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# Mapping the Mitotic Clock by Phase Perturbation

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In synchronized V79 cells perturbed by serum, heat shock, or ionizing radiation at half-hour intervals through a modal 8.5-hour cell cycle, phase-response curves show a characteristic biphasic pattern of advances and delays in subsequent cell divisions. These observations, together with previous observations of quantizement of generation times in this and other cell lines have led us to consider a model incorporating, in the simplest case, a two-component oscillator with two threshold crossings required per cell cycle. By assuming that oscillator variables respond in a simple way to the experimental perturbations, for example, by first order destruction due to heat shock, a map of the qualitative features of the oscillator can be obtained by matching simulated with experimental phase response curves. Random fluctuations in oscillator variables about a fixed trajectory lead to subthreshold oscillations and result in a distribution of generation times which is roughly a negative exponential, but quantized within this exponential envelope. The extent of the random fluctuations can be determined from comparison with data on desynchronization of a cell population after mitotic selection. The same parameters which correctly simulate phase response and the desynchronization data also give good agreement with generation time distribution data.

**Key words: cell cycle, transition probability, limit cycle oscillator, generation time, phase response, division delay, cellular clock**

The response of cells to external influences is often strongly cell cycle phase dependent. This fact has encouraged many workers to attempt to obtain information about the cell cycle timekeeping mechanism by observing the phase change in some marker event (usually mitosis) after administration of a perturbing agent. The rationale behind this approach lies in the hope that the chosen perturbation affects the timekeeping mechanism directly, so that the phase response reflects the properties of the underlying clock. However, since the biochemical nature of the clock, and consequently the effects on this clock of any given perturbation, are largely unknown, it is difficult to confidently identify cellular phase responses with clock responses. Some or all of the observed phase shifts, for example, after any given perturbation may be due to defects in cellular functions normally under the control of an underlying clock, and not due to effects on the clock itself.

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More critically, the very notion of a cellular timekeeper has come into question in recent years as a result of observations made on unperturbed cell populations. Individual cell intermitotic times have been cataloged for many different cell types and have been found to be distributed not in a normal fashion about some mean but in a skewed, roughly negative exponential distribution [1–3]. Such observations have been taken as evidence for a stage in the cell cycle from which cells leave in a perfectly random fashion. The introduction of such a purely stochastic element into the cell cycle has led to an appealingly simple model of the cell cycle first proposed by Burns and Tannock [1] and later revived by Smith and Martin [2] in which the cell cycle is divided into two parts, one which cells enter after mitosis and leave randomly at a constant rate per unit time, and a second state through which cells move uniformly, traversing this state in a constant time $T_B$. The random exit from the "A-state" [2] generates the exponential tail in generation time distribution, and the uniform traversal through the "B-phase" produces a shoulder at time $T_B$ in the graph of fraction of cells undivided vs. age (the "$\alpha$-curve").

To counter the objections that cells do appear to be influenced in subsequent generations by their prior history and that the $\alpha$-curves for most cells are not, strictly speaking, exponential, Brooks et al [4] have convoluted the original model by the addition of a second random transition and a second fixed interval state "L."

We have assembled evidence to argue that the cell cycle is timed by a macromolecular oscillator with limit cycle properties [5, 6]. Such a model is supported by a formal mathematical structure and is based empirically on numerous observations of oscillations in enzyme levels and in the rates of RNA and DNA synthesis [7–9]. Stable limit cycle oscillators have been shown to provide a useful representation of metabolic oscillations in glycolytic intermediates [10], of the mitotic clock of Physarum [11], and of circadian rhythms of eclosion in Drosophila [12, 13]. Even so, in attempting to describe a timekeeping oscillator in a biological system the situation is quite unlike that found in engineering or in the physical sciences, where the state variables are usually well defined and measurable. In biological systems the structure of the oscillator must be imputed by perturbing the organism and then observing some manifestation of the underlying oscillator [14]. By constructing a phase response curve – in this instance, the change in time of mitosis between perturbed and control cultures – an assessment of the relative value of the state variables of the underlying oscillator can be performed. Although our model was developed to explain the phase response curves of cells following perturbation, we recognized that it might also be possible to generate an exponential distribution of generation times using a limit cycle oscillator. Our purpose in this paper is to show that such accurate timekeeping can be reconciled with the apparent stochastic properties of the cell cycle, and to deduce some of the qualitative and quantitative properties of the timekeeping mechanisms from the response of cells to phase perturbations.

## MATERIALS AND METHODS

### Cell Culture, Synchronization and Time-Lapse Video Tape Analysis

V79 cells were synchronized by mitotic selection from roller bottles using the automated synchrony system described in detail elsewhere [6]. Cell divisions were scored by continuously monitoring a field (100–200 cells/field) of each of the synchronous cultures using time lapse video tape microscopy at 50- to 100-fold time compression as described previously [15]. Cell divisions were registered by writing the time of occurrence of each

anaphase directly onto the video screen over the dividing cells. For graphical presentation these times were subsequently tallied and grouped into half-hour classes. Modal class values were determined by inspection. Median values were determined by interpolation within class intervals. Intermitotic times were determined in much the same way by following individual cells and their daughters through several generations.

