ESTIMATING THE MEDIAN GENERATION TIME OF PROLIFERATING CELL SYSTEMS IN STEADY STATE

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ABSTRACT For proliferating cell systems in which the usual "labeled mitoses" method cannot be used to estimate generation times, an alternative scheme is derived. The method presented here is based on observation (by autoradiography) of the median grain count of labeled interphase cells following a pulse of labeled DNA precursor. It is shown that the median generation time of the labeled cells will be equal to the time required for the median grain count to halve, starting from the time when half the labeled cells have completed their first division. This starting time is determined from observation of the first wave of labeled mitoses. The procedure was designed to minimize error resulting from such factors as radiation damage, label reutilization, and the use of a nonzero grain counting threshold. The method is applied to the analysis of two cases of acute leukemia in man.

INTRODUCTION

Most determinations of cellular proliferation parameters such as generation time and the durations of the phases of the mitotic cycle have been based on the technique of labeled mitoses (Quastler and Sherman, 1959; Wimber, 1963; Lajtha and Gilbert, 1967). This method, requiring the measurement of time elapsed between successive peaks in the plot of percentage of labeled mitoses following pulse (flash) labeling of DNA, is perhaps the most satisfactory for most purposes when applicable.

Several situations arise, however, in which this approach cannot be used: (*a*) in the investigation of the kinetics of hemopoiesis in man, it may not be possible to sample the bone marrow sufficiently frequently to insure detection of the second labeled peak; (*b*) if the variability of generation times is sufficiently great, the labeled cells may become so dispersed within the cell cycle that a definite second peak is unobtainable; (*c*) if more than one type of dividing cell occurs in the system, it may not be possible to distinguish them in mitosis. For example, the *types I* and *II myeloblasts* found by Clarkson et al. (1968) in a number of acute leukemia

patients, could be distinguished in interphase but not in mitosis. Unless the different types have identical generation times, it might not be possible to determine to which type the second peak corresponds; (*d*) for studies of chronic leukemia or in hemopoietically normal patients, it may be difficult or impossible to find a sufficient number of mitotic cells to establish an adequate labeled mitoses curve. The problem of distinguishing between cell types in mitosis could also prove serious here.

For cases such as these it is necessary to develop an alternate procedure for estimating generation times. The method to be presented here is dependent on the assumption that upon the division of cells labeled in their DNA (e.g. with tritiated thymidine), the labeled chromosomes (and hence the grains on an autoradiograph) are apportioned approximately equally between the daughter cells (Cronkite et al., 1961). This is the basis of the "grain-count halving" method as used by Killman et al. (1962) and others.

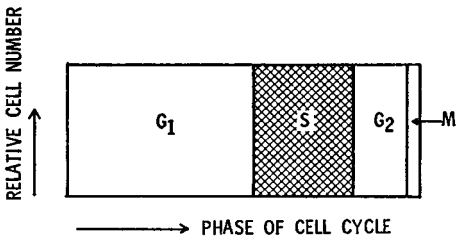

FIGURE 1 Idealized graph of distribution of self-maintaining cell system as a function of phase within the mitotic cycle. It is assumed that only one daughter (on the average) reenters the cycle after division. The system is shown immediately after the injection of a "pulse" of radioactive DNA precursor which labels all cells in the *S* phase; label is indicated by cross hatching.

Consider, for example, an idealized steady-state population of proliferating cells having identical generation times and constant phase density; assume the population is self-maintaining (i.e. there are no precursor or stem cells). Such a system is shown in Fig. 1, where the shading of the *S*-phase cells implies the presence of radioactive DNA tracer (presumably supplied in a "flash" labeling procedure). If autoradiographs are then prepared at frequent intervals and the distribution of nuclear grains determined, Fig. 2 shows the hypothetical plots of median or mean grain counts of labeled cells (on both linear and logarithmic scales) as a function of time. It should be noted that these plots have assumed that background radiation is negligible and that grain counting thresholds are zero. The decreasing portions of the plots are shown as dashed lines because their precise shape will depend on such factors as whether the median or the mean is being considered, whether the grain distribution is dependent on the exact position of the cell within the *S* phase at the time of labeling, and on the particular grain distribution of the population.

It is evident that points separated by intervals equal to one generation time lie on an exponential curve (or on a straight line on a semilogarithmic plot). The time taken for this exponential curve to decrease by a factor of two is equal to the generation time. It seems intuitively reasonable to suppose that if the generation times were variable rather than identical, the plots in Fig. 2 would become relatively smoothed

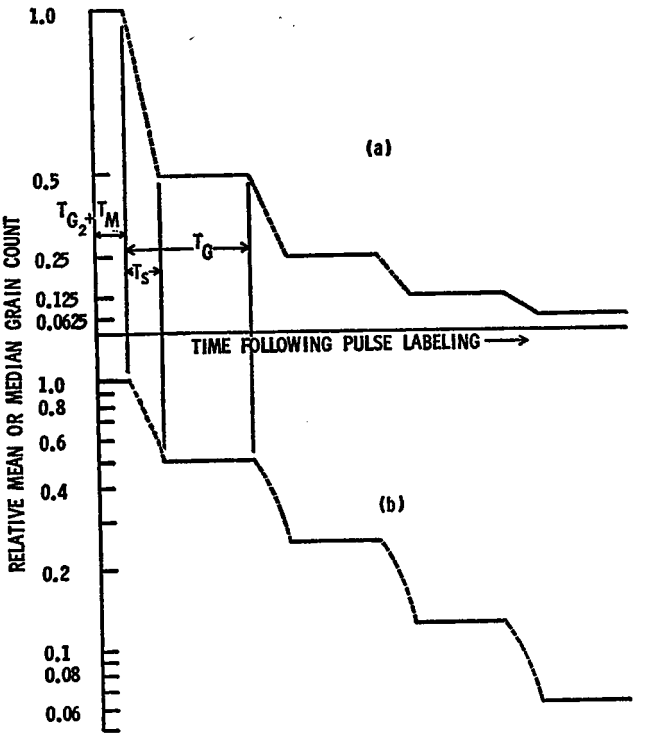

FIGURE 2 Hypothetical plots of median or mean grain counts of labeled cells of system depicted in Fig. 1. T_{G_2}, T_M, T_{G_1}, and T_S are the durations of the premitotic, mitotic, postmitotic, and DNA synthesis phases, respectively. Intercellular variabilities in the durations of these phases are assumed negligible. (*a*) Linear ordinate scale; linear abscissa scale (arbitrary units). (*b*) Logarithmic ordinate scale; linear abscissa scale.

out after one or two cycles; a graph of the logarithm of median or mean grain count vs. time might then be expected to lie on a reasonably straight line, with the grain count halving time equal to the median or mean cellular generation time.

