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Do Cells Cycle?

(cell kinetics/control of growth/DNA replication/cell culture)

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ABSTRACT **We propose that a cell's life is divided into two fundamentally different parts. Some time after mitosis all cells enter a state (A) in which their activity is not directed towards replication. A cell may remain in the A-state for any length of time, throughout which its probability of leaving A-state remains constant. On leaving A-state, cells enter B-phase in which their activities are deterministic, and directed towards replication. Initiation of cell replication processes is thus random, in the sense that radioactive decay is random. Cell population growth rates are determined by the probability with which cells leave the A-state, the duration of the B-phase, and the rate of cell death. Knowledge of these parameters permits precise calculation of the distribution of intermitotic times within populations, the behavior of synchronized cell cultures, and the shape of labeled mitosis curves.**

Subdivision of the intermitotic period into G_1, S, and G_2 (1) has stimulated attempts to analyze the processes controlling the progression of cells from one mitosis to the next. Our aim is to show that much of the information gathered may require reinterpretation because of a fundamental misconception about the nature of G_1.

Analyses of the fraction of labeled mitoses (FLM) at various times after exposure to a pulse of [^{3}H]thymidine ([^{3}H]dT) demonstrate that the durations of S and G_2 are characteristic of particular cell types, and usually do not show much intrapopulation variation (2). Both phases can be regarded as deterministic and specifically related to division. However, the duration of G_1 is extremely variable, both between different cell types and within homogeneous populations (3), and this variability accounts for most of the variation of the intermitotic period. Changes in generation time are also usually attributed to changes in G_1 duration. G_1 has therefore attracted particular interest as the period in which proliferation is regulated. Implicit in the cell-cycle concept is the idea of continuous progression through a chain of events leading to division, for "cycle" implies "an interval during which one sequence of a regularly recurring succession of events is completed" (Webster's Dictionary). There are some rapidly proliferating cells, for which the concept seems appropriate, but even here the distribution of intermitotic times has a curiously wide spread. Applied to slowly proliferating cells, the concept is confused, for although G_1 is regarded as an extensible progression of events leading towards division, the experimental data force the conclusion that this progress can be arrested at some stage or stages (4). Reexamination of published data suggests an alternative interpretation of the nature of G_1. Most of the relevant data are about the *frequency* of division. Now a frequency may reflect a regularly recurring process or it may reflect events occurring randomly with a certain probability (e.g., radioactive decay). Pursuing this thought, we arrived at the model illustrated in Fig. 1.

We propose that the intermitotic period is composed of an A-state and a B-phase. The B-phase includes the conventional S, G_2, and M phases. Whether it also includes part of G_1 is discussed below. Some time after mitosis the cell enters the A-state, in which it is not progressing towards division. It may remain in this state for *any* length of time, throughout which its probability of entering B-phase is constant. This "transition probability" (P) may be supposed to be a characteristic of the cell type but capable of modification by environmental factors.

Distribution of Generation Times. The proposed hypothesis *predicts* wide variation in the duration of G_1 within populations. This variability has had to be considered when trying to estimate mean cycle times, etc., and considerable effort has gone into determining its statistical distribution. The data have not led to any precise formulation, and the most that can be claimed is that the distribution is usually skewed to the right and the variance is large (2). Nevertheless, information about individual generation times can be used to decide whether a "probability" model is reasonable. If the intermitotic period were of uniform duration in a population, the cells would all divide at the same age. If the proportion (α) of the initial population (N) remaining in interphase were plotted against age, Fig. 2*A* would result. If intermitotic times were normally distributed, Fig. 2*B* would result. This curve, plotted with a logarithmic ordinate (Fig. 2*C*) shows that the probability of division increases continuously with age. This result is implicit in the cell-cycle concept. However, if the population were characterized by a transition probability (P) and a B-phase of duration T_B, α would decline expo-

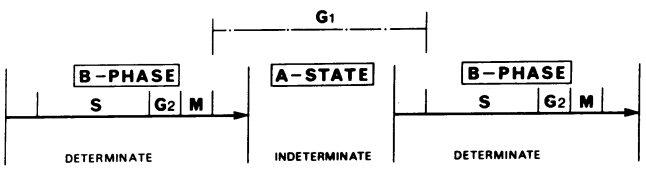

FIG. 1.