### Phase Response Curves

Phase response curves were generated by determining the midpoints of the first mitotic wave following selection synchrony. Midpoints were compared for each pair of perturbed and control cultures as a function of time in the cycle at which the perturbation was begun. The change in phase, $\Delta\phi$, was calculated as the algebraic sum of the median of the generation time of the control cultures minus the median of the paired perturbed culture, $\Delta\phi = Tgc - Tgp$. Positive values of $\Delta\phi$ indicate that shocked cultures divided sooner than the controls, while negative values indicate that division occurred later than the controls.

## RESULTS

### Dynamics of the Oscillator

Several models of oscillating chemical reactions which generate limit cycles in the X,Y phase plane have been described. In our stimulations we have tried different reaction schemes and have found that the qualitative behavior of our model system is not strongly dependent on the particular scheme used. Results presented here use the trimolecular model of Prigogine and Lefevre [16], which has since been called the Brusselator [17]. It can be represented by the following reaction scheme:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X$$

$$B + X \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Y + D$$

$$2X + Y \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 3X$$

$$X \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}}$$

In this system the "precursors" A are assumed to be in vast excess, and therefore $k_1 \simeq 0$. It is also assumed that the final "products" D and E are instantly removed from the vicinity and therefore that effectively $k_{-2} = k_{-4} = 0$, and that $k_{-3} = 0$. If for further simplicity the remaining forward rate constants are set equal to 1 then the dynamical properties of the oscillator are given by the set of differential equations

$$dX/dt = A - BX + X^2 Y - X$$
$$dY/dt = BX - X^2 Y$$

The steady state of this system is given by the equations

$$O = A - (B+1)X + X^2Y$$

$$O = BX - X^2$$

from which we obtain the solutions X = A and Y = B/A. Assuming A and B are both positive as required by the chemical equations, the term under the radical is always less in magnitude than $B - A^2 - 1$ or is negative. Therefore, if $B - A^2 - 1$ is greater than zero the steady state is unstable. For $B > A^2 + 1$ stable oscillations of the variables X and Y are obtained. A detailed analysis of this system will be presented elsewhere.

### General Behavior of the Oscillator

Two extreme forms of the Brusselator are shown in Figures 1A and B. As the value of A increases such that $A^2 \rightarrow B-1$, oscillations become more sinusoidal and the time required for initial values of X and Y to approach the limiting values likewise increases. Such an oscillator is described as "soft" and perturbations to the system may be righted only after multiple loops or oscillator periods are accomplished. This is in contrast to the situation where A decreases such that $A^2 \ll B - 1$, causing the oscillator to assume properties much like a relaxation oscillator. Such a "hard" oscillator is righted almost immediately following peturbation. If cellular timekeeping were accomplished by means of a soft oscillation, then long, multiple cycle delays in cell division would be expected for appropriate choice of time and intensity of the perturbing stimulus. Alternatively, if a hard oscillation is involved, delays longer than one cycle should be difficult to achieve. Work to date suggests that if there is an oscillator it is more hard than soft. We assume that cells travel around a limit cycle shown in Figure 1B with a critical event in the cycle being triggered when Y exceeds a threshold value $\theta$. We assume further that cells are not confined strictly to the locus of the cycle; rather they travel around the cycle in a cloud. If the uppermost part of the cloud is only slightly above the triggering threshold, then the cells in the lower part of the cloud will fail to reach threshold and will not proceed through the cycle. If these cells continue to be distributed in a random cloud, some fraction will have the possibility of skipping yet another threshold event in the next cycle. A phase plane trajectory of the oscillator used in this study is shown in Figure 1C. For high threshold values of $\theta$, and sufficiently large random walk parameters, $D_x$ and $D_y$, the cloud may be broadened enough so that only a small fraction of cells will cross threshold. In Figure 1C the path of a single cell and one of its daughters following each division is tracked through a total of five cell divisions. A cell may pass below threshold in a given cycle and may therefore require two or more circuits before exceeding threshold, as shown. The cell cycle generation time distribution will be polymodal, with peaks at multiples of the limit cycle time, but the envelope enclosing these peaks will be exponential or nearly so. Further, for cells with long generation times the cloud of cells may be very broad and can yield a distribution which is very nearly a simple exponential.

### The Two-Loop Model

The above discussion has been limited to the case of one limit cycle period per cell cycle. However, our observations of subcyclic phenomena suggests that more than one loop is required per cycle. We envision that X and Y are macromolecules, most probably proteins, with constants for synthesis and degradation of a value to give oscillations with a nominal 4-hr period. The values of such constants and the half times required for synthesis and degradation to give such a periodicity are in agreement with values generally seen in animal

