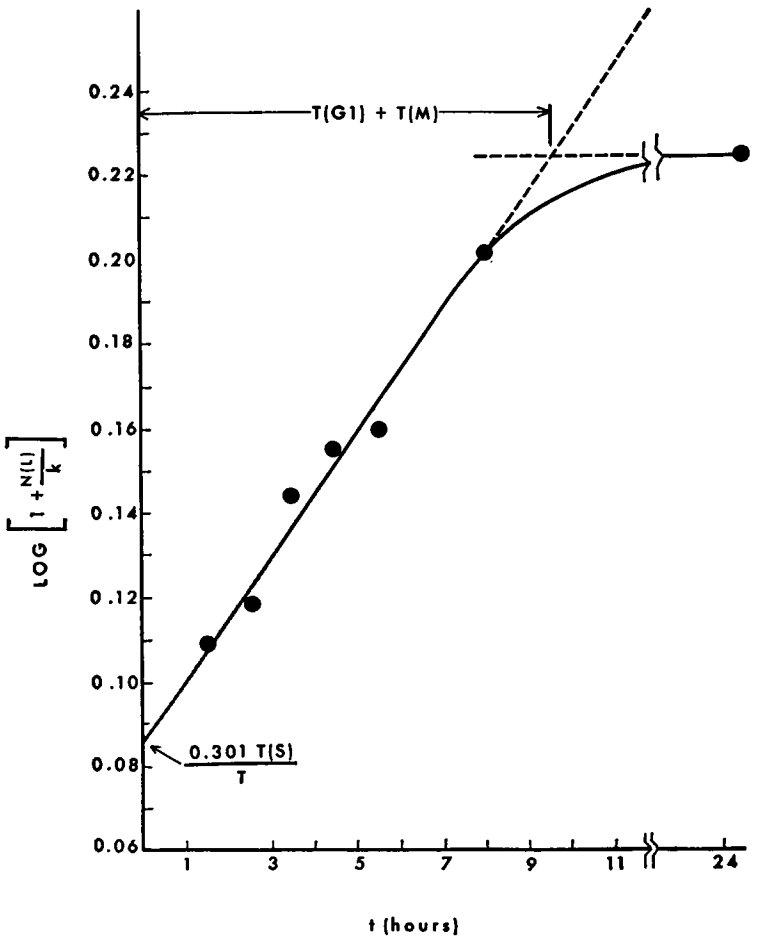


FIGURE 4 Plot of the collection function, log $[1 + N(L)/k]$ where $N(L)$ is the fraction of the total population which has taken up the label (H^3-thymidine) and k is $2^{T(GD)/T}$. Both colcemide and H^3-thymidine were added at $t = 0$. The analysis of the resulting data is discussed in the text.

However, the lag of these cells was appreciably smaller (about 0.01 to 0.03 hours per rad) than that of the cells which were already in G2 at the time of irradiation. This differential behavior of cells in different parts of the life cycle accounted for the large oscillations in mitotic index following irradiation of mammalian cell populations, and was attributed to chromosomal damage which must be repaired before G2 period can be completed (Yamada and Puck, 1961; Puck and Yamada, 1962).

The observation that the delay in completion of G2 is considerably greater for S3 cells already in G2 at the time of irradiation than for cells irradiated in S or G1, has been qualitatively confirmed by Terasima and Tolmach (1963), but these investigators found a maximum lag for cells irradiated in G2 of only about 0.03 hours per rad, which is definitely smaller than that which we reported. The latter investigators for the most part used considerably higher doses (300 rads) and also employed phased cell cultures for their studies. It was desirable to re-examine the lag of cells irradiated while in the G2 period by means of the technique here described, in order to determine whether a more precise figure for the delay per rad can be obtained, what fraction of the G2 population is held back, and at how low a dose the effect can be elicited. The last point is of theoretical importance: if this particular lag disappears at doses well above those known to produce chromosomal damage, then the explanation of the lag as a reflection of chromosomal damage becomes less likely.