This method was basically the one used by Clarkson et al. (1967, 1968) in investigations of the proliferation kinetics in acute leukemia in man. There are, however, some significant drawbacks to the use of this method:

(*a*) The median (or mean) grain count must be determined over a period of several cycles so that the "steps" can become desynchronized. Since this means that a very high level of radioactivity must be employed, the more highly labeled cells might suffer serious damage during the period of observation; this could result in a significant alteration of their generation times.

(*b*) If the amount of radioactive material injected were sufficiently low to insure that the behavior of even the most highly labeled cells would not be altered by radiation damage, it is likely that the median grain count would fall below the counting threshold after only a single cycle. Although this difficulty could be avoided by

applying the method to the median grain count of only a (highly labeled) fraction of the cells (Clarkson et al., 1967, 1968), the possibility remains that this highly labeled fraction is not truly representative of the entire cell population (or even of the entire labeled cell population).

(*c*) Since the background radiation is usually significant, a nonzero counting threshold must be employed. However, lightly labeled cells may fall below this threshold upon division, resulting in an erroneously high estimate of median grain count. A correction factor, based on the experimentally observed decrease in labeling index (i.e. fraction of cells labeled), was therefore introduced (Clarkson et al., 1967, 1968). In many cases, however, the required correction would become so large during the latter part of the computation interval that the accuracy of the final result would depend as much on the validity of the correction procedure as on the data itself; the estimated generation time might therefore be highly sensitive to small changes or errors in the labeling index correction factor.

To overcome these difficulties, it is necessary to devise a procedure which eliminates the requirement that the computation extend over several "grain count halving" cycles. This would insure that (*a*) the entire labeled population could be used rather than only a highly labeled fraction, (*b*) the relative number of cells passing below the grain threshold during the computation interval would be relatively small; hence the correction procedure, even if inaccurate, would probably not seriously alter the result, and (*c*) the dose of radioactivity received by the cells would be lower; thus the possibility that proliferative behavior were altered during the time interval of interest would be minimized.

The method presented here requires data extending over only a single such cycle. Based on the observed decrease of median grain count (MGC), it yields an estimate of the median generation time of the variable cell population. In this procedure, the proper starting time for the computation (relative to the time of labeling) is essential.

Since the exact procedure to be followed will depend on the particular model assumed, it will be necessary to present the derivation in terms of a specific model. However, it should become evident that the method can be adapted to the analysis of certain other models as well.

ASSUMPTIONS OF THE MODEL

The following are the major assumptions that will be made regarding the nature of the cell population under study and the type of experiment to be performed:

1. The system consists of a self-maintained population of proliferating cells at steady state.

2. Cells which leave the system do so immediately following division; the probability of removal is the same for every cell. Because of the assumption of steady state, it follows that half the daughter cells leave, and half reenter the G_1 phase.

3. The probability distribution of generation times (T_G) is assumed unimodal and symmetrical about the mean (and hence about the median as well); the consequences of the elimination of these assumptions will be considered later. The generation time of a daughter cell is assumed independent of that of the mother. It will also be assumed that the standard deviations of the durations of the DNA synthesis phase S, the premitotic phase G_2, and the mitotic phase M, are small compared with the mean or median generation time. In addition, these standard deviations must be sufficiently small relative to the mean duration of S to enable the first wave of labeled mitoses to be determined with reasonable accuracy. It has been shown experimentally that in many cases, most of the variability in the generation time is in fact due to that in the G_1 phase; however, this is not universally true (Baserga, 1965).

For purposes of clarity in the derivations to follow, the above assumptions will be extended such that only the G_1 phase is assumed to be variable; the error introduced by applying the resulting procedure to a system which satisfies only the assumptions given in the preceding paragraph will probably be of the order of magnitude (or less) of the actual standard deviations of the other phases.

4. Incorporation of radioactive DNA precursor into the cell and the DNA can occur only during the presumably well-delineated DNA synthesis phase S. Once incorporated, the metabolic turnover must be negligible (see, however, Pelc [1963]).

5. Upon division, the radioactive label is distributed equally between the daughter cells.

6. Reutilization of tracer from degraded molecules and cells is negligible (Rubini et al., 1960).

7. The amount of tracer incorporated by a cell is independent of both its generation time and its position within the S phase at the moment of labeling. The latter assumption can be verified in part by examination of the grain distribution of the first wave of labeled mitoses. The former assumption appears reasonable provided that the variabilities in the durations of the S phase and in the DNA content of cells in G_2 (or G_1) are small.

Alpen and Johnston (1967) have suggested that grain count variability may be due mainly to (random) differences in the number of chromosomes being replicated at the time of labeling. If this is the case, then the label taken up by a cell would not be greatly influenced by its generation time. They have also found that, in the case of labeled normoblasts in the dog, the mean grain count of labeled cells is relatively independent of the position of the cells within the S phase (except near the beginning and the end).

8. Very few labeled cells will have completed as many as three mitoses by the end of the computation interval for the determination of median generation time. This means, in effect, that few cells will pass through two complete mitotic cycles in less time than the majority requires to pass through one.

9. The radioactive tracer is to be administered in a flash or pulse of label; if not incorporated immediately, it will be degraded rapidly to an unusable form. In their

studies of the metabolism of tritiated thymidine in man, Rubini et al. (1960) found that the available tracer had effectively disappeared by about 30 min after injection.

10. The approximate shape of the first wave of labeled mitoses is obtainable.

11. The accuracy and reproducibility of the technical procedures (e.g. autoradiography, cell classification, grain counting, etc.) are also assumed. Most of these points (as well as many of those listed above) have been discussed in detail by other authors (e.g. Robertson, Bond, and Cronkite, 1959; Killman et al., 1962; Koburg, 1963; Rubini, Keller, and Cronkite, 1965).

While certain of these assumptions may be at best only approximately correct, it will be shown that the proposed procedure for estimating the median generation time will have the effect of minimizing the resulting error.