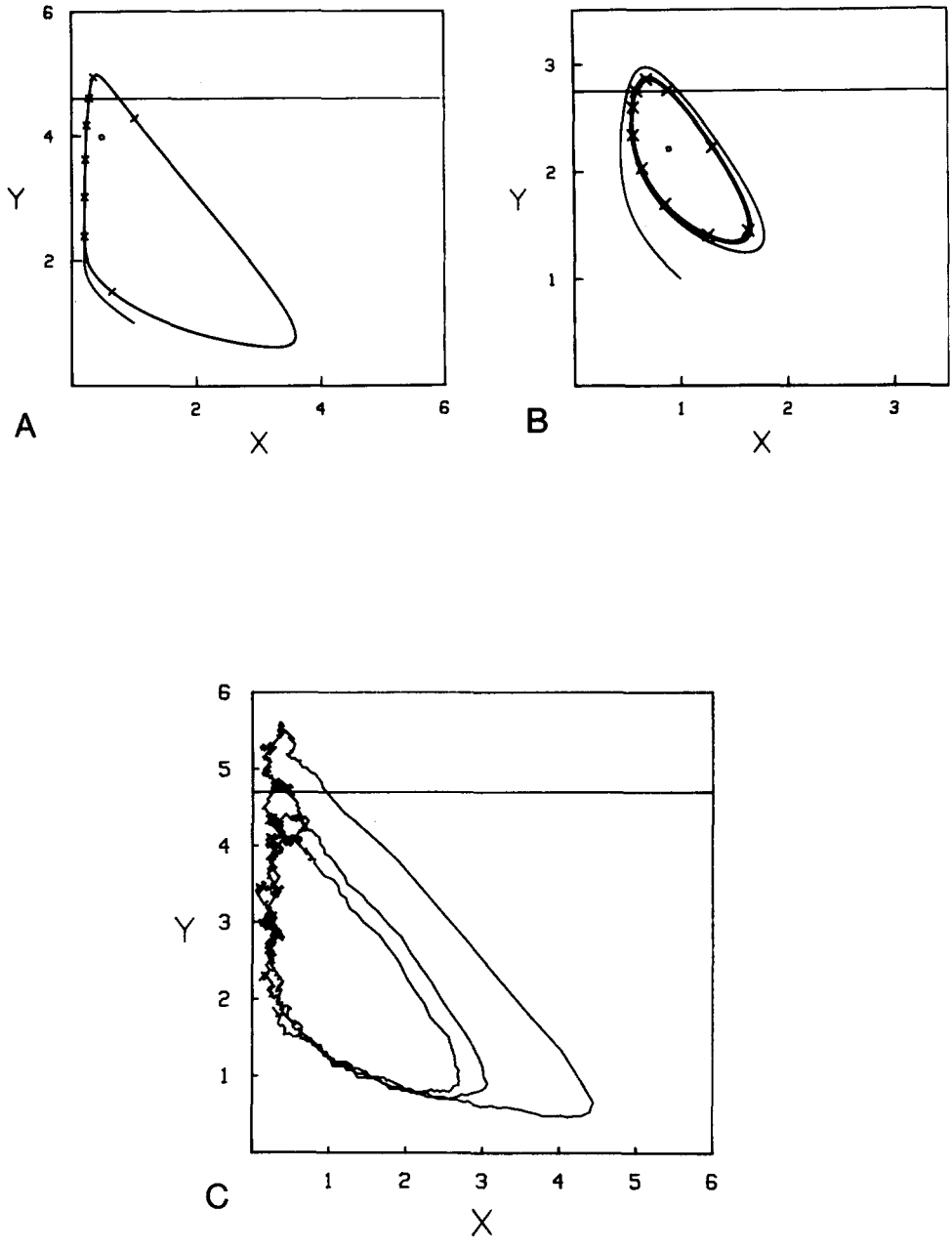


Fig. 1. Phase plane trajectories of the Brusselator. Stable oscillations in the variables X and Y occur for values of parameters $B > A^2 + 1$. In the oscillatory region, the system moves in a clockwise direction about the steady state ($X_{SS} = A$, $Y_{SS} = B/A$). For different values of A and B, the behavior can approximate that of a relaxation oscillator [(1A) A = 0.5, B = 2.0] or can be more "sinusoidal" [1B) A = 0.9, B = 2.0]. Phase plane portrait of the oscillator used to simulate results in this study is shown in 1C (A = 0.5, B = 2.0, $X_0 = 1$, $Y_0 = 1$ Random walk; $D_X = D_Y = 0.024$). Threshold $\theta$ is indicated by the horizontal line. Crosses indicate intervals of 1/10 cycle. All simulations were done using the system in Figure 1C.

cell systems. We consider that in the shortest mammalian cell cycle (8 hr), two crossings of threshold must be accomplished, the first possibly associated with the initiation of a cycle of DNA replication and the second associated with the triggering of mitosis. As we will show later, phase perturbation data also suggests that there are two rather than one "clock" periods per mitotic cycle. Lengthy prereplication stages seen in cells at confluence or in serum or amino acid depleted media occur in this model as a consequence either of altered levels of the "precursors" A, or increased threshold $\theta$, resulting in repeated subthreshold oscillations. Notice that if the threshold value in the first loop is higher than that in the second, then cells will show greater asynchrony in the execution of early versus later cell cycle events. The results presented here will be qualitatively similar if the two thresholds are either sufficiently different or are equal; for the sake of simplicity we have performed all simulations with the assumption of equal thresholds.

There is evidence for approximately 4-hr subcycling behavior in cell lines with generation times considerably longer than 8 hr [9, 18]. It may be, therefore, that these cells