The dose needed to produce, on the average, one visible chromosome aberration is not known for certainty for S3 cells which were in G2 at the time of irradiation, but can safely be taken to be less than 100 rads and may even lie in the neighborhood of 20 rads (Yamada and Puck, 1961). Hence, experiments were carried out at 9 rads, which is the lowest dose for which visible chromosomal anomalies have been reported with any appreciable frequency (Yamada and Puck, 1961) and is still one for which an unequivocal lag measurement should be feasible if the delay efficiency of 0.1 rad per hour is maintained.

In these experiments, the use of H^3-thymidine was omitted, since incorporation of this isotope, even in amounts as small as 0.10 μc/ml, in the medium, causes a significant G2 delay in the labeled cells. Indeed, as will be shown later, a G2 delay resulting from chromosomal incorporation of the radioisotope is to be expected on the basis of a chromosomal theory of G2 lag (Puck, data to be published).

In Fig. 5 is presented a typical set of curves comparing the G2 period of a normal culture and one irradiated with 9.0 rads of 230 kv x-rays. The following points emerge:

(*a*) The present method of life cycle analysis can easily detect the mitotic lag produced in S3 cells by irradiation with doses of 9 rads, demonstrating that the lag exists at doses at least as small as those producing visible chromosome damage.

(*b*) The reversible nature of the lag is revealed by the fact that the radiation-delayed curve eventually returns to meet the normal curve.

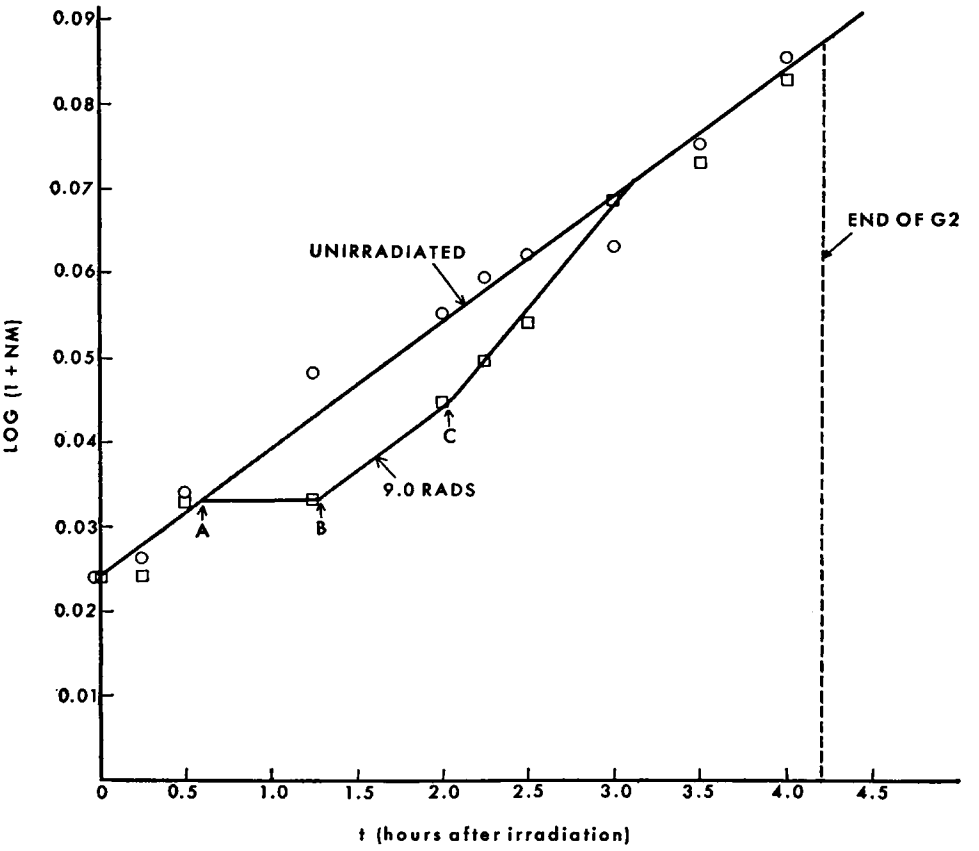


FIGURE 5 Test showing that the method here described can demonstrate and measure a lag of cells in the central part of the G2 region, resulting from exposure to 9.0 rads of x-rays. Both cultures received colcemide, but only one was irradiated. In this case the zero time is that at which the irradiation was delivered, which occurred approximately 3 hours after the colcemide addition, so that the curve begins at a point above the origin. This procedure was adopted in order to avoid complication due to the lag in action of the colcemide. The total generation time was 19.4 hours. The end of G2, calculated from this value and Table II, is indicated.