CORRECTION FOR GRAIN COUNTING THRESHOLD

The model under consideration assumes that cells can leave the system only upon division, and that effectively one daughter of each pair reenters the cycle. This implies that for every labeled cell passing through mitosis, one labeled cell (at half the grain count) reenters the G_1 phase. The only change in the observed labeling index that can then occur is due to the division of lightly labeled cells which fall below the counting threshold. Since this would have the effect of spuriously increasing the observed median grain count of the remaining labeled cells, a correction procedure must be devised.

Define the following:

$I_o(t)$ Observed labeling index at time t.

$I_c(t)$ "Corrected" labeling index at time t, i.e. the labeling index that would theoretically be observed if cells passing below the counting threshold could still be detected as labeled.

$N_o(t)$ Number of labeled cells (above threshold) in sample taken at time t.

$N_c(t)$ Corrected total number of "labeled" cells in sample (including those below the threshold but whose progenitors had grain counts above the threshold at the time of labeling).

The "corrected" labeling index at an arbitrary time t must be equal to the observed labeling index immediately after labeling (at time $t = 0$); that is, $I_c(t) = I_o(0)$. The expression for $I_c(t)$ is $I_c(t) = I_o(t) \cdot N_c(t)/N_o(t)$. Therefore, if a sample of $N_o(t)$ labeled cells is counted, the corrected total that should be used in determining the median grain count is $N_c(t) = N_o(t) \cdot I_o(0)/I_o(t)$, of which $N_c(t) - N_o(t) = [I_o(0)/I_o(t) - 1]N_o(t)$ are below the counting threshold.

DETERMINATION OF MEDIAN GENERATION TIME

In the following discussion it will be assumed that the grain-count data has been corrected for the threshold effect as described in the previous section. The term

"labeled cells" will therefore include not only cells above the counting threshold at a given moment, but also the "false negatives" whose progenitors were above the threshold at the time of labeling.

It was stated earlier that the method to be presented here depends on the hypothesis that the halving of the MGC of the labeled cells can, under the proper circumstances, yield the correct value of the median generation time ($T_{G_{md}}$).

The derivation of the method will proceed according to the following sequence of steps:

1. It will be shown that the correct value of $T_{G_{md}}$ cannot be obtained from observation of the MGC data unless the time (t_0) at which the initial MGC determination is made is within certain specified limits.

2. Given that t_0 is within the limits specified, it will be demonstrated that in general, the generation times of only a portion of the labeled cells will determine the halving time of the MGC of the entire labeled population. These cells are separated into groups depending on their grain counts and on their phase positions relative to mitosis at t_0.

3. An equation will then be derived expressing the functional relationship between an arbitrary (within the limits specified above) value of t_0 and the resulting MGC halving time. The equation will be formulated in terms of the relative sizes of the cell groups of step 2.

4. Utilizing the equation derived above, the precise value of t_0 will be determined such that the $T_{G_{md}}$ of the cells specified in step 2 is equal to the MGC halving time of the entire labeled cell population.

Step 1 Allowed Limits of t_0

If the starting time for the computation were chosen prior to the time when the first labeled cells reached mitosis, the entire labeled population would divide (resulting in the halving of the MGC) within a time equal to $T_S + T_{G_2} + T_M$. (In this notation, T_S, T_{G_2}, and T_M are respectively, the durations of the S, G_2, and M phases.) On the other hand, if t_0 were taken as the time when most of the labeled cells had completed their first mitosis, then the MGC would not subsequently halve until most cells had completed their second mitosis. The resulting generation time estimate would in this case approximate the maximal rather than the median value.

Therefore, t_0 must be chosen such that only a fraction α of the labeled cells have passed through mitosis. The required value of this fraction will be determined in step 4.

Step 2 Cells Influencing the MGC Halving Time

Consider an arbitrary grain distribution curve as depicted in Fig. 3 (shown for convenience as a continuous function). Suppose this is the distribution at the starting time t_0 for the computation of $T_{G_{md}}$. If M denotes the starting median, it follows

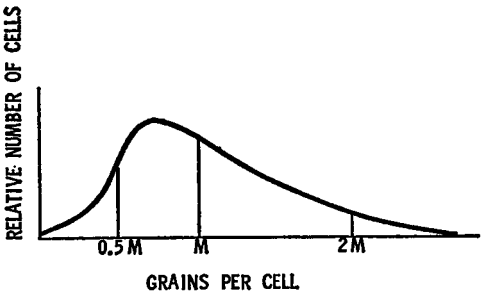

FIGURE 3 Example of hypothetical probability distribution of grain counts on autoradiograph following administration of DNA label. This is assumed to be the distribution at time $t = t_0$, the starting time for the computation of median generation time. M = median grain count of illustrated distribution.

that the median would fall to 0.5 M if all the cells were to divide exactly once. However, since the generation times are assumed variable, it is likely that some labeled cells will have divided twice (subsequent to t_0) before all the cells have divided once.

Because of the assumption that only one daughter, on the average, reenters the mitotic cycle, the division of cells having initial grain counts below 0.5 M will not change the number of cells below this level; hence these cells will not influence the MGC halving time. Furthermore, since few cells will have divided more than twice during the computation interval (assumption 8), cells with initial grain counts above 2 M at $t = t_0$ will similarly not affect the halving time.

Thus the MGC halving time (and hence the $T_{G_{md}}$ estimate) will be independent of the behavior of cells initially (i.e. at $t = t_0$) outside the interval 0.5–2 M grains (this interval could of course include all the labeled cells if the variance of the grain count distribution were sufficiently small).

Even this, however, does not completely specify the cells whose generation times determine the MGC halving time. This specification will depend in part on the phase positions of the labeled cells relative to mitosis at time t_0.

Fig. 4*a* shows the age distribution of a hypothetical population of cells at the time of labeling; the crosshatched area represents the labeled cells. The ordinate gives the relative number of cells having an age τ. Because of the assumption that only the G_1 phase is significantly variable, the system is shown for convenience with this phase at the right-hand part of the cycle. Cells are therefore considered here to be "born" upon entrance into the S phase. The abscissa value $\tau = \tau_1$ corresponds to the end of mitosis.