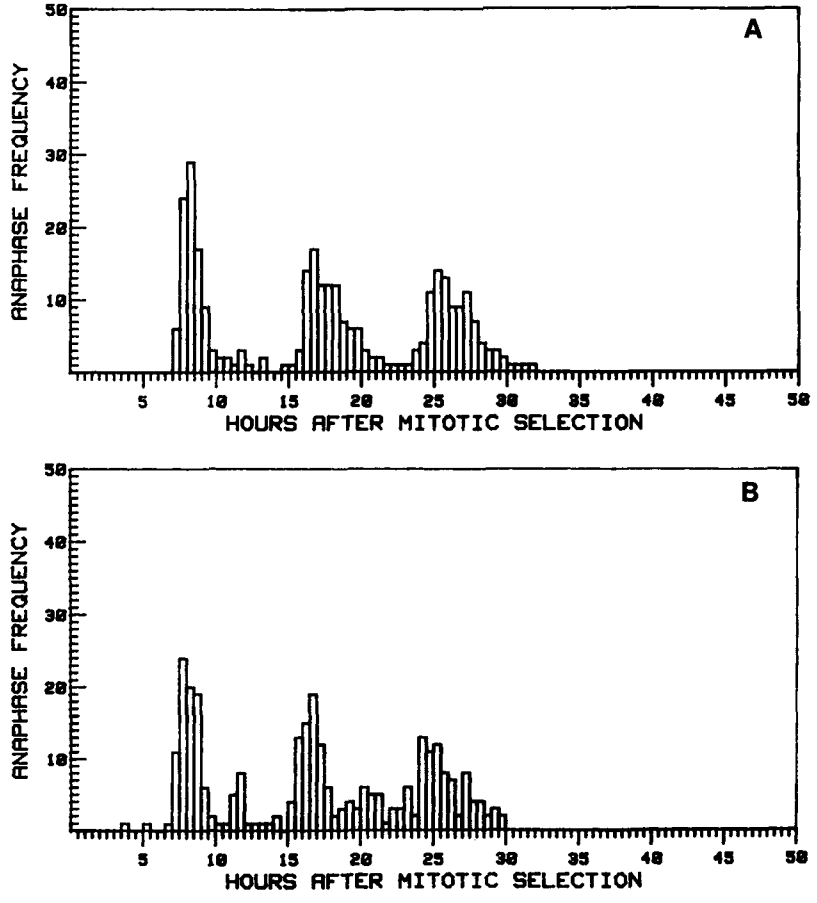


Fig. 2. Desynchronization histograms. Histograms of anaphase frequency for several generations of synchronous V79 cells are shown under conditions of slow (2A) and rapid (2C) decay of synchrony. 100–200 cells were followed for two or three modal generation times after mitotic selection. Figures 2B and D are paired simulations of 2A and C which vary only in the choice of threshold $\theta$. In B, $\theta = 4.5$, in D, $\theta = 4.7$.

require more than two threshold crossings per cycle (see Fig. 3C), each crossing triggering successive cycle events. Alternatively, execution of events normally triggered by a threshold crossing may be delayed until some independent cell parameter, perhaps related to cell size, reaches a critical level. In this way, additional clock periods are inserted into the cell cycle.

### Desynchronization of Mitotic Waves

We have simulated the behavior of a population of 100 cells following a limit cycle trajectory by supposing that two thresholds must be crossed per cell cycle. Simulated cell populations were initiated synchronously just after mitosis, and allowed a random walk with equal magnitude steps in both the X and Y direction in each of 200 time intervals per loop of the limit cycle. After each step in the random walk the dynamical motion in that time interval was calculated. Preliminary calculations and simulations were done to determine a threshold value such that an appropriate fraction of cells failed to reach threshold on any given pass. Each individual cycle time was monitored and a histogram of cycle times was plotted.

In Figures 2A and B, the decay of synchrony in synchronous populations of V79 cells and simulations of the decay of synchrony are shown. The loss of synchrony in a population is accelerated by a number of things including temperature shifts or cold collection,

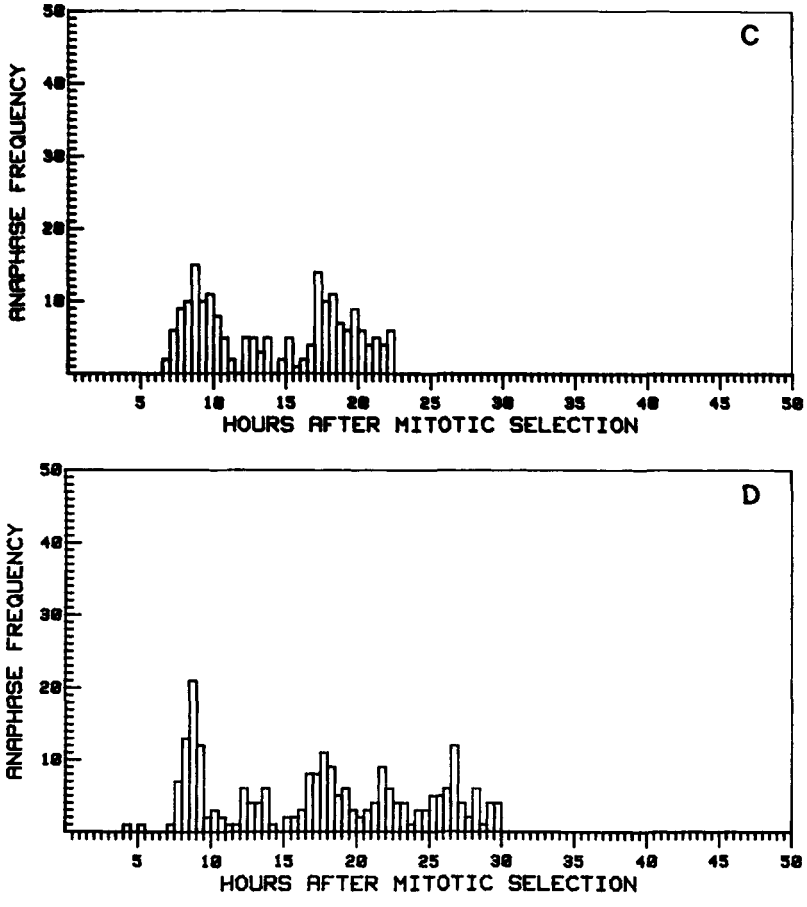


Fig. 2 (continued).