(*c*) While calculation of the exact duration of the lag, and the numbers of cells affected, involves some difficulty because of uncertainty about the form of the distribution of lag among the cells involved, some deductions appear reasonable: The entire abnormality in the curve is confined to a central interval in G2 of 2.4 hours' duration. Hence, this constitutes an upper limit for the duration of the lag produced. The first cells to be affected are those located 0.60 hours before prophase, (point *A*, Fig. 5) and the flatness of the curve during the subsequent 0.70 hours indicates a complete cessation of progress for at least this interval, (point *B*). Hence, this latter figure constitutes a lower limit for the lag. Thereafter, the curve rises, and by 0.65

hours later, (point C) the slope has become greater than that of the normal curve, indicating that the lag of at least some of the cells has ended.

By the use of the simplifying assumption that all cells are either stationary, or else are moving around the cycle at the normal rate, it becomes possible to calculate a virtual lag time which may be useful in this system. This assumption is embodied in the curve of Fig. 5, in which the points on the irradiated plot have been connected by a series of straight lines instead of a smooth curve. The resumption of mitosis collection at point B, 1.3 hours after irradiation would thus indicate the arrival in prophase of cells which were not impeded at all by the irradiation, so these follow the normal slope as shown in the figure. At the 1.95 hour point, (C) such cells are joined by those recovering from the radiation-induced block, producing a slope which is greater than that of the unirradiated control. By this interpretation the cells experiencing delay are confined to the region of G2, beginning at 0.60 hours before mitosis (point A) and extending back until 1.3 hours before mitosis (point B). The cells on either side of this 0.7 hour region would not have been delayed at all. The magnitude of the "effective" delay so calculated for the impeded cells is 1.4 hours (distance from A to C) which corresponds to an efficiency of 0.16 hours per rad, which agrees well with the estimate obtained by other methods, utilizing doses in the range of 35 to 100 rads (Puck and Yamada, 1962). In the present experiment, the generation time was 19.4 hours, from which the normal G2 period can be approximated from Table II as 4.2 hours. The cells "effectively delayed" by this small dose of irradiation constituted only 14 per cent of the original G2 population or 2.3 per cent of the total population. Despite the small cell numbers involved, the results of such an experiment are readily reproducible.

DISCUSSION

Other methods for determining the degree of randomness (or synchrony) of a cell population have been proposed (Engelberg, 1961). The method of analysis here described is capable of identifying separately individual departures from randomness which are spaced more than an hour apart in time, and of locating with reasonably high resolution their positions in the cycle. Indeed, the obeying of the relationships of equations text (4) and text (6) under the appropriate experimental conditions may be taken as a test of the randomness of any cell population.

The fact that the summation of all the subtotals in Table II falls somewhat short of 100 per cent, may be a reflection of the fact that the variance in T is not zero. The existence of a dispersion in T would tend to increase slightly the per cent of cells in each division of the life cycle over that calculated, because at the end of one average life cycle period, the total number of cells would have slightly more than doubled. Greater refinement of the equations to correct for the existing dispersion can be carried out if necessary.

The method for life cycle analysis here described also can find application as a tool for more rapid determination of specific nutritional deficiencies of various mammalian cells and action of inhibitory metabolites, as well as pinpointing their sites of action. Both single cell plating and massive cell inocula methodologies require approximately 5 to 10 days before the growth behavior of the cells in a par-

ticular medium can be ascertained. By means of the present technique, 24 hours may suffice. This method should also be useful in achieving finer discriminations in the comparison between metabolic patterns of normal and malignant cells, and the effects thereon of various agents. Finally, it appears applicable to life cycle analysis of selected cell populations *in vivo*.