Fig. 4*b* shows the system at $t = t_0$, at which time the fraction α of labeled cells has passed through mitosis. These (divided) cells will be called *group* I; those not yet divided by the time t_0 constitute *group* II. Progeny of *groups* I and II cells will retain the designation of *groups* I and II, respectively.

Referring to Figs. 3 and 4*b*, and taking note of assumptions 3 and 8, it can be ascertained that the only cells whose generation times will affect the $T_{G_{md}}$ estimate are those in *group* I with grain counts between 0.5 M and M, and those in *group* II in the range M to 2 M. Let the subscripts b and h denote cells with grain counts within these respective ranges. All the cells in *group* II_h will pass into the *group* II_b

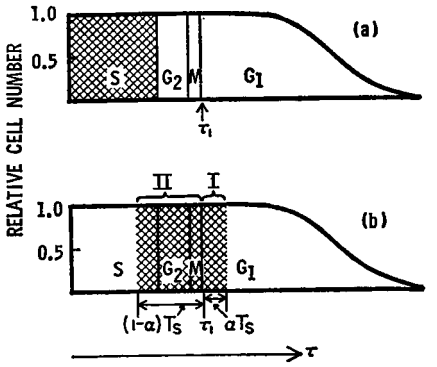

FIGURE 4 (*a*) Hypothetical cell population at time of labeling ($t = 0$). Plot of the relative number of cells (in arbitrary units) of age τ, where $\tau = 0$ corresponds to the beginning of the S phase. Cells in the various phases S, G_2, M, and G_1 are separated by vertical lines. Labeled cells are indicated by crosshatching. For convenience in visualizing the system, the G_1 phase (the only one assumed variable in this figure) is indicated on the right. It should be noted that half of all daughter cells leave the system upon division at ($\tau = \tau_1$) and therefore the ordinate remains constant at this point. (*b*) The system at $t = t_0$. α = fraction of labeled cells that have divided by this time; *group* I cells = labeled cells that have divided and *group* II = labeled cells not divided by $t = t_0$.

compartment shortly after t_0 ; some of these with sufficiently short generation times may divide again during the $T_{G_{md}}$ computation period and hence would affect the MGC halving time.

Although cells in *group* II_b at time t_0 will all fall below the 0.5 M grain level and thus will contribute to the decrease in the MGC, this will occur shortly following t_0 and will be independent of their generation times. This means that the generation times of these cells will not affect the $T_{G_{md}}$ estimate.

It may therefore be concluded that the MGC halving time will be determined by the generation times of the *groups* I_b and II_b cells only. So long as assumption 7 holds, however, the resulting $T_{G_{md}}$ estimate will be valid for the entire population. In an actual system, this computed $T_{G_{md}}$ may in fact be a more valid estimate of the entire (labeled plus unlabeled) population than would an estimate affected by all of the labeled cells (e.g. using the *mean* grain count). This is because of the possibilities both of (*a*) radiation damage to cells having the highest grain counts, and (*b*) error at the lowest grain levels due to possible reutilization of label. In addition, the relative grain-count error due to background is higher at lower grain levels.

Step 3 Relationship Between Specified t_0 and Corresponding MGC Halving Time

Define the following:

$N_{I_b}(t_0)$ ≡ number of cells in *group* I_b at $t = t_0$

$N_{II_b}(t_0)$ ≡ number of cells in *group* II_b at $t = t_0$

$N_{T_b}(t_0)$ $\equiv N_{I_b}(t_0) + N_{II_b}(t_0)$

$N_{I_b}^{(1)}(t_0, t_1 - t_0)$ $\equiv$ number of *group* I_b cells that will have divided once during the interval $t_0 \leqq t \leqq t_1$

$N_{II_b}^{(1)}(t_0, t_1 - t_0)$ $\equiv$ number of *group* II_b cells that will have divided at least once during the interval $t_0 \leqq t \leqq t_1$

$N_{II_b}^{(2)}(t_0, t_1 - t_0)$ $\equiv$ number of *group* II_b cells that will have divided twice during the interval $t_0 \leqq t \leqq t_1$

$T_H(t_0)$ $\equiv$ time interval required for the median grain count of the labeled population to decrease to half of its value at t_0.

Since the total number of cells in the range 0.5 M to M grains at time t_0 is equal to $N_{T_b}(t_0)$, the MGC halving time $[T_H(t_0)]$ will be equal to the time required for $N_{T_b}(t_0)$ cells to pass below the 0.5 M grain level.

Therefore, at the time $t_1 = t_0 + T_H(t_0)$, when the MGC of labeled cells has halved, we have

$$N_{I_b}^{(1)}[t_0, T_H(t_0)] + N_{II_b}^{(1)}[t_0, T_H(t_0)] + N_{II_b}^{(2)}[t_0, T_H(t_0)] = N_{T_b}(t_0) = N_{I_b}(t_0) + N_{II_b}(t_0). \quad (1)$$

All the *group* II_b cells will have divided within a short time $[= (1 - \alpha)T_S]$ after t_0; thus $N_{II_b}^{(1)}[t_0, T_H(t_0)] = N_{II_b}(t_0)$ and equation 1 becomes

$$N_{I_b}^{(1)}[t_0, T_H(t_0)] + N_{II_b}^{(2)}[t_0, T_H(t_0)] = N_{I_b}(t_0). \quad (2)$$

This equation defines an implicit relationship between t_0 and $T_H(t_0)$, where t_0 must be within the limits given in step 1. In the next step, $T_H(t_0)$ will be equated to the $T_{G_{md}}$ of the cells specified in step 2, thereby uniquely determining the values of t_0 and α. It should be noted that owing to the assumption that intercellular variability in the durations of the S, G_2, and M phases is negligible, α and t_0 are related by the expression

$$t_0 = \alpha T_S + T_{G_2} + T_M. \quad (3)$$

Step 4. Determination of α and t_0

It was found above that the MGC halving time, $T_H(t_0)$, must satisfy equation 2. In order to relate $T_H(t_0)$ to $T_{G_{md}}$, an expression for the quantity

$$N_{I_b}^{(1)}(t_0, t - t_0) + N_{II_b}^{(2)}(t_0, t - t_0)$$

in terms of $T_{G_{md}}$ will first be obtained. This expression will then be substituted into equation 2 with $T_H(t_0)$ set equal to $T_{G_{md}}$ to determine the required values of t_0 and α.