changes in serum concentration and the method of achieving synchrony. In this study a synchronous culture obtained under optimized conditions (Fig. 2A and B) is compared with a culture that experienced a serum concentration shift prior to mitotic selection (Fig. 2C and D). Desynchronization can occur in the model as a result of either an increase in random step size, or an increase in the threshold value $\theta$. In the latter, the loss of synchrony occurs due to subthreshold oscillations and consequent skipping of threshold crossings. The change in $\theta$ from 4.5 to 4.7 was sufficient to generate the increase in desynchronization.

### Generation Time Distributions – $\alpha$ and $\beta$ Curves

Generation time distributions of V79 cells were obtained by following individual cells and their progeny in video tape recordings. These intermitotic times were then tallied. In the case of WI-38 the pedigree data of Absher et al [19] were replotted as intermitotic times. In Figure 3A, B, and C, the distribution of generation times of individual cells in populations of V79 and WI-38 are plotted as the fraction of the population that has not yet divided vs. the generation time (the $\alpha$ curve [2]). Simulated distributions generated by the oscillator model are shown for comparison. Simulations using the 2-loop oscillator were performed using the parameter values obtained from desynchronization experiments shown in Figure 2. For $\theta = 4.5$, nearly all cells cross both thresholds on the first attempt and generation times are tightly distributed about the modal value. For slightly higher values of the threshold, significant numbers of cells fail to cross one or both of the thresholds in the first pass and hence the distribution shows considerable skewing and some quantizement.

Additional simulations were done to obtain the distribution of differences in generation times between sister cells (the $\beta$-curve [4]). As expected, in this model, sister cell generation times are uncorrelated, and the $\beta$-curves parallel the tails of the $\alpha$-curves (data not shown). In order that cell populations show correlated sister cell generation times as well as exponential $\beta$-curves, there must be an aspect of the cellular timekeeping which is variable in the population but constant between sister cells, and another aspect which is purely random. We speculate that here again cell size may play a role in that sister cells will both tend to be smaller or larger than normal according to whether the mother is smaller or larger than normal. In the picture we present here, a sub- or supra-normal size at birth may lead to a greater or lesser number of oscillator loops, respectively, before first threshold crossing is reached. This will tend to correlate sister generation times, while subsequent random subthreshold passages will generate nearly exponential $\beta$-curves.

### Phase Response to Perturbation

Figure 4 shows a series of synchronized V79 cells given single high serum pulses, heat shocks, or ionizing radiation treatment at half-hour intervals through a modal 8.5-hr cell cycle. Each data point represents the difference between paired treated and control cultures of 100–200 cells/field.

The phase response curves display a biphasic pattern of advances and/or delays in subsequent cell divisions. A characteristic phase response curve with a repeating 4-hr periodicity was generated. In Figure 4a the times to the first and second synchronous waves following synchronization were compared for each pair of serum pulsed and control cultures as a function of time from the beginning of the serum pulse. Some differences were noted in the phase shift accomplished by different serum lots. This may be reflected in the fact that the phase response curve shows a relatively broad band of responses. Beginning 0.5 hr after mitotic selection, pulses with serum produce delays in the midpoint