Abbreviations: G_1, the interval between mitosis (M) and DNA synthesis; S, the period of DNA synthesis; G_2, the interval between S and M; T_x, the duration of a period x; FLM, the fraction of labeled mitoses; LI, labeling index.

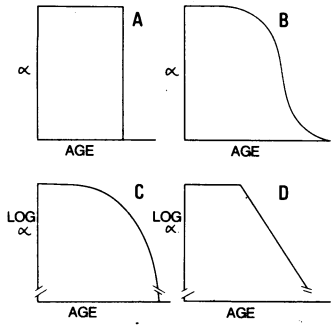

FIG. 2. The proportion of cells remaining in interphase as a function of age. Explanation in text.

$$\alpha = [N - (\text{no. cells already divided})]/N \quad [1]$$

nentially beginning at time T_B. On a logarithmic ordinate this gives a straight line (Fig. 2*D*), and the probability of division remains constant.

Fig. 3 shows the frequency distributions of intermitotic times for various cells obtained by time-lapse cinematography of exponentially growing cultures 6–10. We have expressed these data as log α against age. All show exponential decay after a lag, demonstrating that initiation of cell replication processes occurs at random and not at the end of a "regularly recurring succession of events." The term "cell cycle" is therefore inappropriate.

Experimental Determination of T_B and P. The above examples were rapidly proliferating cells. No similar studies seem to have been attempted with slowly growing populations. However, if one assumes that the transition probability can take any value and is environmentally modifiable, the model can be used to describe many types of proliferative behavior. P clearly *is* variable in different populations, as is T_B (see above). That both are also subject to environmental modification can be found from data (11) for *Euglena* grown in different culture conditions. Clearly T_B and P are of great significance for the kinetic analysis of growth. Both can be obtained by the method described above where

$$P = [(\alpha_t - \alpha_{t+\Delta t})/\Delta t]/\alpha_t \quad [2]$$

The data given below in Fig. 3 do not fit the predicted curve (Fig. 2*D*) exactly, since there is an initial downward curvature before linearity is reached. This curvature is due to

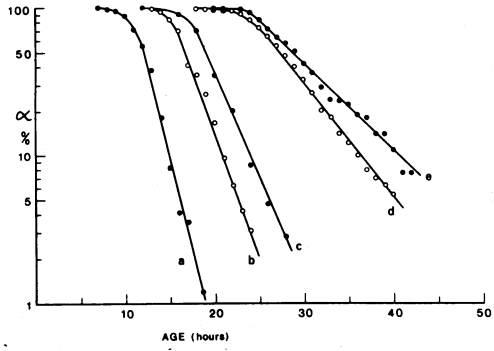

FIG. 3. Distribution of generation times of various cell types in culture, (*a*) rat sarcoma (6); $P(hr^{-1}) = 0.45$; $T_B(hr) = 9.5 \pm 1.0$, (*b*) HeLa S3 (7); $P = 0.32$; $T_B = 14 \pm 0.8$, (*c*) mouse fibroblasts (8); $P = 0.30$; $T_B = 15 \pm 1.2$, (*d*) L5 cells (9); $P = 0.18$; $T_B = 22.5 \pm 1.4$, (*e*) HeLa (10); $P = 0.14$; $T_B = 23 \pm 0.8$.

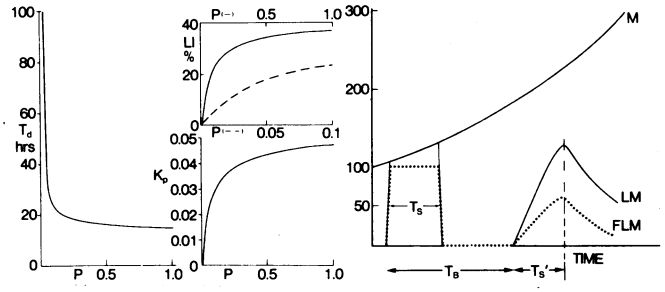

FIG. 4. (*left*) Curves for a hypothetical cell type relating transition probability (P), population doubling time (T_d), labeling index (LI), and cell production rate constant (K_p), calculated with $T_B = 15$ hr and $T_S = 8$ hr, from Eqs. **3**, **4**, **10**, and **12**.