Refer again to Fig. 4*b*. All the labeled cells of ages $\tau \leqq \tau_1$ at $t = t_0$ (*group* II) will have divided once within a time interval $(1 - \alpha)T_S$. The numbers, both of

these cells that will have divided a second time and of cells with $\tau > \tau_1$ at $t = t_0$ (*group* I) that will have divided once by the time t, are given by the numbers of such cells with generation times shorter than $t - t_0 - \tau_1 + \tau$.

Let the probability distribution of generation times for cells in the system be assumed continuous and denoted by $f(x)$, where x is the generation time and

$$\int_0^\infty f(x)\,dx = 1.$$

The age densities of *groups* I_b and II_h at time t_0 are identical and equal to a constant (assumptions 2 and 7); call this constant k. Then

$$N_{I_b}^{(1)}(t_0, t - t_0) + N_{II_h}^{(2)}(t_0, t - t_0) = \int_{\tau_1-(1-\alpha)T_s}^{\tau_1+\alpha T_s} k \int_0^{t-t_0-\tau_1+\tau} f(x)\,dx\,d\tau. \quad (4)$$

Let $t - t_0 = T_{G_{md}} + \delta$; then equation 4 becomes

$$\begin{aligned} N_{I_b}^{(1)}(t_0, T_{G_{md}} + \delta) &+ N_{II_h}^{(2)}(t_0, T_{G_{md}} + \delta) \\ &= k \int_{\tau_1-(1-\alpha)T_s}^{\tau_1+\alpha T_s} \int_0^{T_{G_{md}}+\delta-\tau_1+\tau} f(x)\,dx\,d\tau \\ &= k \left[\int_{\tau_1-(1-\alpha)T_s}^{\tau_1-\alpha T_s} \int_0^{T_{G_{md}}+\delta-\tau_1+\tau} f(x)\,dx\,d\tau \right. \\ &\quad \left. + \int_{\tau_1-\alpha T_s}^{\tau_1+\alpha T_s} \int_0^{T_{G_{md}}+\delta-\tau_1+\tau} f(x)\,dx\,d\tau \right]. \end{aligned} \quad (5)$$

The second integral in this expression becomes

$$\begin{aligned} \int_{-\alpha T_s}^{\alpha T_s} \int_0^{T_{G_{md}}+\delta+\tau} f(x)\,dx d\tau &= \int_0^{\alpha T_s} \left[\int_0^{T_{G_{md}}+\delta+\tau} f(x)\,dx \right. \\ &\quad \left. + \int_0^{T_{G_{md}}+\delta-\tau} f(x)\,dx \right] d\tau = \int_0^{\alpha T_s} \left[2\int_0^{T_{G_{md}}} f(x)\,dx \right. \\ &\quad + \int_{T_{G_{md}}}^{T_{G_{md}}+\tau} f(x)\,dx + \int_{T_{G_{md}}}^{T_{G_{md}}-\tau} f(x)\,dx \\ &\quad \left. + \int_{T_{G_{md}}+\tau}^{T_{G_{md}}+\tau+\delta} f(x)\,dx + \int_{T_{G_{md}}-\tau}^{T_{G_{md}}-\tau+} f(x)\,dx \right] d\tau. \end{aligned} \quad (6)$$

The sum of the second and third integrals of the last expression becomes

$$\int_0^\tau [f(T_{G_{md}} + x) - f(T_{G_{md}} - x)]\,dx; \quad (7)$$

the sum of the fourth and fifth is

$$\int_0^\delta [f(x + T_{G_{md}} + \tau) + f(x + T_{G_{md}} - \tau)]\,dx. \quad (8)$$

If expression 6 (with 7 and 8 substituted) is substituted into expression 5, the result is

$$N_{I_b}^{(1)}(t_0, T_{G_{md}} + \delta) + N_{II_b}^{(2)}(t_0, T_{G_{md}} + \delta)$$
$$= k\int_{\tau_1-(1-\alpha)T_s}^{\tau_1-\alpha T_s}\int_0^{T_{G_{md}}+\delta-\tau_1+\tau} f(x)\,dx\,d\tau + 2k\int_0^{\alpha T_s}\int_0^{T_{G_{md}}} f(x)\,dx\,d\tau$$
$$+ k\int_0^{\alpha T_s}\int_0^{\tau}[f(T_{G_{md}} + x) - f(T_{G_{md}} - x)]\,dx\,d\tau$$
$$+ \int_0^{\alpha T_s}\int_0^{\delta}[f(x + T_{G_{md}} + \tau) + f(x + T_{G_{md}} - \tau)]\,dx\,d\tau. \quad (9)$$

Because $f(T_{G_{md}} + x) = f(T_{G_{md}} - x)$ for all x (assumption 3), the third integral in equation 9 vanishes. Using the same assumption, it follows that $\int_0^{T_{G_{md}}} f(x)\,dx = 0.5$ and therefore the value of the second integral is $k\alpha T_S$. Finally, at a time equal to one median generation time following t_0 ($t = t_0 + T_{G_{md}}$), $\delta = 0$ and the last integral is equal to zero. Equation 9 then reduces to

$$N_{I_b}^{(1)}(t_0, T_{G_{md}}) + N_{II_b}^{(2)}(t_0, T_{G_{md}})$$
$$= k\int_{\tau_1-(1-\alpha)T_s}^{\tau_1-\alpha T_s}\int_0^{T_{G_{md}}-\tau_1+\tau} f(x)\,dx\,d\tau + k\alpha T_S. \quad (10)$$

The number of *group* I_b cells originally present at time t_0 was $N_{I_b}(t_0) = k\alpha T_S$; if we now require that $T_H(t_0) = T_{G_{md}}$, equation 10 becomes

$$N_{I_b}^{(1)}[t_0, T_H(t_0)] + N_{II_b}^{(2)}[t_0, T_H(t_0)]$$
$$= k\int_{\tau_1-(1-\alpha)T_s}^{\tau_1-\alpha T_s}\int_0^{T_{G_{md}}-\tau_1+\tau} f(x)\,dx\,d\tau + N_{I_b}(t_0). \quad (11)$$

Comparing equation 11 with 2, it is evident that $k\int_{\tau_1-(1-\alpha)T_s}^{\tau_1-\alpha T_s}\int_0^{T_{G_{md}}-\tau_1+\tau} f(x)\,dx\,d\tau = 0$. Because the integral $\int_0^{T_{G_{md}}-\tau_1+\tau} f(x)\,dx$ is not in general equal to zero, the following relationship must hold: $\tau_1 - (1-\alpha)T_S = \tau_1 - \alpha T_S$, or $\alpha = 0.5$. It can therefore be concluded that under the conditions given, the median generation time of the cells specified will be equal to the MGC halving time provided that the starting MGC is taken at the moment when half the labeled cells have passed through mitosis. Utilizing equation 3, this means that $t_0 = 0.5T_S + T_{G_2} + T_M$.

