KINETIC CELL-CYCLE ANALYSIS OF A CULTURED MAMMALIAN CELL POPULATION

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ABSTRACT The parameters of the cell cycle are analyzed in terms of the stochastic theory of cell proliferation for a murine mastocytoma line. The cells were grown in suspension culture under steady-state conditions in a chemostat. Initial estimates of the parameters from synchronous growth indicate that agreement of the data with the model is obtained only if the model is modified to include an initial proliferating fraction of less than 100%, and a cell loss continuing throughout the course of the experiment. The analysis verifies that the modified theory adequately describes the data, and that similar parameters are obtained from both desynchronization and percent labeled mitosis experiments. The average cycle time from 10 desynchronization experiments was 8.24 ± 0.52 h with a cellular standard deviation of 1.28 ± 0.18. The combined parameter obtained by dividing the cellular standard deviation by the cycle time is shown to be a useful measure of biological variability well defined over many different experiments. The rate constant for cell loss is about 0.009 which gives an 8% cell loss per cycle. The cell loss is sufficient to account for the apparent deficit in initially proliferating cells. The initial distribution of the synchronous cells is qualitatively examined and is found to be peaked late in G_1 or early in S.

INTRODUCTION

Several authors have explored the application of a mathematical model to experiments involving the induction or decay of synchrony of a population of cells (1, 2). In this model one assumes that the time required for a given cell to traverse the division cycle and to produce two daughter cells is an independent[1] random variable obtained from a distribution with average cycle time T_c and variance σ^2. In the present paper the model is applied to and tested on a particular experimental system in which mammalian cells are kept at a chosen optimal population density under constant conditions in a continuous culture. Since under these experimental conditions synchrony is relatively well maintained (4), the consequences of the model can be tested over long periods of time covering many cell generations.

[1] i.e. successive generation times are statistically independent. This assumption can be relaxed to include the effect of correlations between daughter cells (3). Consideration of such correlations introduces additional parameters and is apparently unnecessary for obtaining consistent results from long term experiments with the system discussed here.

The mathematical model has proven to be useful in studying and parameterizing cell growth in in vivo situations as in tumors (eg., reference 5). Since the experiments studied here are highly controlled, the analysis should help us better understand the limitations of conclusions drawn with the model in more complex in vivo situations as well as improving our understanding of experiments with mammalian cells in culture.

MATERIAL AND METHODS

A. Cell Line, Culture Techniques, and Preparation of Synchronous Suspension Cultures (Exps. 1–10).

Suspension cultures of a transplantable murine mastocytoma (cell line P-815X2) were used. The origin of the cell line and the general culture techniques have been previously reported (6). The medium has been described as medium I (7) and contained 10% undialysed horse serum. The general method of preparing partially synchronous cell populations by centrifugation of cells in isotonic sucrose gradients and collection of slowly sedimenting early interphase cells has been reported in detail previously (6). After preincubation in spinner culture for 48 h, the cells were centrifuged, suspended in a small volume of medium, placed on isotonic sucrose gradients, and centrifuged. The fraction of cells sedimenting most slowly and corresponding to 2–5% of the original cell number was collected. These cells which are mainly in early interphase are then suspended in sucrose-free medium to obtain a cell density of $1–3 \times 10^5$ cells/ml, and incubated under steady-state conditions (6, 8). Under these conditions, the cell suspension is continuously being diluted with fresh medium in order to compensate for the increase in cell number due to cell division.

Characterization of Synchrony. In order to measure cell multiplication as a function of incubation time, samples were withdrawn from synchronous cultures at intervals of 1 h and mixed with an equal volume of a fixing solution containing 50% ethanol and 50% of a 0.05 N HCl solution. The fixed cells were counted with a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and the values obtained were multiplied with the cumulative dilution ratio of the steady-state culture (4).

The method used for determining mitotic indices has been previously described (6). Similarly, the proportion of DNA-synthesizing cells was determined by pulse-labeling. The pulse consisted of a 20 min incubation of an aliquot of the suspension culture with [^{3}H]thymidine as described before (6).

B. Kinetic Analysis of Asynchronous Cultures (Exps. 11 and 12)

A clonal subline (termed P-815X-d) was derived from the P-815X2 cells. This subline is characterized by a near diploid chromosome number and a relative homogeneity of its cell population (9). The cells were incubated during several days under steady-state culture conditions (6) in medium I (7) containing 10% dialysed horse serum. For pulse-labeling, the cells of a steady-state culture were centrifuged and suspended in 10–15 ml of medium. This suspension was incubated during 20 min with 0.2 μCi/ml of [^{3}H]thymidine (5 Ci/mmol, the Radiochemical Centre, Amersham, England). Subsequently the cell suspension was diluted to 30 ml with medium, centrifuged, and the cells were washed with medium and reincubated under steady-state conditions in medium containing unlabeled thymidine (0.01 μmol/ml). After reincubation of pulse-labeled cells, samples were withdrawn at intervals of 1 h and fixed by mixing with an equal volume of ethanol-acetic acid-water (5:2:3, vol/vol). Smears were prepared as previously described (9), stained by the Feulgen reaction, and processed for autoradiography. In the autoradiographs, cells were considered to be labeled if covered by more than 5 grains.

C. *Mathematical Model*

In references 1 and 10 it was shown that most of the observed effects of synchrony are independent of the particular form of the distribution function for the transit times. This is because as an experiment proceeds the sum of several transit times is the important random variable in determining the increase of the population (see eq. 9 reference 1), and the behavior of such a sum approaches a Gaussian distribution regardless of the initial distribution postulated. Now as a practical matter, distributions such as have been observed for cells (11, 12) with a well defined peak and width give results for desynchronization which become indistinguishable from those given by a truncated Gaussian after the first generation or two.[2]

In the Results section (part A), we find that cell loss is observed and the model must be modified to include this fact. We assume for simplicity that cell loss is uniform over the cell cycle and throughout the experiment. Hence, the probability for cell loss per unit time is κdt where κ is a constant.[3] By a modification of the methods of reference 18 it can be shown[2] that the analysis of reference 1 holds with the slight changes given in Eqs. 2 and 11.