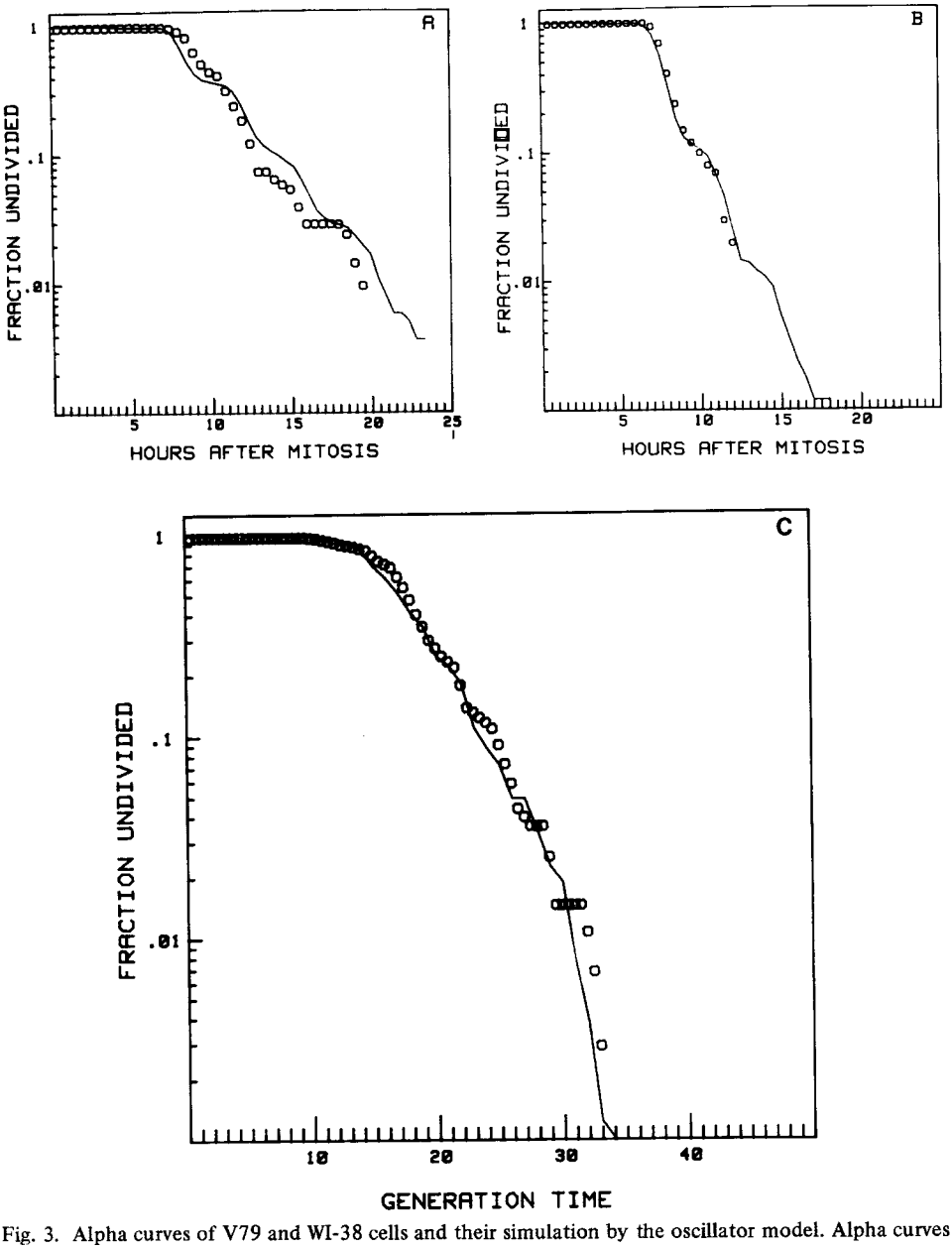


Fig. 3. Alpha curves of V79 and WI-38 cells and their simulation by the oscillator model. Alpha curves describing the undivided fraction of cells in generation time distribution curves are shown in Figures 3A, B and C. 200 ± 50 generation times are represented in each curve. In A and B, circles indicate distributions of generation times in V79 cells growing under suboptimal (A) and optimal (B) conditions. Simulation of these curves (solid lines) shows that the distribution of generation times is exponential but quantized within the exponential envelope. In A threshold $\theta = 4.7$, in B $\theta = 4.5$. All other parameters are unchanged from those in Figure 1C. In Figure C, circles indicate generation time distributions of WI-38 cells and the lines give the simulated distributions. Here parameters are the same except that cycle time is increased by requiring that the number of oscillator loops in one cycle is increased from 2 to 4. Note that with the long cell cycle generated by this model, the resulting distribution of generation times is smoother and approaches a straight line at long generation times.

of the subsequent mitotic waves (delay is maximum at 1.5 hr). Delays give way abruptly to advances at 2.5 hr and the amount of advance then decreases as pulses are given between 3 and 5 hr into the cycle. At 5 hr decreasing advances become delays which increase for serum pulses occurring between 5 and 6 hr. Delays again give way abruptly to advances at 6 hr and again the amount of advance decreases through the late portion of the cycle. Pulses very late in the cycle appear to generate phase delays.

Similarly in Figure 4B the results of 10-min 45°C heat shocks given at 0.5-hr intervals through the cycle are shown. Cells pulsed soon after mitosis are slightly delayed in the subsequent mitoses relative to the paired unshocked control. Minimum delay, and in some

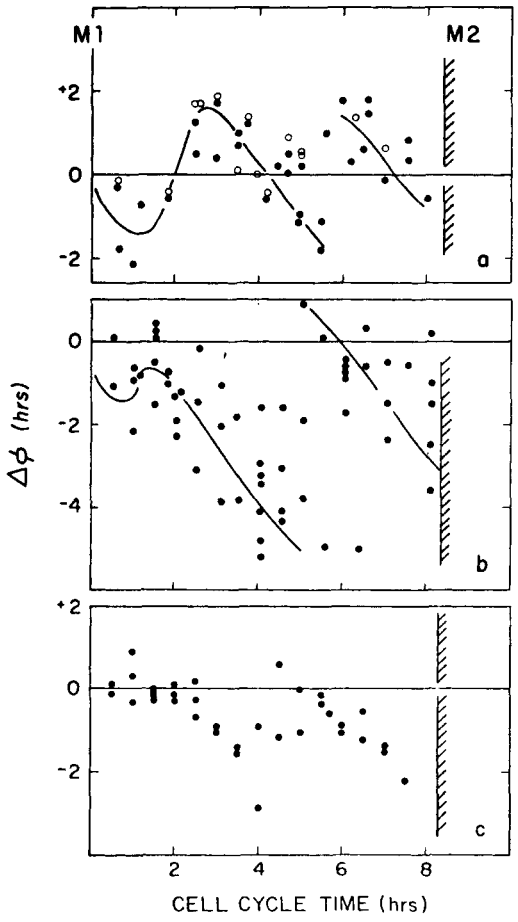


Fig. 4. Phase response of synchronous V79 cells to perturbation by serum, heat shock and ionizing radiation. (A) Serum pulses. At intervals following mitotic selection, serum concentration in the medium was increased from 5 to 20%. Midpoints of first (•) and second (○) mitotic waves. (B) Heat shock. Midpoints of the first mitotic wave following synchronization and a 10-min 45°C heat shock are compared for each pair of heat shocked and control cultures as described in (a). (C) Ionizing radiation. Synchronous V79 cells were exposed to 150 rads from a Cobalt-60 source at 30-min intervals through the first synchronous cell cycle. Analyses of division advance or delay were determined as described in (A) and (B).