DISCUSSION

The preceding result would seem to resolve the problem of determining the median generation time of the labeled cell population. However, questions arise with respect to its applicability to an actual system in which the various assumptions may be satisfied only approximately if at all.

Of course the accuracy of the procedure cannot be guaranteed unless the assumptions are valid; however not all of them are crucial. Furthermore, some of them can be tested directly or indirectly.

The first assumption, that the system be self-maintained, precludes the existence of precursor or "stem" cells. Although the existence of multipotent hemopoietic stem cells has been demonstrated (Wu et al., 1967), it may be that in many cases, the stem cells are ordinarily in a "resting" or G_0 phase and do not supply the system unless it becomes depleted. Even if there is a steady influx of precursor cells, however, their relative contribution to the cell type under investigation could well be negligible.

Another source of error is the possible presence of a nonproliferating fraction of cells (Mendelsohn, 1962). If some of the labeled cells enter a nonproliferating state (from which they presumably do not reenter the cell cycle), this would be expected to lead to an overestimate of the median generation time of the proliferating cells. One might expect that the presence of such a nonproliferating state could be detected simply by noting whether the initial labeling index were equal to the ratio of T_S (obtained by an independent method) to $T_{G_{md}}$. This, however, is subject to error because of the variability of generation times (Quastler, 1963); depending on the actual values of T_S and $T_{G_{md}}$ as well as on the shape of the generation time distribution, the labeling index might be less than, equal to, or greater than the ratio $T_S/T_{G_{md}}$. Unless either the T_G distribution were known, or the ratio of T_S to T_G were the same for all cells, the test would not be very reliable for determining the presence of nonproliferating cells.

Even if such a nonproliferating fraction were present, the computed $T_{G_{md}}$ might be a valid estimate for the entire labeled population if one regards the cells entering the nonproliferating compartment as possessing an infinite generation time. The accuracy of the result would depend, however, on whether the rate at which cells enter this compartment were small compared with the rate of cell passage through the mitotic cycle. If this is the case, then the relative number of labeled cells entering the nonproliferating fraction during the period of the $T_{G_{md}}$ computation would be small (even if the size of this fraction as a whole were large) and the resulting generation time estimate might be quite accurate.

If the fraction of cells entering the nonproliferating compartment upon division were large, however, assumption 8 might be violated. This would probably result in a generation time estimate larger than the true $T_{G_{md}}$ of the proliferating fraction, but smaller than that of the "combined" labeled population.

The validity of assumption 2 could be tested in part by noting whether the labeling index increases subsequent to the input of label. This of course is dependent on the validity of assumption 9 that the available label has been utilized or degraded by the time the first labeled cells have reached mitosis. If the interphase labeling index then increases in correlation with the first wave of labeled mitoses, it is reasonable to infer that both daughter cells reenter the cycle after division.

Unless this is merely a transient phenomenon (as indicated by a rapid decrease of the labeling index to its initial level), this would invalidate assumption 2. The major error in applying the procedure to such a system would be in the estimate of the labeling index correction factor. Because of the relatively short period during which the correction is applied, however, it would in many cases be of small magnitude and cause little error.

The symmetry of the T_G distribution was required to insure that *group* I cells with generation times too long to have divided during the interval

$$t_0 \leqq t \leqq t_0 + T_{G_{md}}$$

would be balanced by *group* II cells with generation times sufficiently short to have divided twice within this interval. If the distribution were not symmetrical, but skewed to the right or left (towards longer or shorter T_G's, respectively), then these two groups would not in general be equal at $t = t_0 + T_{G_{md}}$, and the MGC halving time would be respectively longer or shorter than the true $T_{G_{md}}$.

If the distribution were bimodal, the effects of asymmetry would be aggravated; in fact, even a symmetrical bimodal distribution might lead to analogous error.

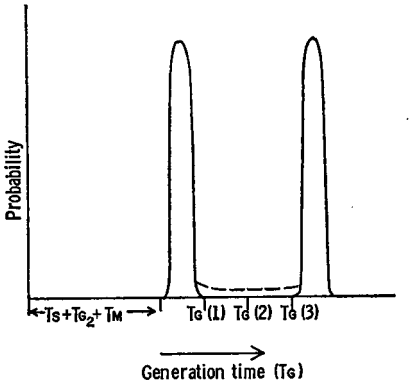

FIGURE 5 Example of a bimodal probability distribution of generation times. The median is not unique unless the dashed line forms part of the distribution; if so, the median is equal to $T_G(2)$. It should be noted, however, that very minor changes in the shape of the distribution could shift the median to any point between $T_G(1)$ and $T_G(3)$.

These points can be illustrated with reference to the bimodal generation time distribution of Fig. 5. Although the median of such a distribution would not be a very meaningful parameter with which to characterize the cell population, this case is considered here simply to illustrate the limit of the error that may be encountered.

First, assume that the solid line represents the actual distribution, with the two peaks having equal areas. It is evident that the $T_{G_{md}}$ is not unique; any value between $T_G(1)$ and $T_G(3)$ will be a median of the distribution. If the "desired" median generation time is $T_G(2)$, then the suggested method for determining $T_{G_{md}}$ would not in general give this desired result.

If the dashed line in Fig. 5 formed part of the distribution, the median would then be unique, and the method would in theory yield the correct value of $T_{G_{md}}$ [$= T_G(2)$].

In practice, however, the plot of the median grain count of such a system would probably be indistinguishable from the preceding one, yielding a similar $T_{G_{md}}$ estimate.

Furthermore, by making very slight changes in the shape of the distribution, the true median could be shifted to any point between $T_G(1)$ and $T_G(3)$ without appreciably altering the computed $T_{G_{md}}$. The location of the true $T_{G_{md}}$ at $T_G(1)$ or $T_G(3)$ might represent limiting cases of right-handed or left-handed "skewness." (Since the illustrated distribution is bimodal, it might be preferable to refer to "positive or negative third moments about the median" rather than "right-handed or left-handed skewness.")