FIG. 5. (*right*) Theoretical basis for the form of FLM curves. After a pulse of [^{3}H]dT, no mitoses will be labeled before time T_G; then, in a period = T_M all mitoses become labeled. Throughout the period T_S, only labeled cells enter mitosis. After this the number of labeled mitoses (LM) falls to zero. By this time the number of labeled *cells* will have doubled, by division. No cells re-enter mitosis before a period = T_B. If LM at time t_i is n, then the number of these cells in mitosis during a later time interval is $2N_{t_i}\{\exp(-K_{trans}[t + 1]) - \exp(-K_{trans}t)\}$ where $t = 0$ when $t - t_i = T_B$. This equation is used to calculate the contribution of successive groups of labeled mitoses in the first peak to the second. It is convenient to take groups at hourly intervals (note that one uses the *area* under the curve). FLM = LM/M. M increases exponentially as $M_0 \exp(K_p t)$.

variation in T_B. The minimum T_B is the time the first cells enter division, and the maximum the time at which the curve becomes linear. If we assume a normal distribution between these limits, the coefficients of variation were about 10%.

T_B and P can also be obtained from cells synchronized by the procedure of Terasima and Tolmach (12), by counting the cells at intervals after seeding. This procedure gives a cumulative curve of generation times that can be treated in the same way as the time-lapse data. These methods can be used only for cells in culture. However T_B can be obtained from FLM curves, provided the second peak is sufficiently well defined (see Fig. 5). T_B is then the time between the beginning of the first and second peaks of labeled mitoses. Then P may be computed from the cell production rate constant (K_p) (13) and T_B.

$$K_p = \ln 2/T_d \quad [3] \qquad K_p = LI/T_s \quad [4]$$

We shall describe the derivation of P in detail. It is simplest if B-phase is all premitotic and cell loss is negligible. Growth is then exponential, and the increase in cell number with time is:

$$N_{t_1} - N_{t_0} = N_{t_0} \exp\{K_p(t_1 - t_0)\} - N_{t_0} \quad [5]$$

where N_{t_1} and N_{t_0} are the number of cells at times t_1 and t_0. At any given time a fraction of the cells, N_A/N, is in the A-state. With constant K_p, N_A/N remains constant, and the increase in N_A is:

$$N_{A_{t_1}} - N_{A_{t_0}} = N_{A_{t_0}} \exp\{K_p(t_1 - t_0)\} - N_{A_{t_0}} \quad [6]$$

For derivation of P it is necessary to know N_A/N as a function of K_p and T_B. Consider an interval $t_1 - t_0 = T_B$. During this time all cells that divide must have been at some stage of

B-phase at time t_0; therefore

$$N_{t_1} - N_{t_0} = N_{Bt_0} = N_{t_0} \exp(K_p T_B) - N_{t_0} \quad [7]$$

By definition $N_B + N_A = N$, so, from [7]

$$\{N \exp(K_p T_B) - N\} + N_A = N$$

Dividing through by N,

$$\{\exp(K_p T_B) - 1\} + N_A/N = 1$$

Rearranged, this becomes

$$N_A/N = 2 - \exp(K_p T_B) \quad [8]$$

(This equation shows that N_A/N is a function of both K_p and T_B, and also, as $K_p \to 0$, $N_A/N \to 1$, i.e., when growth rate is zero, all cells are in A-state. Conversely, when $\exp(K_p T_B) \to 2$, $N_A/N \to 0$, i.e., virtually all cells will be in B-phase when growth is maximal.)