The behavior of colcemide deserves further study, particularly as it may depend upon a relative impermeability of the cell to at least some outside metabolites during mitosis. If this is indeed the case, particular care will be required in designing experiments measuring metabolic phenomena during this interval.

The demonstration that this methodology permits detection of the delay produced in cells of the G2 phase by an x-irradiation of only 9 rads, as well as location of the specific cells so affected, is an index of the power of the method. In effect, this phenomenon divides G2 into three distinct parts. The fact that at this low dose, the delayed cells are largely confined to a period which may be as small as 1.4 hours furnishes a possible explanation for the lower delay values observed with the phased cell technique (Terasima and Tolmach, 1963): unless the phased cell population selected for irradiation happened to coincide accurately with this particular period of the life cycle, the delay observed would be less than maximal. However, there is also the distinct possibility that the delay efficiency decreases with increasing doses of irradiation. If the mechanism of the delay is indeed chromosomal in origin, as we have suggested (Yamada and Puck, 1961), a fall in delay efficiency might conceivably occur with increasing doses as one proceeds from the lowest doses in which damage is confined to a single chromosome, to higher ones where it involves multiple chromosomes. If the repair process proceeds independently in each chromosome, the delay efficiency will fall; if there is a linear order of chromosome repair, the efficiency could remain constant. There is a suggestion that the efficiency does actually fall with increasing dose, but more study of these phenomena is required.

The change in the number of affected cells with dose also requires further study. Our experiments are unable to detect with certainty a lag at the G2 stage of cells irradiated, while they are in the G1 or S states, with 9 rads. This is to be expected if the cells irradiated at an earlier point exhibit a lag on arriving in G2 only 1/4 or 1/5 as great as that of the cells already in G2 when irradiated (Puck and Yamada, 1962).

It is important to search for correlation between chromosomal damage and the differential sensitivity to lag of cells irradiated at different parts of the life cycle. Dewey and Humphrey (1962) looked for such a relationship in mouse L cells but found the results to be equivocal. However, they employed doses of 250 rads, and it is possible that the situation is much more complex at these high doses. Chu, *et al.* (1961), however, who studied human cells exposed to 25 to 100 roentgens of x-irradiation, reported that the sensitivity to chromosomal aberration is 5 times greater during the G2 period than in S or G1, a ratio which corresponds remarkably

well to the difference we find in the extent of the subsequent G2 lag. These studies are continuing.

APPENDIX

Consider a randomized cell culture growing with a mean doubling time, T. If the variance of T is small, the number of cells, n, present at any moment is:

$$n = n^0 2^{t/T} \qquad (1 \text{ Appendix})$$

where n^0 is the cell number at $t = 0$.

Let $N(1)$ be the fraction of the population contained within the interval of the life cycle, $T(1)$, defined as the period extending from the end of mitosis to a given point, 1, and measured counterclockwise around the cycle, as shown in Text Fig. 1. Then, for the four intervals, G1, S, G2 and M (itosis)

$$T(\text{G1}) + T(\text{S}) + T(\text{G2}) + T(\text{M}) = T \qquad (2 \text{ Appendix})$$

$$N(\text{G1}) + N(\text{S}) + N(\text{G2}) + N(\text{M}) = 1 \qquad (3 \text{ Appendix})$$

Obviously, each of these divisions can be subdivided further if desired. The fraction of the cell population contained in the interval $T(1)$ of Text Fig. 1 is constant for a random culture, and has been shown by other investigators (Crick, 1952) (Stanner and Till, 1960) (Smith and Dendy, 1962) to be.[4]

$$N(1) = 2^{T(1)/T} - 1 \qquad (4 \text{ Appendix})$$

This well known equation makes it clear that the cell population is not distributed uniformly over the period of the life cycle, but is most dense immediately after mitosis and falls smoothly to a minimum just before cell division.

Consider the change in distribution which results on addition of a blocking agent which acts only at a specific point in the cycle. It is assumed that the cells contained in a given interval preceding this point can be recognized by a microscopic test.