instances a slight advance, occurs when shocks are given 1.5 hr after mitosis. There follows a pattern of increasing delays up to 5 hr, when an abrupt shift in response occurs giving a second minimum in delay at 5.5–6 hr of the cell cycle. Pulses given later than 6 hr in the cycle give a pattern of increasing delays up to the subsequent mitosis. The response curve appears as two parallel lines sloping downward to the right, with a small cluster of values between 4 and 6 hr of the cycle showing a constant 2-hr delay. In some experiments heat shocks given after 4 hr showed a splitting of the anaphase frequency histogram, suggesting that at these values of time and perturbing stimulus member cells may be either slightly advanced or delayed, or quantally delayed by $\Delta\phi + 4$ hr. It is often the case in calculating the mean or midpoint of the population that the value will be found to lie between the two discrete peaks [6]. This may serve to explain why in earlier division delay studies the results were described as showing a transition point with constant delays in response to perturbations late in the cycle. This capacity to phase jump is further shown when on occasion the midpoint of a population shocked at 7 hr is delayed, not by 0.5–1.0 hr, but by 5 hr, or a full subcycle.

Figure 4C shows the phase response to 150 rads of ionizing radiation applied at one-half-hour intervals through the V79 cell cycle. Cells pulsed up to 2 hr after mitotic selection are slightly delayed or advanced in the subsequent mitosis relative to the paired untreated control. There follows a pattern of increasing delays up to 4 hr when an abrupt shift in response occurs, giving a second minimum delay, and in some cases slight advances at 4.5 and 5 hr. Pulses given after 6 hr in the cycle show increasing delays up to the initiation of the first postselection mitotic wave.

### Simulation of Phase Response to Heat Shock

To simulate the phase response behavior of V79 cells to heat shock, oscillator variables X and Y were reduced by 90% at various intervals and the system was then allowed to evolve from this new state. In each case the system relaxed toward the stable trajectory, and eventually crossed threshold. Phase shifts in the time of mitotic threshold crossings were computed and compared with the heat shock data of Figure 4. The results are shown in Figure 5. Note that any extensive first order destruction of oscillator variables would place the system close to the origin and yield qualitatively similar phase response curves.

## DISCUSSION

It does not appear necessary to resort to a strictly stochastic model in order to generate the commonly observed distribution of cell generation times. The limit cycle model simulates an exponential or pseudo-exponential distribution by permitting random walk of individual cells below threshold. The oscillator model predicts (and fits) a biphasic response to heat shock and other perturbations in V79 cells with 8-hr generation times. The model suggests that prolonged failure to execute early cycle processes will occur when the value of the parameter A, the precursor material in this reaction scheme, changes, moving the steady state and trajectory and causing repeated subthreshold oscillations.

The tendency of many cellular processes to oscillate with a common period that is shorter than that of the cell cycle [7–9, 20–23] and the consequent quantizement of generation times [23] suggests that cellular timekeeping involves an oscillatory mechanism having much in common with other biological rhythms. That such periodicities are not routinely observed in heterogeneous tumor cell populations or in cells with relatively long generation times, may be more a reflection of the choice of experimental system and

synchrony technique than an intrinsic difference between cell types. Setting aside the existence of oscillations of periods less than one-cell generation time, it seems appropriate to require the crossing of two thresholds for cell division to take place since there are by most accounts a minimum of two dependent events in the cell cycle. DNA synthesis must occur before cell division and cell division must occur between rounds of DNA synthesis, although exceptions to those two conditions can be found. It should also be mentioned that pseudo-exponential generation time distributions (but not the correct phase response curves) also were achieved in a series of simulations in which the limit cycle period equaled the cell cycle period.

The model presented here does not quantitatively describe the common observation that big mother cells give rise to relatively larger daughter cells which therefore divide faster than their more ordinary sized counterparts, nor does it account for the need for a cell to achieve some minimum size before DNA synthesis can occur. Elsewhere we have extended the model by adding cell size along a third (Z) axis [6]. In that model a threshold crossing will only initiate an event such as DNA synthesis if such minimum size has already been obtained. This more general model predicts a correlation of sister generation times and may possibly explain the negative correlation of mother/daughter cell generation times. In addition exponential $\beta$-curves are generated.