The rate of cell production at time t is $K_p N_t$. Clearly, all the cells dividing at time t must have undergone the A–B transition at time $t - T_B$. If we introduce a rate constant for transition, K_{trans}:

$$K_{trans} N_{A_{t-T_B}} = K_p N_t \quad [9]$$

From [8]

$$N_{A_{t-T_B}} = 2N_{t-T_B} - N_{t-T_B} \exp(K_p T_B) \quad [a]$$

$$N_t = N_{t-T_B} \exp(K_p T_B) \quad [b]$$

Substituting **a** and **b** in [9] and dispensing with subscripts to N

$$K_{trans} = K_p \exp(K_p T_B)/2 - \exp(K_p T_B) \quad [10]^*$$

The cohort of cells in the A-state at time t_0 will decay exponentially according to:

$$N_{A_t} = N_{A_{t_0}} \exp(-K_{trans} t) \quad [11]$$

P equals the proportion of cells "lost" from A-state per unit time:

$$P = 1 - \exp(-K_{trans}) \quad [12]$$

If cell loss occurs randomly, its only effect is to reduce the rates of increase of N and N_A equally. Thus N_A/N remains the same, and the derivation of P is unchanged. In practice, cell loss must be taken into account when K_p is estimated (13, 14).

Fig. 4 shows that when P is high, variations in P have relatively little effect on growth rate. Fine control of growth by varying P would therefore require fairly low Ps. It is interesting that its values, even in rapidly growing cells in culture (Fig. 3), were all below 0.5 hr^{-1}.

Constituents of B-Phase and "Position" of the A-State. The above data say nothing about the position of the A-state, but it is accepted that variation in generation times occurs mainly in G_1. B-phase obviously includes S, G_2, M, and probably some part of G_1. This is so for human amnion cells in which T_B was 16 hr and S + G_2 + M only 9 hr, and in which no cells less than 6 hr old incorporated [^{3}H]dT (5). In

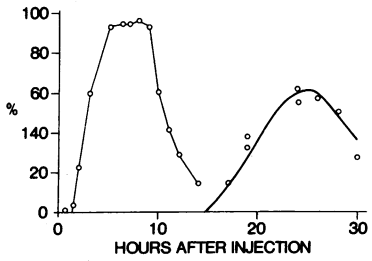

FIG. 6. FLM curve for BICR/M1 tumor (18). Cell loss was negligible. Volume doubling time was 23 hr. Assuming this to equal T_d, $K_p = 0.03\ hr^{-1}$ (Eq. 3). From the experimental points, $T_B = 14$ hr, $K_{trans} = 0.1\ hr^{-1}$ (Eq. 10). The second peak was calculated from successive hourly groups of labeled mitoses in the first peak, as described in Fig. 5.

synchronous cultures, also, some minimum time is required between mitosis and the beginning of S. This part of G_1 could be either a postmitotic period or one immediately preceding DNA synthesis, or both. The need for postmitotic reorganization seems intuitively probable, and there is evidence currently taken to indicate that a significant set of events immediately precedes DNA synthesis (15). This has been regarded as an important phase of the cycle in the regulation of growth. Our thesis, of course, is that the initiation of DNA synthesis is random and not regulated at all in the usual sense. It is nevertheless important to know when the A–B transition occurs, because the sequence of events in B-phase is interesting in its own right and because one wishes to know what occurs at transition. Unfortunately, we cannot find any data enabling us to decide whether B-phase begins before initiation of DNA synthesis or not.

Reinterpretation of FLM Curves. This technique consists in labeling cells in S with a "pulse" of [^{3}H]dT and discovering the time course with which they subsequently pass through mitosis (16). At a time equal to G_2, cells that were at the end of S when labeled enter mitosis; thereafter, for a time equal to T_S, only labeled cells enter M, so the fraction of labeled mitoses (FLM) = 100%. Once cells labeled early in S have passed through mitosis, the FLM falls to zero and remains so until cells labeled late in S again enter mitosis. If the cell cycle is invariant, there follows a peak of labeled mitoses identical with the first, and so on *ad infinitum*. In practice, no such thing happens (16). The first peak of labeled mitoses usually fits reasonably well, but the second seldom if ever reaches 100% and is more widely spread. Such "damping" is always observed and may be of any degree. In extreme cases, no second peak occurs at the expected time (17–19). This result is attributed to variability of cell-cycle time. It is nevertheless maintained that such data yield values for the mean cycle time, measured as the time between the maxima of the first and second peaks. Analysis of this kind of data has reached a high level of sophistication (2, 20–23).