RESULTS

A. *Initial Estimates of the Parameters by Approximate Methods*

In this section simple approximate or "hand" methods of obtaining the parameters of cell growth are discussed and applied to experimental data. These methods were used in making the initial estimates of parameters which were used as starting values for the computer analysis presented later. The methods are interesting in that while little more than an understanding of the formulas and some graph paper is necessary for their use, the values obtained (Table I) are reasonably close to the final estimates. The param-

TABLE I

APPROXIMATE ANALYSIS OF LONG RUNS (FIG. 1)

Parameters	Exp. 1	Exp. 2
T_c	8.5 h	7.7
$\alpha = \ln 2/T_c'$ (see Eq. 2)	0.081 h^{-1}	0.090
m (measured log phase growth rate)	0.0704 h^{-1}	0.0832
$\kappa = \alpha - m$ (loss rate)	0.011 h^{-1}	0.007
T_d	9.85 h	8.33
σ	1.01–1.26 h	0.90–0.95
σ/T_c	0.118–0.148	0.117–0.123
P_0 (from Eq. 8)	2.0×10^5 cells	1.3×10^5
P_0 (from Eqs. 1 and 2)	2.1×10^5 cells	1.1×10^5
Initial growth fraction	0.80	0.75

[2]Bronk, B. V. To be published.

[3]This modification has already been considered for labeled mitosis in reference 7 where it was shown that the curve for the fraction of labeled mitotic cells was not affected for a uniform cell loss and in fact is not affected very much even if the cell loss is concentrated at the end of the cell cycle. The log-phase fractions-in-state are also unaffected by a cell loss which is *uniform* over the cycle, but may be substantially affected by a loss concentrated at the end of the cycle.

eters estimated are T_c, the cycle or generation time; σ, the standard deviation of the cycle time; m, the asymptotic growth rate; P_0, the initial viable cell population.

The formula for the population at time t, $P(t)$, is for log-phase growth given by

$$P(t) = Ie^{mt}, \tag{1}$$

where I may be obtained by integration of the asymptotic formula (Eq. 11) and has a value[4]

$$I = P_0/2^a T_c(\alpha - \kappa), \tag{2}$$

with

$$a = (1 - \sigma^2/T_c^2)$$

and where[4] $\alpha = \ln 2/T_c'$ and $T_c' = T_c/(1 + (\ln 2)\,\sigma^2/2T_c^2)$, κ is the rate constant for cell loss and m is equal to $(\alpha - \kappa)$. Values for κ; T_d, the doubling time; P_d, the initial non-proliferating population; and the initial growth fraction are obtained from the estimated parameters, and are also listed in Table I.

The growth curves are obtained as explained in reference 4 by multiplying measured cell number by a volume factor which accounts for dilution. The "carpeted staircase" growth curve characteristic of a synchronized population is shown in Fig. 1 for exps. 1 and 2. Since the data are taken hourly, a time interval short compared with T_c, (dP/dt) may be obtained simply by taking the difference between adjacent data points. The derivatives are plotted against time in Fig. 2 for the first 35 h of exp. 1. The derivative function is more convenient than $P(t)$ for use with the methods of reference 1 and also illustrates the persistence of synchrony longer (see Fig. 4) than is apparent from the growth curves. Taking differences tends to amplify fluctuations so that smoothing was done by averaging the data between adjacent hours for presentation of data in Figs. 4–6 and 9. This amounts to taking the differences between cell numbers every 2 h. The smoothing is reasonable since the continuous culture setup would tend to be self-correcting for fluctuations in dilution or sample size from one hour to the next. While the smoothed data were used for diagnosis and presentation, the best-fit parameters agreed with those obtained from the raw derivatives.

The solid line of Fig. 2 is a hand-drawn curve used to locate the peaks, their heights, and the widths for the first three peaks for exp. 1. The first peak is at 8 h and the seventh peak is at 59 h. Averaging the differences between the peaks gives 8.5 h as an estimate for T_c. (The minimum difference was 8.0 h and the maximum, 9.0 h.) Next, the formula (see reference 1) for the half width at half maximum, HW, is

$$\mathrm{HW} = 1.18\sqrt{n}\,\sigma, \tag{3}$$

[4]Since σ^2/T^2 turns out to be small for the present experiments, we may set $T_c' = T_c$ and $a = 1$ for these estimates.

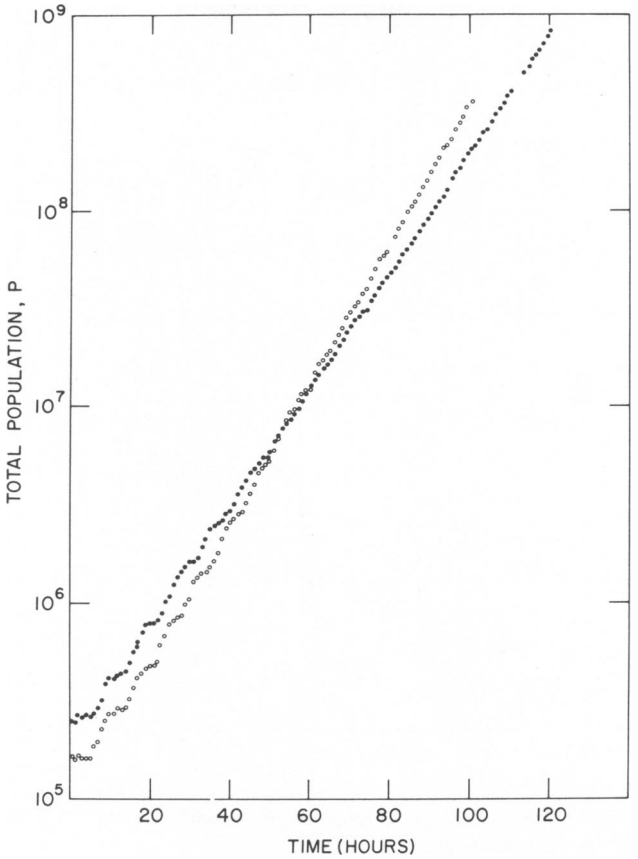

FIGURE 1 Logarithmic growth curves, · · · exp. 1 and ooo exp. 2.

where n is the number of the peak. Eq. 3 is used with the first three peaks to estimate σ for exp. 1. The values obtained for σ are 1.01, 1.09, and 1.26 h.