In order to estimate the maximum possible error that might result from the invalidity of the assumptions of unimodality and symmetry about the median (the other assumptions will still be considered valid), refer to Figs. 4 and 5. In the following discussion it will be assumed that changes in the T_G distribution required to shift the median betweeen $T_G(1)$ and $T_G(3)$ are sufficiently slight so that the computed value of the $T_{G_{md}}$ will be unchanged.

During the time interval $t_0 \leqq t \leqq t_0 + T_G(1)$, all of the *group* II cells, plus approximately half of the *group* I cells, will divide. Half of the second generation *group* II cells will then have divided by the time $t = t_0 + T_G(1) + 0.5\ T_S$. Because the T_G distribution and the initial ($t = 0$) grain distribution were assumed independent of age (τ) and of each other (assumption 7), it follows that the MGC will have halved by this time. The computed $T_{G_{md}}$ will therefore be $T_G(1) + 0.5\ T_S$ (unless $T_G(3) - T_G(1) < 0.5\ T_S$, in which case the estimated median will be approximately equal to $T_G(3)$).

If the true median were at $T_G(1)$, it would thus be overestimated by at most $0.5\ T_S$. If the median were $T_G(3)$, it would be underestimated by the amount $T_G(3) - T_G(1) - 0.5\ T_S$ if $T_G(3) - T_G(1) > 0.5\ T_S$; otherwise the computed median would be correct. Since $T_G(1) > T_S + T_{G_2} + T_M$, the median could be underestimated by at most the median duration of the G_1 phase, less $0.5\ T_S$.

Finally, if the actual median were between the values $T_G(1)$ and $T_G(3)$, the indicated method for determining $T_{G_{md}}$ might result in either an overestimate or an underestimate, depending on the values of $T_G(1)$, $T_G(3)$, T_S, and $T_{G_{md}}$ itself.

If the distribution of generation times were asymmetrical but unimodal, the error would be qualitatively similar to the above cases, but of much smaller magnitude. In practice, it is much more likely that the distribution would be skewed to the right rather than the left; several investigators have in fact found this to be the case (Dawson, Madoc-Jones, and Field, 1965; Kubitschek, 1962). Since assumption 8, if valid, would limit the degree of skewness, it appears likely that the method presented here will overestimate the true $T_{G_{md}}$ by an amount significantly less than $0.5\ T_S$.

The preceding discussions were based on the tacit understanding that the median grain count would be determined sufficiently frequently to allow detection of sig-

nificant changes at the moment of their occurrence. In fact, this is likely to be true only during the early period after labeling; hence the uncertainty as to the correct value of $T_{G_{md}}$ will also be dependent on the time interval between samples.

It was mentioned in the Introduction that in certain situations it is impossible to obtain a plot of even the first wave of labeled mitoses. If not, one cannot determine the starting time t_0 of the $T_{G_{md}}$ computation interval by the method indicated.

An alternate method would be to plot the *mean* grain count of interphase cells following the injection of label. This curve would be expected to decrease linearly by a factor of two between the times that the first and last labeled cells pass through mitosis. The proper value of t_0 can then be taken as the time that the mean grain count has fallen to 0.75 of its maximum value. This method has the disadvantage that the mean cannot be determined accurately if many cells have high grain levels; furthermore, such cells may be damaged by the presumably high level of radioactivity and may fail to divide. The *median* grain count, while less affected by these drawbacks, may possibly decrease to half of its initial value prior to the first passage of all the labeled cells through mitosis.

Another possible method would be to simply note the interval during which the interphase grain distribution shifts towards lower grain values in the period following the administration of label. This interval would be associated with the passage of cells through mitosis, and t_0 would coincide with the moment when this process was judged to be half completed.

These alternate procedures, although probably satisfactory in many cases, are not as precise as the preferred method of utilizing the first wave of labeled mitotic cells. They are also more critically dependent on the validity of certain of the assumptions listed earlier.

An important matter which has not been considered here is the determination of the T_G distribution function. Although the variance, if not the skewness, could at least be determined with fair accuracy if a second peak could be detected on the labeled mitoses curve (Barrett, 1966; Takahashi, 1966), these methods are not applicable in the present case. If, however, it could be assumed that the failure to detect the second peak was caused by loss of synchrony (with respect to labeling) owing to a large variance of generation times, a lower bound for the variance might be obtained.

Another approach would be to hypothesize a detailed mathematical model of the system, based upon either the assumptions listed here or on modifications of them. By comparing the grain distributions predicted by this model (using a range of values of parameters representing the second and third moments of the generation time distribution) with the observed distribution, it might be possible to obtain a reasonably accurate estimate of the variance and shape of the probability distribution of generation times.

A possible pitfall inherent in this method is that minor, uncontrollable differences

in the preparation of the successive samples for autoradiographic analysis may lead to significant (random) changes in the second and third order moments of the grain count distribution. These changes, if not erroneously attributed to variability in the generation time distribution, may nevertheless make it difficult or impossible to separate variability resulting from technique from that owing to true differences in the proliferative behavior of the cells.

EXAMPLE: ESTIMATE OF GENERATION TIMES IN ACUTE LEUKEMIA

The results of the preceding sections will now be applied to two cases of acute leukemia in man. These cases have been described and analyzed previously (Clarkson et al., 1967) and a number of proliferation parameters were estimated. The generation time estimates were based on the least-squares fit of a straight line to the semilog plot of the median grain count data, as discussed earlier.

The same data will now be analyzed by the method presented here.

The experimental procedure, described in detail in the reference cited, was as follows: 20 mc of tritiated thymidine (6.6 c/mmole) were injected intravenously; smears were then made from bone marrow aspirates at subsequent intervals. Autoradiographs were made using Kodak AR10 stripping film (Eastman Kodak Co., Rochester, N. Y.). Cells having fewer than five grains were classified as unlabeled.

Patient I. M. T.

Fig. 6*a* shows the labeling indexes of mitotic and interphase blasts vs. time; Fig. 6*b* is a plot of the median grain count (corrected for labeling index changes) of interphase blasts.