In the hypothesis proposed here the first peak of labeled mitoses is interpreted as before, but thereafter the analysis is different. The cells labeled at the end of S appear first in the FLM curve. Since T_B is the minimum intermitotic time, it is only after this that these cells will again enter mitosis. These cells will contribute to the pool of labeled mitoses at a rate determined by P. Their contribution will rise abruptly after T_B and then decline exponentially. Successive groups will behave in the same way; thus the total of labeled mitoses in

* If B phase is divided into post- and premitotic phases, a different expression for K_{trans} is obtained, but numerical substitution gives the same answer as Eq. 10.

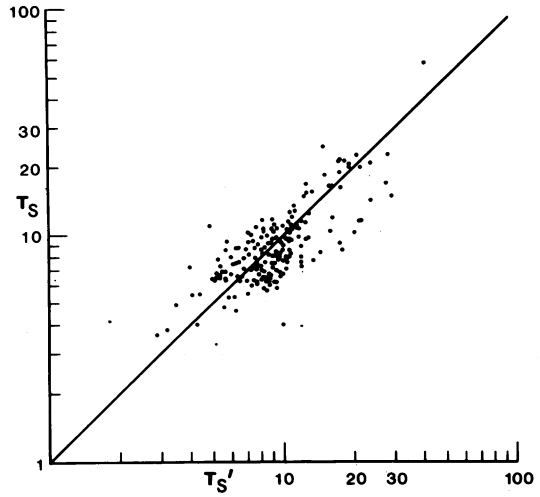

FIG. 7. Correlation between T_S and $T_{S'}$ in FLM curves. Of 348 published, 199 contained sufficient data to estimate both T_S and $T_{S'}$. The *line* shows the theoretical regression if T_B were invariate. Variation in T_B spreads the second peak, thus leading to higher estimates of $T_{S'}$ and lowering the slope of the regression of T_S on $T_{S'}$. Calculated slopes were 0.73 and 0.79 for normal and neoplastic tissues, respectively.

the second peak at any time can be calculated as the sum of a staggered set of curves of this type. The total rises for a period equal to T_S, whatever value P may take; the maximum height of the second peak is, however, a function of P.

Since the distribution of generation times is defined by P and T_B, it follows that FLM curves can be calculated from data obtained by other methods (Fig. 5). In practice it is easier to calculate the second peak from points on the first (Fig. 6). This method also takes some account of variability in B-phase. Published FLM curves confirm the predicted relationship between T_S and the time ($T_{S'}$) taken for the second peak to reach a maximum (Fig. 7).

"Proliferative Pools." Since variation in P alone simply alters the height of the second FLM peak without changing its position, the distance between peaks is not equal to nor a function of "mean generation time." Clearly, we must expect discrepancies between estimates of generation time based on FLM curves and those on, e.g., knowledge of T_S and labeling index. Where growth rate is high the discrepancy may be slight, since as P increases, the FLM curve approaches the "classical." With slowly proliferating tissues, large discrepancies have frequently been observed (13) and interpreted in terms of "resting phase," "proliferative pool," and "growth fraction." Such concepts propose that within morphologically homogeneous populations only some cells are cycling. The remainder are conceived as fertile, "out of cycle," but capable of re-entry after appropriate stimulation. They are sometimes considered to be in a special "G_0" phase (13).

If the existence of the A-state is admitted, it is unnecessary to postulate two distinct modes of behavior for cells in a homogeneous population, and the method commonly used for their detection is invalid. This is not to say that proliferative pools do not exist, and it is worth considering other methods of detection. One is to label cells in S and wait for the labeled group to lose its synchrony. The fraction of labeled mitoses should then approach the labeling index, unless there is a subpopulation of nonproliferative cells (24). A second is to label continuously with [^{3}H]dT. The labeling index will eventually reach 100% unless there is a subpopulation of nondividing cells. There are well-recognized methodological difficulties in both methods (13). In practice the "growth fraction" so measured tends towards unity as the interval between labeling and estimation increases. Although this result is expected, because the "nonproliferating" fraction is supposed to arise from the "decycling" of proliferating cells, it makes the choice of times for estimation of growth fraction arbitrary. In any case, unless "nonproliferating cells" can be shown to be fertile, they may be regarded as moribund.