In Fig. 3 we plot P_{max}/P for the first six peaks of exp. 1 for comparison with Eq. 27 and Fig. 3 of reference 1. The first two points (+ signs) bend rather far below a possible straight-line fit. These points would be raised if the denominator were reduced. This suggests that a portion of the initial population is nonproliferating. An estimate of a number of nonproliferating cells, called P_d, is given below.[5] When this number, 0.56×10^5 is subtracted from P, a reasonable fit to a straight line results, as shown by the circles in Fig. 3. According to the formula,

$$P_{max}/P = 2/3\sqrt{2\pi\sigma^2 n}, \tag{4}$$

[5]We assume here that P_d is initially nonproliferating. If these cells started dividing later, say after the first two cycles, they would by then constitute such a small portion of the population that they wouldn't affect the estimates of parameters given in this paper. Similar remarks would apply if P_d were a *very* slowly proliferating population.

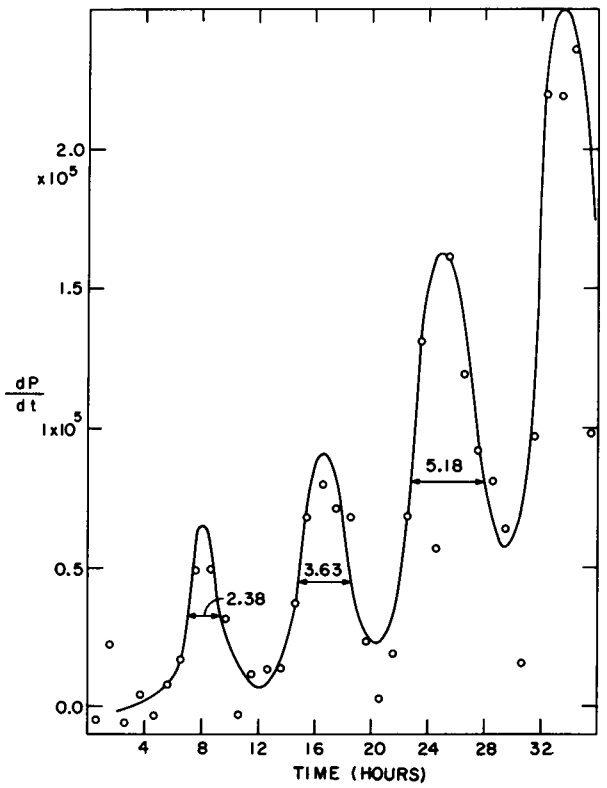

FIGURE 2 The time derivative of the population, $\dot{P}$, vs. time for exp. 1. The curve was hand-drawn through the experimental data.

the slope of the straight line of Fig. 3 may be divided into 0.266 to give a value for σ. We find $\sigma = 1.07$ h from this method for exp. 1. A similar analysis for exp. 2 gives the results indicated in Table I.

A value for m is obtained from the log-phase portion of the growth curve (Fig. 1). The derivative of P divided by its value (interpolated) at the time of the derivative gives according to Eq. 1, $\dot{P}/P = m$. If we average $\dot{P}/P$ for the last 16 values obtained for exp. 1, we obtain

$$<\dot{P}/P> = m = 0.708. \tag{5}$$

The standard deviation of this quantity over the same 16 values is 0.023. A 2% error in volume from one sample to the next would give this sort of fluctuation in m. Actually the oscillatory term in P still gives changes of this magnitude for the time interval where the above computation is made (between 100 and 120 h). The values of m listed in Table I are actually taken from a straight-line fit of the natural log of the population vs. time (Fig. 1) for log-phase growth. P_0 is also obtained from this procedure by the use of Eqs. 1 and 2. The results of least squares fits by computer for these

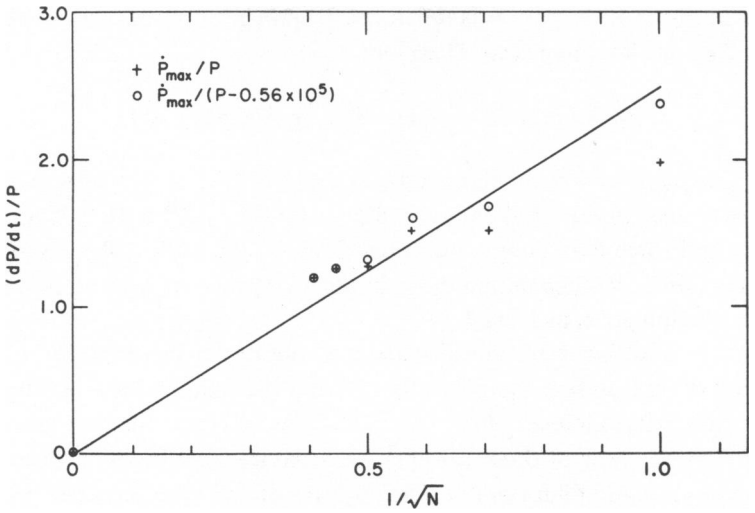

FIGURE 3 $\dot{P}_{max}/P_{viable}$ plotted against the reciprocal square root of the order of the peak for exp 1. The values of $\dot{P}_{max}$ are estimated from the hand-drawn curve shown in Fig. 2. The slope of the line fitting the data is divided into the number (0.266) to give $\sigma \approx 1.07$. For the three points with highest N, o coincides with +.

parameters for the last 20 h for both exps. 1 and 2 are presented in Table I. The fit is excellent since the computed points agree with the experimentally measured values to within almost four significant figures in the natural log at each point. Since α may be obtained using our estimate for T_c, we obtain κ, the cell loss rate constant from $m = (\alpha - \kappa)$.

The value of P_0 obtained from the intercept of the fit to the log-phase portion of the growth curve is 2.1×10^5 for exp. 1. An independent estimate for P_0 was obtained as follows. Choosing a convenient time point, t_i, on the initial level portion of the growth curve, compare the cell population at that point with the population one cycle time later (this initial time, t_i, is chosen as 4 h, then $t_i + T_c$ occurs at 12.5 h for exp. 1, and at 11.7 h for exp. 2). We assume that the nonproliferating portion neither lyses nor begins to divide by 12.5 h and that P viable at time, t_i equals P_0. Then

$$P_d + P_{viable}(t_i) = P_{total}(t_i), \tag{6}$$

$$P_d + P_{viable}(t_i + T_c) = P_{total}(t_i + T_c). \tag{7}$$

This estimate assumes that cells enter and leave (by lysis) the nonviable population at compensating rates so that P_d remains roughly constant over the particular time period involved.