Case I. The blocking agent acts instantaneously, and only at the end of mitosis (*i.e.* at $T = 0$), so that cell multiplications stops when the agent is added. In this case, the total cell number, n, is constant:

$$n = n^0 = \text{constant} \qquad (5 \text{ Appendix})$$

Since mitosis itself is the period immediately preceding the block, and can be readily scored visually, the accumulation of cells in mitosis is measured. If the blocking agent is added at $t = 0$, and the proportion of cells in mitosis, $N(\text{M})$, scored at different intervals, the relationship will be obtained in which $N(1)$ is $N(\text{M})$:

$$N(1) = 2^{[T(1)+t]/T} - 1 \qquad (6 \text{ Appendix})$$

This situation differs from that of equation (4) because, as time progresses, cells are prevented from leaving the region $T(1)$ (Text Fig. 1). Hence, the effect is as though

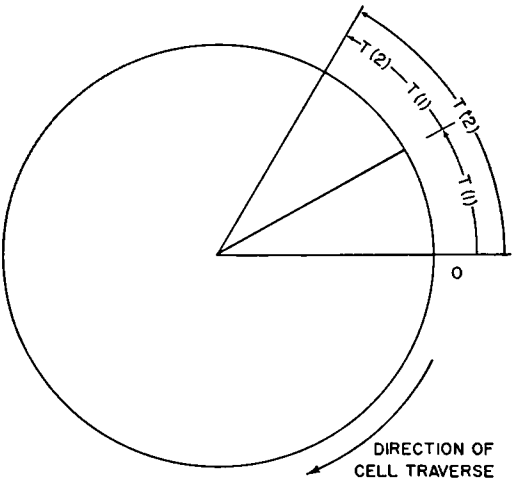
[4] At $t = 0$, the cell number is n^0, and the fraction of cells in $T(1)$ is $N(1)$. If a time, $t = T(1)$, is now allowed to elapse, all the cells in $T(1)$ will double, and the new cell population is $n = n^0\, 2^{T(1)/T}$. The increase in cell number, Δn, is equal to those originally present in $T(1)$, and is $n^0\,(2^{T(1)/T} - 1)$. Hence, $N(1) = \Delta n/n^0 = 2^{T(1)/T} - 1$.

the upper limit of $T(1)$ is increasing with time so as to sweep out a progressively larger region. By rearrangement (6) becomes

$$\log_{10}[1 + N(1)] = \frac{0.301}{T}[T(1) + t] \qquad (7 \text{ Appendix})$$

We call the quantity, log $(1 + N)$ the collection function. Thus, for an inhibitor acting at $t = 0$, a plot of log $[1 + N(\mathrm{M})]$ against the time, t, would yield a straight line with intercept equal to 0.301 $(T(1))/T$, as shown by the dashed line of Text Fig. 2, where the interval $T(1)$ is $T(\mathrm{M})$, the time of mitosis. Given the type of inhibitor postulated, the straight line behavior establishes that the cell population was indeed random at $t = 0$, and provides values for the time of mitosis, $T(1)$, and the total generation time, T, from the intercept and the slope.

Case II. Next, consider an agent which instantaneously and quantitatively produces a block at a point, $T(1)$, in the life cycle which precedes mitosis (Appendix Fig. 1). The cells in the interval, $T(2) - T(1) = \Delta T$, are presumed to be scorable microscopically.



APPENDIX FIGURE 1 Life cycle diagram for a culture in which the cell population of the interval, $T(2) - T(1)$, which extends from the end of $T(1)$ to the end of $T(2)$, is to be determined. The fraction of the population of a random culture contained in the interval, $T(2) - T(1)$, is given by equation (8) of the Appendix.