Our proposal could be regarded as postulating proliferative pools as an inherent property of all cell populations, in that there will always be some cells engaged in processes leading to division and some that are not. Clearly this is not what is usually meant; namely that a distinct subpopulation with zero transition probability coexists with the proliferating population. In attempting to overcome certain difficulties in the "G_0" concept Burns and Tannock (17) proposed a model formally identical to ours. Unfortunately they failed to generalize their model, applying it only to slowly proliferating cells. Neither did they adduce firm evidence in its favor, and their paper has not received the attention it deserves, even in the restricted field to which it was applied.

Synchronous Cell Culture. Our hypothesis makes the kinetics of synchronized cultures predictable and increases their usefulness. Methods for synchronizing cells in culture have been extensively used in the study of biochemical changes through the cycle (25). However, it has not been

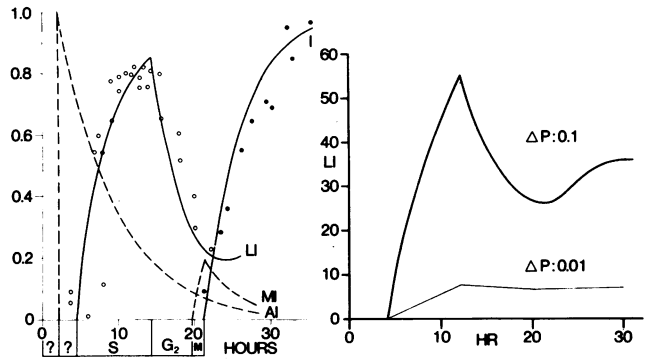

FIG. 8. (*left*) Kinetics of cells synchronized at mitosis. AI is the proportion of cells in A-state. Cells enter A-state some unknown time after mitosis. AI declines according to Eq. 11. Labeling index (*LI*) rises for a period = T_S as $1 - \exp(-K_{trans} \cdot t)$, where t is the time after the *minimum* G_1 period. Thereafter LI is given by $\{1 - \exp(-K_{trans} \cdot t)\} - \{1 - \exp(-K_{trans} \cdot [t - T_S])\}$. Similar curves can be calculated for the mitotic or G_2 indices. The proportional increase in cell number (I) beginning at time T_B is $1 - \exp(-K_{trans}[t - T_B])$. Curves I and *LI* were calculated from data for L5 cells (9). The value of $P = 0.18$ hr^{-1} was obtained from the distribution of intermitotic times (see Fig. 3). T_S was taken as 10 hr, by examination. MI = mitotic index.

FIG. 9. (*right*) Changes in LI when P, initially 0, is increased. Calculation of the first peak is as described in Fig. 8 and of the secondary rise as in Fig. 5, with $T_S = 8$ hr and $T_B = 14$ hr. The lag between stimulation and increased *LI* could be the time required to increase P, a fixed pre-S period, or both.

possible to maintain good synchrony, and attempts to improve synchrony by cloning (26) or fractionation of mitotic cells according to size (27) have failed. The existence of an indeterminate A-state accounts for this failure; the degree of synchrony is a function of P, and complete synchrony is possible only if $P = 1$. After synchronizing of cells in mitosis, the proportions of cells in S, G_2, or M rise to maxima in times equal to the durations of the phases (Fig. 8) because the number of cells entering decreases continuously, while the number leaving at a given time is the number that entered at a previous time equal to the duration of the phase. In Fig. 8, the cells behave as predicted.

Changes in Transition Probability. The growth rate of a population of cells is determined by P, T_B, and the rate of cell loss. It seems likely that variation in P is the major means of regulation. Fig. 9 shows the effects of small and large abrupt changes in P on the [^{3}H]dT labeling index. The larger change produces a quasi-synchronous burst of DNA synthesis. The response to the smaller change is so heavily damped that the labeling index simply rises to a new level. Both kinds of response have been observed (28–31).

Although it is premature to inquire closely into the mechanisms underlying the transition probability, it is pertinent to ask whether changes in P arise from generalized changes in cellular economy or from specific processes that "set" its value. In the simplest case, transition from A-state to B-phase could depend on a critical amount of a single substance, regulated by a number of linked feedback loops. The instantaneous amount of the "initiator" would vary cyclically (32). If the "initiator" were rare, the variation would be subject to considerable biochemical noise, and the threshold would be exceeded at irregular intervals. The mean amount of "initiator" and the amplitude of its fluctuations, and hence P, could be modified in many ways.

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