Subtracting Eq. 6 from Eq. 7 we have

$$P_{viable}(t_i + T_c) - P_{viable}(t_i) = P_{total}(t_i + T_c) - P_{total}(t_i),$$

$$= \text{increase in the viable population over one cycle,}$$

but this is equal to $P_0(2e^{-\kappa Tc} - 1)$ where the exponential factor accounts for the cell loss during one doubling time. Therefore

$$P_0 = [P_{\text{total}}(t_i + T_c) - P_{\text{total}}(t_i)]/(2e^{-\kappa Tc} - 1). \tag{8}$$

For exp. 1, taking t_i as 4 h, the numerator in Eq. 8 is 1.67×10^5 cells and using the value of m obtained in Eq. 5 to give $\kappa = 0.010$ we find $P_0 \approx 2.0 \times 10^5$. If for exp. 1 we average the first three data points, we obtain 2.56×10^5 for $(P_0 + P_d)$, the initial number of cells present. We then obtain the estimate for P_d that we used to plot Fig. 3 and the growth fraction given in Table I.

There is one additional graphical method to obtain an estimate for σ which we describe, but do not present the graph for. Taking the estimate of m as the value for $<\dot{P}/P>_{\text{ave}}$, the natural log of $|P/P - <\dot{P}/P>|$ is plotted for the maxima and minima of P vs. the time of those extrema. A separate straight line is drawn through the maximum and minimum points. The negative of this slope is called λ (1, 10) and is given by

$$\lambda = 2\pi^2\sigma^2/T_c^3. \tag{9}$$

Averaging the two slopes for exp. 1 gave $\lambda = 0.0368$, which together with $T_c = 8.5$ h gave a value of $\sigma = 1.07$ h from Eq. 9 in reasonable agreement with our other estimates.

Our further "eye ball" estimate may be made before we turn to the results of the computer analysis. In reference 10 it is shown that the asymptotic value for the percent of mitotic cells labeled is t_2/T_c where t_2 is the transit time for the S phase. In Fig. 6, the data points for exp. 11 are shown. Laying a straightedge across the graph we may estimate 52% as midway between the peaks and valleys. The cycle time is estimated from midrise to midrise as about 9.2 h so that $t_2 \approx 4.8$ h, which is close to the final estimate.

The results of this section are summarized in Table I. The doubling time, $T_d = \ln 2/m$ is also included. The above section shows that an easy and rapid analysis can yield approximate values for the important parameters. It is clear that the data cannot be fitted without allowing for cell loss, since α and m differ significantly.

B. Determination of Optimal Parameters

In order to determine the best set of cell cycle and growth parameters for each experiment including growth experiments for synchronized cells (exps. 1–10) and labeled mitosis experiments (exps. 11, 12) it is necessary to use a computer fitting procedure in which the parameters are varied until deviation (Dev) is minimized.

$$\text{Dev} = \sqrt{\Sigma(F_i - \bar{F}_i)^2/(n - 1)}. \tag{10}$$

F_i is the value of the data at the ith point, $\bar{F}_i$ is the value given by theory for that point, and n is the number of points fitted.

Synchronous Growth Experiments. The analysis of section A indicated that

cell loss and an initial growth fraction less than one are indicated for the two long experiments (exps. 1, 2). It can be shown[2,3] that incorporation of cell loss into the model does not affect the asymptotic formula derived in Eq. 1 and therefore we may set

$$(dP/dt)/e^{mt} = (P_0/2^a T_c)[1 + \cos([2\pi t/T_0] + \delta)e^{-\lambda t}] \qquad (11)$$

where $T_0 = T_c/[1 + \sigma^2 \ln 2/T_c^2]$ and the other constants are defined in Eqs. 2 and 9. A phase constant, δ, has been inserted into the cos term in order to allow for the fact that the peak of the cohort of cells may be advanced beyond the beginning of G_1. δ could be negative if any delay due to handling is greater than this advance. The left-hand side of Eq. 11 is taken from the experimental points. The right-hand side is the theoretical F of Eq. 10 with the parameters T_C, σ, δ, and P_0 to be obtained from the fit (see Figs. 4 and 5). The results are shown in Table II, along with the other parameters obtained from these four. The parameter m obtained from log-phase growth is also given. Eq. 11 is an asymptotic approximation and is appropriately fit to the later oscillations for exps. 1 and 2. In Fig. 4 we show the fit to Eq. 11 for the data of exp. 1. The data taken over more than two days demonstrate both the value of the asymptotic formula and the adequacy of the experimental methods to maintain constant conditions. In Fig. 5 the corresponding fit is shown for exp. 6 which is typical of the shorter experimental runs. A fit between about 8–24 h is adequate for the eight shorter runs (exps. 3–10). We demonstrated this (Table II) by obtaining essentially the same results for exps. 1 and 2 when these experiments were fit for that time domain as when they were fit over the much longer runs.

For the two long runs, m has already been obtained from log-phase growth as described in Results (part A) and is therefore not obtained from the fit but included in the experimental side of Eq. 11. This tends to assign equal weight to all points fit. For the short runs where this information wasn't available fits were obtained for several different values of m varying between 0.06–0.1. It was found that even for this

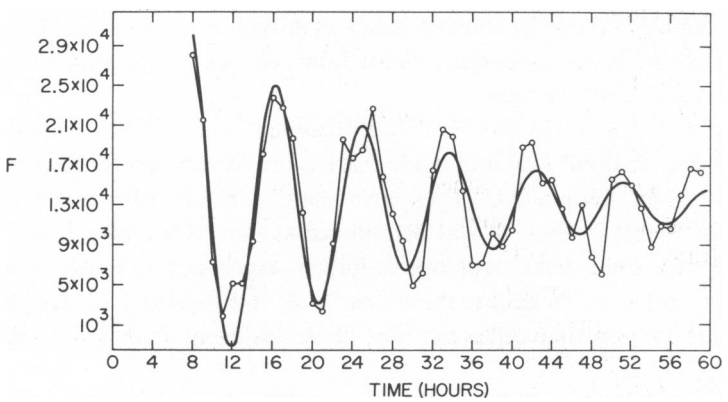

FIGURE 4 F (theory and experiment) vs. time for exp. 1 (long run). (F is defined as $\dot{P}/e^{mt}$. See Eq. 11)