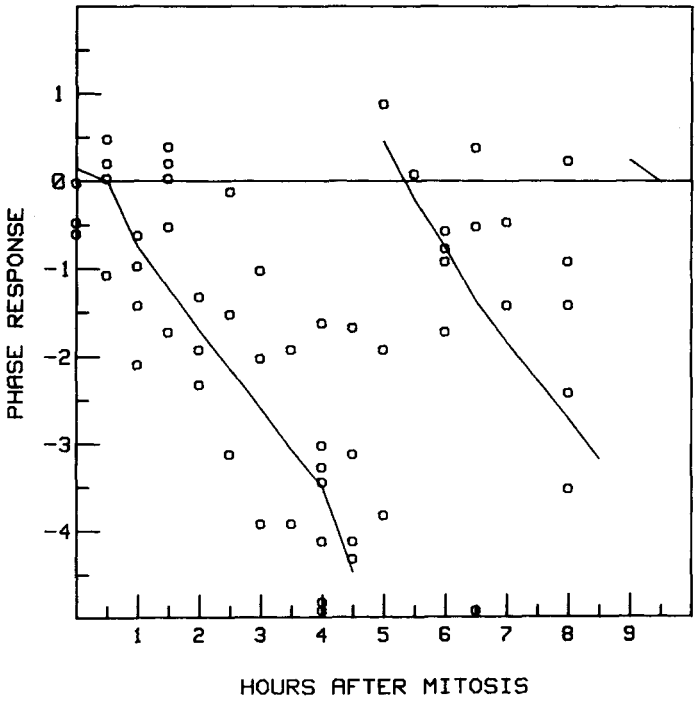


Fig. 5. Simulation of phase response of V79 cells to heat shock. The heat shock phase response curve generated in Figure 4 by treatment of synchronous cell cultures for 10 min at 45°C through the cycle is shown as open circles (○). Solid lines indicate a simulation performed using the oscillator model with parameter values of A = 0.5, B = 2, $\theta$ = 4.7. Perturbation at each half-hour point in the cycle was assumed to destroy 90% of X and Y by first order kinetics.

Limit cycle oscillators are attractive as models for timekeeping because they can combine both stochastic and deterministic elements. In unperturbed conditions, in the absence of any noise or random walk, the trajectory followed by a cell would be fixed and the time to pass an arbitrary threshold would be constant in each loop. Either normal variation or a perturbation to the state variables, or a change in the system parameters will alter the course of a cell on the trajectory in ways that often seem very "real" from the biological perspective. This property of phase responsiveness led to the initial experiments on phase perturbation and the resulting 2-loop model for the minimum cell cycle.

The set of strong perturbations described above provide information about the period and about the asymmetry and continuity properties of the clock. All the curves are consistent with two repeated response curves, each with period roughly equal to half of the 8-hr cell generation time. This suggests that there is a 4-hr timekeeper, responsible for the quantizement of generation times, which is being affected by the perturbations. For heat shock or serum perturbations, each of the two branches of the phase response curves have slope of approximately $-1$, suggesting that the perturbations drive the timekeeping system to a constant phase in the cycle, after which all perturbed cells take an equal time to pass through mitosis. This provides evidence that it is the clock which is being affected by the perturbation, since a response not based on clock perturbation would be highly unlikely to yield a delay exactly proportional to cycle phase.

The phase response data in Figure 4 provide information about the shape of the oscillator cycle. If, for example, heat destroys each of the oscillator variables with first-order kinetics, the system will be driven toward the origin as a result of the application of heat. After heat shock all cells are found in a small area in X-Y space, and have only a small range of phases. Consequently, when cells resume their cycling they take nearly the same time to reach threshold. Since cells which the have just passed threshold show small advances and those which are just reaching threshold show large delays, the origin (X = Y = O) must be at a phase representing a very early part of the cycle. Therefore, as shown here, the part of the cycle just after threshold, during which the system moves through decreasing values of X and Y, must be traversed very rapidly. Conversely, the part of the cycle during which the system rises toward threshold must be traversed relatively slowly (Fig. 1A).

Evidence for a continuous oscillator, rather than a strict relaxation oscillator comes from the capacity of cells to skip an event such as mitosis and display quantized generation times [6]. Furthermore, at the junction between the two repeated branches of the response curves for heat shock and serum pulses, the phase difference rapidly changes by approximately 4 hr, equal to the period of the assumed underlying timekeeper. This magnitude of change is exactly what is expected for a clock with a threshold triggering of some cell cycle event (ie, a "point of no return"). To visualize this, consider two cells, one with phase just prior to the threshold crossing and one just after. If the clock is continuous and the perturbation affects clock variables only, both cells should be driven to very nearly the same phase by identical perturbations. Since one cell is at the end of its cycle and the other is at the beginning, the difference in phase change due to the perturbation is one period of the timekeeper, ie, different by $\simeq 4$ hr. This 4-hr discontinuity should be seen for all "large" perturbations of a continuous oscillator, but will occur in a relaxation oscillator only if a particular perturbation resets the clock variable to exactly the same level starting from either its high prerelaxation or low postrelaxation value.

The above discussion points out the fact that phase response data as commonly presented are often not sufficiently stringent in their assessment of oscillator dynamics. Perturbations are characteristically chosen because they give maximum phase response

consistent with viability. Under such conditions variables are reset to nearly identical phase and the response curve so generated shows an increasing delay with increasing progress through the cycle up to some execution point. In our experiments, two such points appeared – one 4.5 hr into the cycle, after the initiation of DNA synthesis and coincident with the replication of the bulk of the DNA, and the other at or near mitosis. To properly assess the "interior" of the oscillator the cells should be subject to gentler perturbations where, as Winfree [13] has done for Drosophila eclosion rhythms, for appropriate choice of phase and perturbing stimulus an apparent equilibrium point or singular state is approached, with the consequence that arrhythmic emergences are observed. It is important to state explicitly that the apparent discontinuity observed here in the phase response curves does not imply that timekeeping is accomplished by a relaxation oscillator. Rather it is more consistent with a continuous oscillator which gates certain mutually dependent, largely irreversible and therefore discontinuous cellular events such as DNA synthesis and mitosis.

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