The cell fraction present in the interval, ΔT, in a random, non-inhibited population is given from Appendix equation (4) by

$$\begin{aligned} N(2) - N(1) &= N(\Delta T) \\ &= 2^{T(2)/T} - 1 - (2^{T(1)/T} - 1) \\ &= 2^{T(2)/T} - 2^{T(1)/T} \\ &= 2^{T(1)/T}(2^{[T(2)-T(1)]/T} - 1) \end{aligned} \qquad (8 \text{ Appendix})$$

If the agent is added at $t = 0$, the cells continue to enter the region ΔT, but cannot

leave it, and so accumulate. However, the cells initially in $T(1)$, being unaffected, continued uninterruptedly around the cycle, so that $T(1)$ is gradually depleted. Moreover, all of these latter cells will divide, so that the cell number does not remain constant, as in Case I, but increases over the period, $T(1) \geq t \geq 0$, to reach a constant limit at $t = T(1)$:

$$n = n^0 2^{t/T} \quad \text{for} \quad T(1) \geq t \geq 0$$

$$n = n^0 2^{T(1)/T} \quad \text{for} \quad t \geq T(1) \qquad (9 \text{ Appendix})$$

The expression for the fraction of the cell population in ΔT at any time after addition of the inhibitor can be found by separately determining the cell fraction of $T(2)$ and $T(1)$ and then subtracting the latter from the former.

If the cell block had been at $T = 0$ in Appendix Fig. 1, the fraction of the cell population in the entire region $T(2)$ would be

$$\frac{n(T2)}{n^0} = 2^{[T(2)+t]/T} - 1.$$

Since, however, the block is at $T(1)$, the actual value will be decreased because of the passage of the cells out of $T(1)$. Let us first consider only the time, $t \leq T(1)$.

$$\frac{n(T2)}{n^0} = (2^{[T(2)+t]/T} - 1) \quad \text{minus cell loss from} \quad T(1).$$

Since cells continue to leave $T(1)$ but no new cells enter,

$$\frac{n(T1)}{n^0} = 2^{[T(1)-t]/T} - 1 \qquad (10 \text{ Appendix})$$

Therefore, at any time, t, the cell loss from $T(1)$ is

$$2^{T(1)/T} - 1 - (2^{[T(1)-t]/T} - 1) = 2^{T(1)/T} - 2^{[T(1)-t]/T}$$

Therefore,

$$\frac{n(T2)}{n^0} = 2^{[T(2)+t]/T} - 1 - 2^{T(1)/T} + 2^{[T(1)-t]/T} \qquad (11 \text{ Appendix})$$

Now, the cell number in the interval, $T(2) - T(1) = \Delta T$ is found by subtracting equations Appendix (10) from Appendix (11):

$$\frac{n(\Delta T)}{n^0} = \frac{n(T2)}{n^0} - \frac{n(T1)}{n^0} = 2^{[T(2)+t]/T} - 2^{T(1)/T} \qquad (12 \text{ Appendix})$$

Since the cell number is increasing by virtue of the doubling of the cells passing out of $T(1)$, the observed fraction of the cells in the interval, ΔT, must be corrected by the factor, $\frac{1}{2^{t/T}}$.

Therefore,

$$N(\Delta T) = N2 - N1$$

$$= \frac{1}{2^{t/T}} (2^{[T(2)+t]/T} - 2^{T(1)/T})$$

$$= 2^{[T(1)-t]/T}(2^{[T(2)-T(1)+t]/T} - 1) \quad \text{for} \quad T1 \geq t \geq 0 \qquad (13a \text{ Appendix})$$

$$N(\Delta T) = \frac{1}{2^{T(1)/T}} (2^{[T(2)+t]/T} - 2^{T(1)/T})$$

$$= 2^{[T(2)-T(1)+t]/T} - 1 \quad \text{for} \quad t \geq T1 \qquad (13b \text{ Appendix})$$

To obtain appropriate linear functions, equations (13*a*) and (13*b*) can be transformed as follows:

$$\log \left(1 + \frac{N(\Delta T)}{2^{[T(1)-t]/T}}\right) = \frac{0.301}{T} (T(2) - T(1) + t)$$

$$\text{for} \quad T(1) \geq t \geq 0 \qquad (14a \text{ Appendix})$$

$$(\log (1 + N(\Delta T)) = \frac{0.301}{T} (T(2) - T(1) + t) \quad \text{for} \quad t \geq T(1). \qquad (14b \text{ Appendix})$$

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