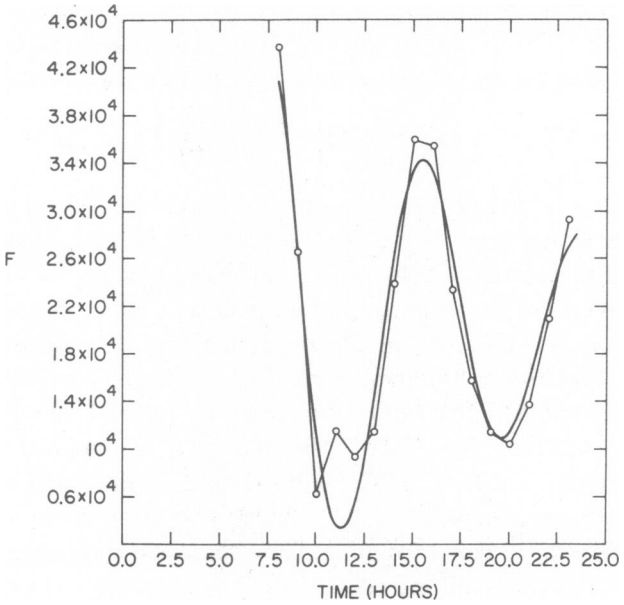

FIGURE 5 F vs. time for exp. 6 (short run).

wide range of m, the four fitted parameters did not vary widely. Typically, σ varied less than 5%, T_c less than 1%, δ and P_0, 25–50%.

The actual range of m is probably much less than this. If κ has a value in the range 0.009 ± 0.002 as is reasonable from exps. 1 and 2, then δ and P_0 vary by less than about 5% and σ and T_c by less than 1%.

C. *Percentage Labeled Mitosis*

Labeled mitosis curves were analyzed for two experiments which we denote by numbers 11 and 12. (See Table III.) The experiments were performed under steady-state conditions similar to those for the synchronous growth experiments. This allows estimates to be made for the individual transit times as well as the generation time, T_c, and σ^2, the variance for the cycle.

The theoretical curve was obtained by a procedure which first obtained a fit using the Fourier ring method[6] (13) and finally varied the parameters to obtain the final fit by the age-transfer method (1). (These give identical results when the first four data points are omitted.) The best fit was determined as that which gave a minimum value for Dev (Eq. 10). Interpretation of labeled mitosis experiments involves the additional parameters t_i and σ_i which denote the transit time and its standard deviation for the ith phase of the mammalian cell cycle. The phases as usual, from one to four, are G_1, S, G_2, M.

The parameters were selected with various different assumptions. First all the

[6]This method is based in part on the asymptotic analysis of reference 10 and hence yields the same parameters.

TABLE II

SUMMARY OF PARAMETERS OBTAINED FROM SYNCHRONOUS GROWTH OF P-815 CELLS IN SPINNER CULTURE

Exp. no.	T_c	σ	$(T_0/2\pi)\delta$	P_0 cells $\times 10^5$	m	α	κ	σ/T_c	Initial growth fraction	Dev $\times 10^3$
	h	h	h		h^{-1}	h^{-1}	h^{-1}			
1	8.840	1.282	+1.11	2.26	0.0704	0.0790	0.0086	0.1450	0.88	2.79
2	7.458	0.916	−0.94	0.96	0.0832	0.0934	0.010	0.1228	0.59	1.73
3	8.39	1.32	+0.98	2.80	0.074	0.0835	0.009	0.1573	0.93	3.15
4	8.10	1.26	+0.47	3.37	0.078	0.086	0.008	0.1553	0.99	5.48
5	8.51	1.31	+0.39	2.31	0.074	0.0825	0.0085	0.1536	0.84	3.20
6	8.45	1.49	+0.97	3.44	0.075	0.083	0.008	0.1760	0.97	3.47
7	7.75	1.20	+0.62	2.71	0.083	0.090	0.007	0.1549	0.88	3.10
8	7.83	1.19	+0.38	2.64	0.082	0.089	0.007	0.1526	0.89	3.67
9	7.95	1.22	+0.78	2.89	0.080	0.088	0.008	0.1535	0.98	3.58
10	9.15	1.58	+1.88	2.91	0.069	0.076	0.0075	0.1722	0.86	6.09
1*	8.96	1.44	+1.30	2.35	0.0704	0.0780	0.0076	0.161	0.92	2.78
2*	7.74	1.14	−0.48	1.00	0.0832	0.0902	0.007	0.147	0.61	1.68

Exps. 1 and 2 are fit to the asymptotic formula (Eq. 11) from 8 to 59, and 8 to 49 h, respectively. Exps. 3–10 are all fit from 8 to 23 h inclusive. The asterisked numerals are exps. 1 and 2 fit between 8 and 23 h, and 8 and 28 h, respectively, to test the effect of fitting over a shorter running time.

The parameters fit are T_c, the cycle time; σ, the standard deviation of the cycle time; $(T_0/2\pi)\delta$ the advance in the cycle; P_0, the initially proliferating population.

The parameter α is obtained from Eq. 11, while the growth rate, m, is obtained by the straight-line fit to log-phase growth for exps. 1 and 2. The death rate κ is then $\alpha - m$. For exps. 3–10, no log-phase data is available so m was adjusted arbitrarily (after a trial gave an initial value of α) to give a death rate κ between 0.007 and 0.009 as is consistent with the values from exps. 1 and 2.

The deviation gives a relative measure of goodness of fit (see Eq. 10).

The initial growth fraction is obtained by dividing P_0 by the initial number of cells per cubic centimeter obtained by averaging over the measured number at 0, 1, and 2 h.

smoothed data points were fit against theory. The resulting parameters are given in Table III beside numerals 11* and 12*. The "best fit" obtained (not shown) fits the initial rise well but the first peak of the experimental points seems too broad to fit the theoretical curve, and the best value for T_c is too long to properly fit the descending limb of the third peak.