The starting time t_0 for the computation of $T_{G_{md}}$ will be taken as the point halfway between the 50% levels on the rising and falling portions of the first wave of labeled mitoses. This is computed as $t_0 = 11.4$ hr. From the interphase MGC data and using linear interpolation, the starting "median" grain count is 16.6; the 8.3 grain level occurs at $t = 87.2$ hr. Therefore, the estimated $T_{G_{md}}$ is about 76 hr. If the distribution of generation times were skewed to the right (towards longer T_G's), the actual $T_{G_{md}}$ would be somewhat less than this; under the worst possible case of skewness, the error would be equal to half the duration of S ($T_S = 19$ hr in this patient) and $T_{G_{md}}$ would then be about 66 hr.

This estimate compares with the value of 83 hr (67% confidence interval = 70–102 hr) obtained by the "semilog plot" method (Clarkson et al., 1967, 1968). This confidence interval refers to uncertainty in the true slope of the semilog plot. It might be noted that the proposed method for obtaining $T_{G_{md}}$ does not assume an exponential time dependency; hence this source of uncertainty is not introduced.

Some qualifications to the application of the proposed method to patient M. T. should be noted:

(*a*) The observation (Fig. 6*a*) that the labeling index (*LI*) of interphase blasts

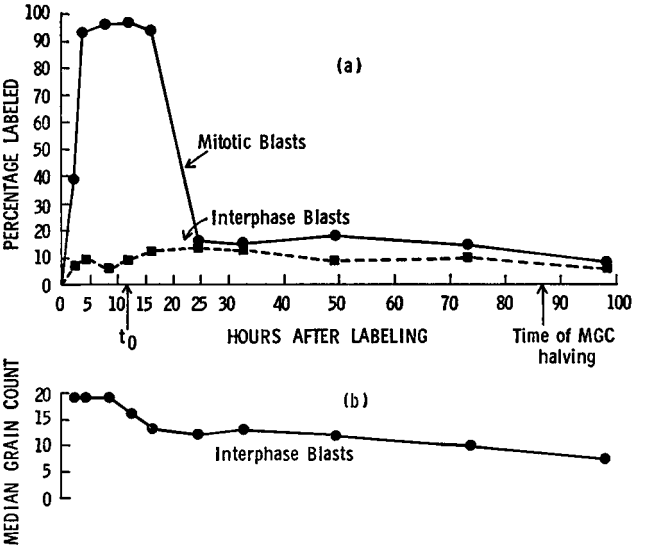

FIGURE 6 Patient I. (M. T.) (*a*) Labeling indexes of mitotic and interphase blasts vs. time. (*b*) Median grain count (corrected for labeling index changes) of interphase blasts.

continues to increase while the labeled cells are passing through mitosis supports the hypothesis that both daughters remain in the system for a time after division. This means that assumption 2 is not satisfied. One error resulting from this is that the threshold correction factors, based on the interphase labeling index observations, will be incorrect. In particular, no correction whatsoever would be made while the labeling index is increasing, although some cells will be falling below the threshold during this time.

(*b*) As stated in the Discussion, discrepancy between the observed labeling index (*LI*) and that predicted from the relationship $LI = T_S/T_{G_{md}}$ does not necessarily imply that a nonproliferating cell fraction is present. In the present case, however, the great discrepancy between the two values (6% vs. 25%, respectively) does seem to imply either that there is a large nonproliferating fraction, or that many of the blasts have generation times so much longer than the median that assumption 8 is violated. This would be expected to result in an overestimate of the true $T_{G_{md}}$.

Patient II. R. R.

Figs. 7*a* and *b* show plots of the labeling indexes of mitotic and interphase blasts, and the median grain count of interphase blasts.

It should be noted that in this patient, the median grain count dips unexplicably at the 12 hr sample. Furthermore, the *LI* rises to four times the initial level after about a day. Although it is possible that these phenomena arise from varying degrees of blood dilution of the marrow aspirates (the initial blood *LI* was extremely low and the average grain count of these few labeled cells was very high), there is no

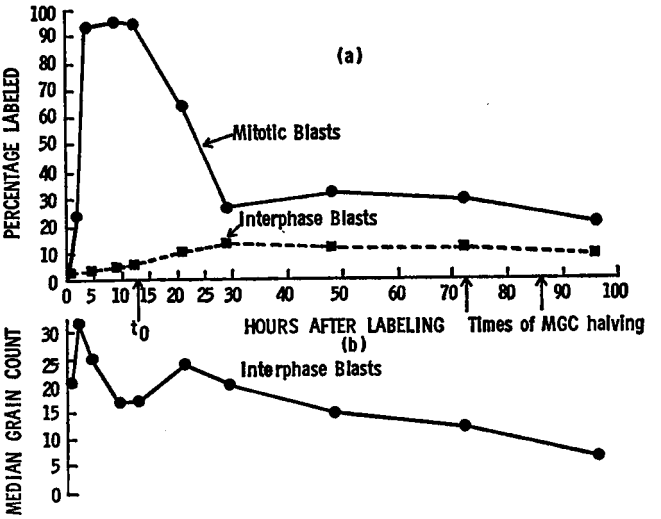

FIGURE 7 Patient II. (R. R.) (*a*) Labeling indexes of mitotic and interphase blasts vs. time. (*b*) Median grain count (corrected for labeling index changes) of interphase blasts.

direct evidence for this. Whatever the cause, it appears unlikely that the proposed method can be applied with any degree of confidence to this case. Nevertheless, an estimate will be made for purposes of comparison.

The initial time t_0 is determined to be 13.2 hr. Although the MGC is 17 at this time, it subsequently increases to a maximum of 24 grains. It seems preferable to choose 24 as the starting MGC; however, $T_{G_{md}}$ estimates will be made using both values.

For a starting MGC of 24, the median halves by 72 hr, yielding $T_{G_{md}} = 59$ hr. If the starting median were 17 grains, the halving time would be $T_{G_{md}} = 73$ hr. Since $T_S = 22$ hr, asymmetry in the generation time distribution might cause an error of up to 11 hr.

By the earlier method of computing generation times, the estimate was 49 hr, with a 67% confidence interval of 42–60 hr. Because of the unresolved peculiarities in the grain count and labeling index curves for this patient, however, it cannot be determined which of the above $T_{G_{md}}$ estimates is more accurate.

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