Our conjecture is that there is a division delay during the first division after labeling due to handling of the cells which gives an exaggerated breadth to the first peak. This idea is tested by dropping the first four data points and obtaining the best fit of the theoretical curve to the rest of the points. The resulting fit is excellent and is shown in Fig. 6 for exp. 11. The resulting parameters are given beside numerals 11** and 12** in Table III.

The values for T_c obtained by deleting the first four points are closer to each other and to those obtained from the synchrony experiments than those obtained otherwise. This is also true for the t_2 values which are only 4% apart for 11** and 12** but are 15% apart for 11* and 12*. The values for t_1 and t_3 are not very close between the

TABLE III

PARAMETERS OBTAINED FROM LABELED MITOSIS EXPERIMENTS

Exp. no.	Δ	t_1	t_2	t_3	t_4	T_c	σ_1	σ_2	σ_{34}	σ	σ/T_c
11*	0.0	1.65	5.2	1.8	0.75	9.4	0.35	1.04	0.47	1.15	0.122
12*	0.0	1.75	6.0	1.9	0.70	10.35	0.25	1.26	0.71	1.47	0.142
11**	0.0	1.41	4.79	2.67	0.0	8.87	0.28	0.96	0.53	1.13	0.127
12**	0.0	0.6	5.00	3.69	0.0	9.36	0.12	1.00	0.74	1.25	0.133
11***	1.75	3.18	4.79	0.91	0.0	8.88	0.65	0.99	0.19	1.20	0.135
12***	2.5	3.20	5.02	1.16	0.0	9.38	0.72	1.12	0.26	1.36	0.145
Best estimates											
11 *b*	0.68	2.09	4.79	1.80	0.19	8.87	0.43	1.00	0.41	1.16	0.131
12 *b*	1.53	2.18	5.02	1.90	0.26	9.36	0.49	1.13	0.49	1.32	0.141

All values are in hours.

*These values are from a fit using all experimental points. The optimal parameters fit only the initial rise well because the optimal cycle time determined is too long for the rest of the data.

These values are from a fit to the labeled mitosis experiments, but deleting the first four data points. In this set as well as for*, the individual σ_i were not varied, but we use the simplifying assumption that σ_i/t_i is the same for each of the three states with values chosen so that the total σ is selected by the fit. Note that G_2 and M are combined in these runs with a combined standard deviation denoted σ_{34}.

***These values are from a fit to the same experimental data without the first four data points, and in addition a delay, Δ, is subtracted from the time of each experimental point to simulate possible delay due to handling.

(*b*) This is a summary of the best parameters using additional information from reference 9 to determine t_4.

two experiments however. (t_4 is set equal to zero for convenience. The meaning of t_4 equal zero is that t_3 includes M as well as G_2). We believe that the discrepancies in these parameters are due to the effect of the initial division delay on the remaining experimental data. To test this idea we again fit the data, deleting the first four points, but allowing a delay, Δ h, which is subtracted from the time of each experimental point.

The results for particular values of Δ are given beside numerals 11*** and 12*** in Table III. The fit is equally good for values of Δ between zero and those given beside numerals 11*** and 12*** and appears identical to that shown in Fig. 6 for exp. 11. The values for t_2 and T_c remain essentially unchanged for Δ in this range, while σ changes only by a few percent. The value of t_3 and Δ are however coupled together by the fitting process so that their sum remains constant. Since T_c and t_2 remain essentially constant and t_4 is fixed at zero by assumption, t_1 is also determined by t_3. The values for t_1, t_3, and Δ therefore cannot be determined by this procedure.

We write down a "best" set of parameters for these experiments beside numerals 11 *b* and 12 *b*. In this set we use the values (invariant for various Δ) for t_2, and T_c obtained for 11** and 12**. Since the fit to the initial rise in the PLM curve is good for 11* and 12* and this depends mainly on t_3, we obtain the value for t_3 using this procedure. A value for t_4/t_c is obtained from reference 9, Table III (exps. 1 and 2 in that table refer to the same data we label 11 and 12 here). Now according to the ob-

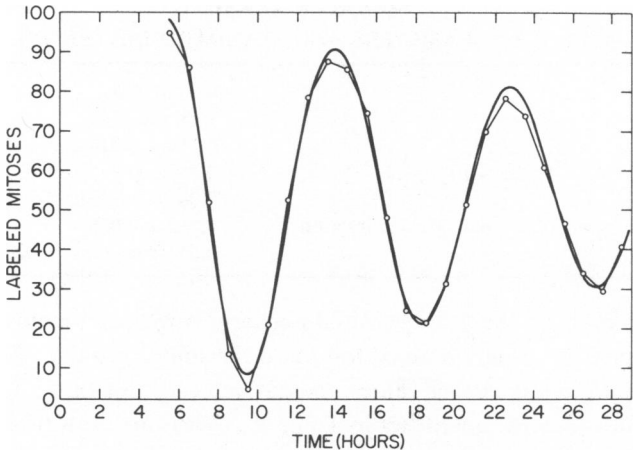

FIGURE 6 Labeled mitosis vs. time for exp. 11. First four data points dropped, but $\Delta = 0$.

servation above, the value for $t_3 + t_4 + \Delta$ is given by the value for t_3 in 11** and 12**, therefore our assumptions give a value for Δ, and we then choose the σ obtained for that Δ. The value for t_1 is given by the difference $(T_c - t_2 - t_3 - t_4)$. The values of the ratios t_2/T_c and $(t_3 + t_4)/T_c$ obtained here are compared in Table IV with the values obtained in reference 9, Table III, values *b* for exps. 1 and 2. The latter values were obtained by the independent methods of percent labeling, and microflouorimetry of unlabeled cells to distinguish G_1 cells from G_2 and M cells by the difference in DNA content per cell.

DISCUSSION AND MODELING

We may now determine average parameters and their standard deviations for the experiments analyzed in the previous section. (See Table II for individual experiments. Only t_2/T_c is averaged from Table IV.) The results are presented in Table V. The

TABLE IV

COMPARISON OF THE VALUES OF THE RATIOS t_2/T_c AND $(t_3 + t_4)/T_c$

	Exp. 11		Exp. 12	
	11 *b*	Ref. 9	12 *b*	Ref. 9
t_2/T_c	0.54	0.51	0.54	0.54
$(t_3 + t_4)/T_c$	0.22	0.21	0.23	0.22

The closeness of the Table III values for the ratio $(t_3 + t_4)/T_c$ to the reference 8 values merely confirms that the use of the * values for t_3 was a good guess. The ratio t_2/T_c is invariant to the various values for Δ however and it is satisfying that it is quite close for both experiments to the ratio for this value obtained by a quite different method of analysis in reference 9. The final values for σ and T_c are very close to those obtained here by analysis of desynchronization experiments. This supports use of the same underlying model to analyze the two different experiments as previously assumed (1).

TABLE V

AVERAGE PARAMETERS AND STANDARD DEVIATIONS

T_c	8.24 ± 0.52
σ	1.28 ± 0.18
σ/T_c	0.154 ± 0.014
$\tau = (T_0/2\pi)\delta$	0.84 ± 0.47
κ	0.0093 ± 0.0010
Initial growth fraction	0.913 ± 0.056
t_2/T_c	0.54 (from exps. 11 and 12)

value for τ contains both the average initial position as well as any possible delay due to handling. Since the value obtained for τ and the initial growth fraction in exp. 2 are quite different from the other values, the parameters from exp. 2 are not included in the average for these two quantities in Table V. With this exception, the values for τ in Table II are positive indicating an average initial position somewhat advanced in the cycle. The average for τ is in good qualitative agreement with the initial position indicated by modeling other data below. The average obtained for the initial dividing fraction is very close to what would be obtained if the nondividing fraction was about equal to the fraction lost during one cycle (about 8% using κ and T_c from Table V.) This leads to the conclusion that cell loss is probably due to loss of viability and that nonviable cells remain in suspension for a time of the order of a cycle without lysing.

The average cycle times have a reproducibility to within about 6%. This small variability is to be expected since it has been previously observed that cell cycle times in different experiments are not necessarily identical. It should be noted that the P-815X-d cell line used for kinetic analysis of asynchronous cultures (variations with time of mitotic labeling indices, exps. 11 and 12) is different from the P-815X2 line used for preparation of synchronous cultures (exps. 1- 10). Furthermore, the medium contained undialysed serum in exps. 1–10 and dialysed serum in exps. 11 and 12. For this reason the results of exps. 11 and 12 were not included in obtaining the average values for T_c and σ given in Table V. Nevertheless, the values obtained from the labeled mitosis experiments (see 11 *b* and 12 *b*, Table III) for T_c and σ, as well as σ/T_c, are quite close to the averages obtained from the desynchronization experiments.

The variation in cell cycle times observed in different individual experiments performed under apparently identical conditions may be attributable to different lots of horse serum used. Variations in generation time of Chinese hamster and HeLa cells attributable to different lots of serum have, in fact, been previously described (15, 16). Furthermore, it cannot be excluded that cell populations with shorter cell cycle times were selected as a result of prolonged culture in vitro.

The average value of the coefficient of variation, σ/T_c, is exactly what we would obtain from $\bar{\sigma}/\bar{T}_c$ if σ and T were independent, although the standard deviation of σ/T_c is slightly less than expected. Since σ/T_c is well defined with a small variance over many different experiments, it can be considered to be a measure of inherent biological variability. This parameter is also important for making predictions about the time response of a synchronous population to outside perturbations (e.g., radiation [17]).

The use of asymptotic formulas for obtaining parameters has the advantage of allowing the use of known procedures for choosing an optimal path in the multi-dimensional parameter space to proceed to the best fit. These procedures require the use of derivatives with respect to the various parameters and therefore make use of specific formulas which we have developed. We now consider the effect of different initial distributions on some experiments involving the synchronous population. This is done by inserting appropriate values of the parameters into the computer model using the cell-transfer method of Kemmy (1) along with several different distributions of the population. Now other effects due to handling for example may have a strong effect on the initial part of the experimental curves and it is the early part of the curves which would have information available about the initial population distribution. We therefore look only for qualitative differences arising from three different *idealized* initial distributions. These are: a delta function in the beginning of G_1; a delta func-

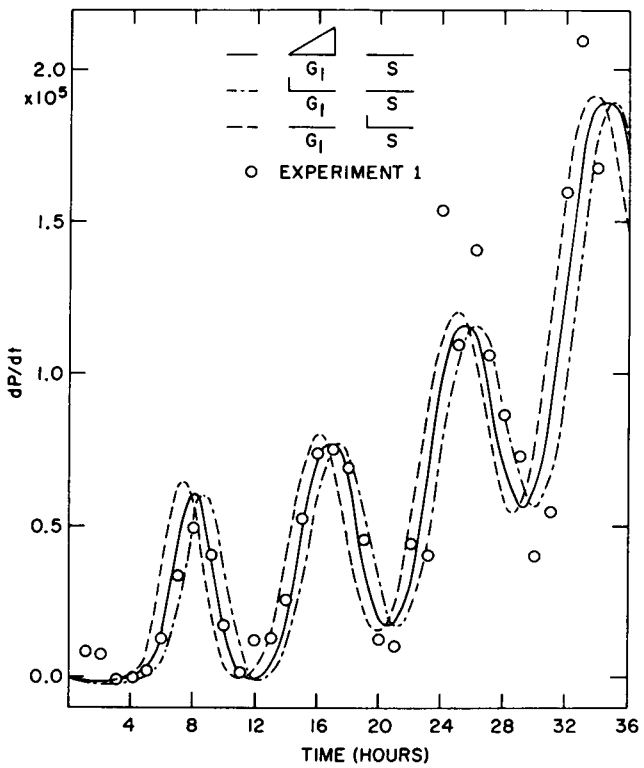

FIGURE 7 Simulation of dP/dt vs. time for exp. 1. The triangle indicates a linear distribution in G_1 starting with 0 cells/h just divided, to approximately 60 cells/h at age 1.35 h. The other two initial distributions are delta functions at the beginning of G_1 and S, respectively. The parameters follow:

$$t_1 = 1.4, t_2 = 4.95, t_3 = 2.32, t_4 = 0.17, T_c = 8.84$$
$$\sigma_1 = 0.45, \sigma_2 = 1.15, \sigma_3 = 0.5, \sigma_4 = 0.05, \sigma = 1.33.$$

tion in the beginning of S; and a linear distribution in G_1 starting with 0.0 cells/h of age zero in G_1 up to 60.0 cells/h of age 1.35 in G_1 with no older cells. In Fig. 7, we have plotted the theoretical values for dP/dt for the first 36 h following synchronization obtained from the three different initial distributions. The experimental points for exp. 1 are also shown. The cycle parameters used were approximately[7] those for exp. 1. The experimental points and curves for percent in mitosis obtained with the same parameters are displayed in Fig. 8. The effect of moving the cells forward in the cycle is to make the peaks come earlier. The difference in shape of the peaks of these two curves due to these drastically different assumed initial distributions is hardly detectable. We can make the qualitative conclusion that the peak of the initial distribution is somewhat advanced in G_1 from these two graphs since the triangle distribution gives the best results. The mean of the triangle distribution is at about 0.9 h, in rough agreement with the value of τ of 1.1 h obtained earlier for exp. 1 (Table II).

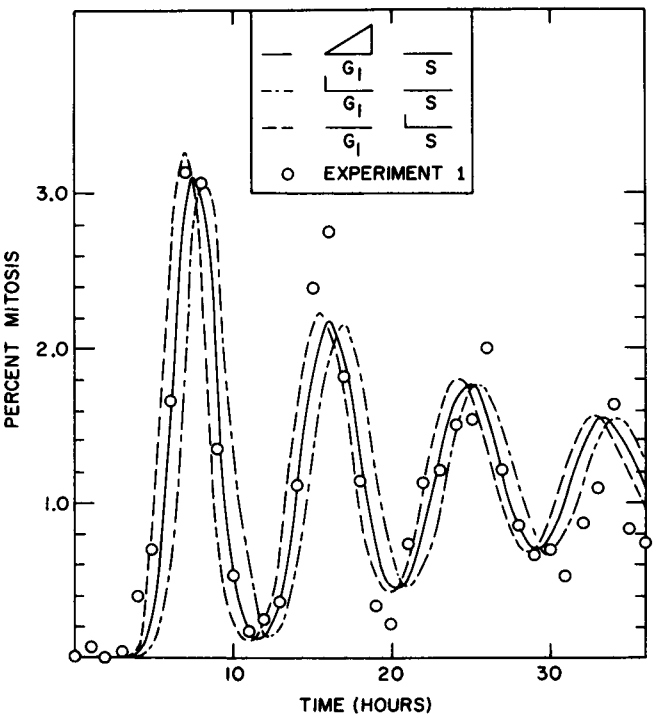

FIGURE 8 Simulation of percent mitosis vs. time for exp. 1 with various initial distributions. Parameters and initial distributions are as described for Fig. 7.

[7] t_1 was somewhat shortened at the expense of t_3 compared with the values obtained in exps. 11 *b* and 12 *b*. This would have very little effect on Figs. 7 and 8, but would tend to shift the graph in Fig. 9 about 1/2 h to the right on the first decrease only.

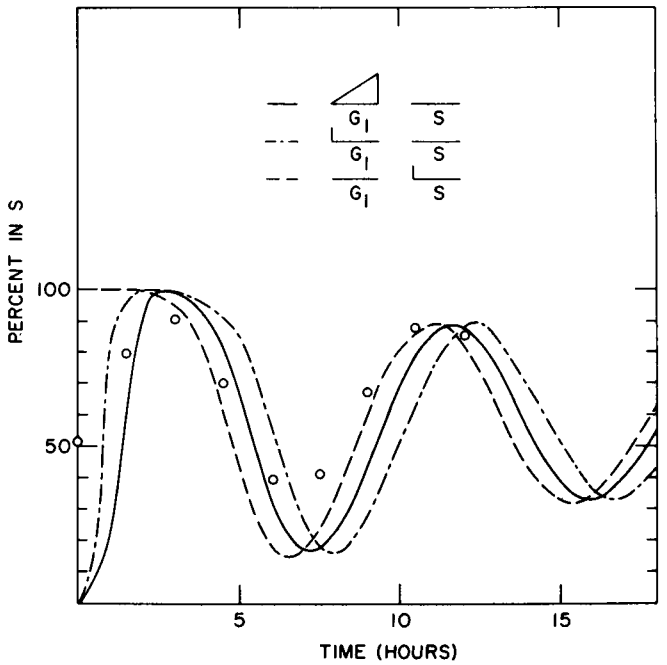

FIGURE 9 Simulation of percent in S vs. time following resuspension of synchronized cells. Parameters and initial distributions are as described for Fig. 7.

The effect of the initial distribution should be more apparent in an experiment which measures labeling index and hence simulations with the same parameters are presented in Fig. 9. The data is from a measurement of labeling index vs. time after resuspension following synchronization that was done as a part of exp. 10 (See Table II).

The theoretical curves are similar in shape to the curve of experimental points except for the initial rise. The maximum labeling index achieved in the experiment (90%) is in rough agreement with the initial dividing fraction for exp. 10 (86%) as might be expected if dead cells are the source of the deficit in both cases.

The experimental points follow a pattern initially appropriate for a mixture of cells late in G_1 and cells early in S as indicated by the initial 52% labeling index. The somewhat shallow minimum of the experimental curve and its too early rise to the second peak are probably explained, at least in part, by cells initially an hour or two into the S phase which arrive in S the second time earlier than would cells from the delta function distribution initially at the beginning of S. This spread into the first part of S would also help explain the shallow first experimental minimum found for almost all the experiments of Table II (see Figs. 4, 5, and 7) as compared with the theoretical curve (Eq. 11) derived from a delta function distribution advanced by an amount τ from the beginning of G_1.

The simulations indicate that the information presently available as to the precise

initial distribution is limited.[8] More detailed labeling index experiments (Fig. 9) could be used to determine initial distribution more precisely when this becomes important. At present the qualitative conclusion that the initial distribution is centered late in G_1 with about half the cells in early S seems justified by the simulations.

The simulations as well as intercomparison of the parameters from the tables indicate that the model is adequate to give a consistent description of data obtained by several different methods. The experimental system gives quite consistent, and within reasonable limitations, reproducible results.

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[8] A further possible complication which was not considered is that there could be a correlation of cycle time with size just after division. Suppose for example that the faster dividing cells are larger than other cells of the same position in the cycle. Then even if we were able to select only G_1 cells, we would tend to get a special population of slow G_1 cells for the first division.