

INTRAPOPULATION KINETICS OF THE MITOTIC CYCLE

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ABSTRACT

Data obtained with time lapse cinemicrographic techniques showed that the distribution of generation times for exponentially proliferating human amnion cells in culture is skewed to the right and that reciprocals of generation times appear normally distributed. As shown for bacteria, the true age distribution is much broader than theoretical distributions which fail to take into account the dispersion of generation times. By means of the technique utilizing autoradiographic detection of tritiated thymidine in cells whose mitotic histories were recorded by time lapse cinemicrography, it was shown that the G1 distribution is similar to the generation time distribution but is more variable. In our experiments, the G2 + prophase distribution resembled the generation time and G1 distributions. The data suggested two possibilities for S: either it is relatively constant, or it is inversely related to the lengths of G1 and G2 + prophase. Since G1 is more variable than the total cycle, and G2 + prophase more variable than the computed sum of S + G2 + prophase + metaphase, it was concluded that the relationships between parts of the cycle are non-random and that compensating mechanisms apparently help regulate the lengths of successive parts of the mitotic cycle in individual cells.

INTRODUCTION

Although some descriptions have been published of intrapopulation variation in generation times for several lines of mammalian cells in culture (8, 15, 22, 27) there are few data on the population dynamics of cells of higher organisms which can be compared with those available for bacteria (12, 13, 18-20). In addition, the mitotic cycle of higher organisms is divisible into several measurable parts, and estimates of the variability of each part have been presented only for the intestinal epithelium of mice (24) and for *Tradescantia* root tips (31). The purpose of this study was to define the proliferation kinetics of mammalian cells in culture and to see how variations in different parts of the

cycle account for intrapopulation variations in generation times.

Presented herein are generation time and age distributions derived from time lapse cinemicrographic studies which demonstrate that these growth characteristics of exponentially growing mammalian cells in culture are similar to those described for microorganisms. We also present determinations of the distributional characteristics of each measurable part of the cycle. Our findings show that the relationships between the lengths of the individual stages of the cycle are non-random and suggest that compensating mechanisms oper-

TABLE I
Summary of Films Used in This Study

Film no.	Mean intermitotic time	No. mitotic cycles measured	SD	Cutoff point to end*	
				Hrs.	Standard deviations in excess of 1 mean generation time
	<i>hr.</i>				
69	19.4	48	±2.7	31.5	4.48
85 (2)	17.9	45	±2.1	23.4	2.58
92	19.4	118	±2.8	27.2	2.82
94	20.7	50	±3.3	28.4	2.33
96	19.9	31	±2.6	26.7	2.59

* Time between birth of last cell to be counted and end of film.

ate which govern the rate at which cells progress through successive parts of the mitotic cycle.

MATERIALS AND METHODS

All studies were carried out on a heteroploid line of human amnion cells (3). The abbreviations for the three parts of interphase are taken from Howard and Pelc (7). G1 is the part of the mitotic cycle from the end of telophase to the beginning of DNA synthesis, S is the period of DNA synthesis, and G2 is the period from the end of DNA synthesis to prophase. P, M, A, and T signify prophase, metaphase, anaphase, and telophase.

Data on A + T + G1 were obtained by a method which allows the radioautographic detection of incorporated tritiated thymidine (T-H³) in individual cells whose previous mitotic histories had been recorded by time lapse cinemicrography (28). Generation time and age distributions were obtained from analyses of these same films. G2 + P was measured by exposing populations of cells grown on coverslips in Leighton tubes to T-H³ (0.25 to 1 μ c/ml; sp. act. 1.9 to 6.7 c/mmole) for 10 to 15 minutes, washing them free of isotope, reincubating, and fixing them at intervals thereafter. The curve obtained by scoring the percentage of labeled metaphases as a function of time since exposure to isotope is used as a measure of G2 + P.

RESULTS

The Total Cycle

GENERATION TIME DISTRIBUTION: Experimental requirements for studying the generation time distribution suggested by Powell (18) and Kubitschek (13) are: (a) Individual cultures which comprise a distribution should be identical. (b) Cultures should be growing exponentially. (c)

TABLE II
Constancy of Generation Time with Aging of Culture

Time period of birth	Mean intermitotic time	No. cycles	SD
<i>hr.</i>	<i>hr.</i>		
0-3.9	17.5	8	±0.8
4.0-7.9	18.8	10	±1.7
8.0-11.9	18.5	14	±2.4
12.0-15.9	18.5	2	±0.6
16.0-19.9	19.1	5	±1.2
20.0-23.9	19.3	9	±1.4
24.0-27.9	20.4	27	±2.5
28.0-31.9	23.7	10	±3.5
32.0-35.9	18.1	3	±3.2
36.0-39.9	18.3	16	±2.9
40.0-43.9	19.4	13	±2.8

The data should not be biased by the method of analysis. To fulfill condition (a), emphasis will be placed upon one film, no. 92, in which 118 generation times were measured. However, this one film is representative of a group of films (Table I), although the number of generation times measurable in each of the others was smaller and there was some variation in generation times between them. Concerning condition (b), all cells in this film, except six which died during the experiment, were actively proliferating and the mean generation time was essentially constant (Table II).

In relation to condition (c), Powell (18) has pointed out that if all mitotic cycles which occur over a finite period of time are included in the findings, the data will be biased toward shorter values, since, toward the end of this period, those cells with longer generation times would not have had

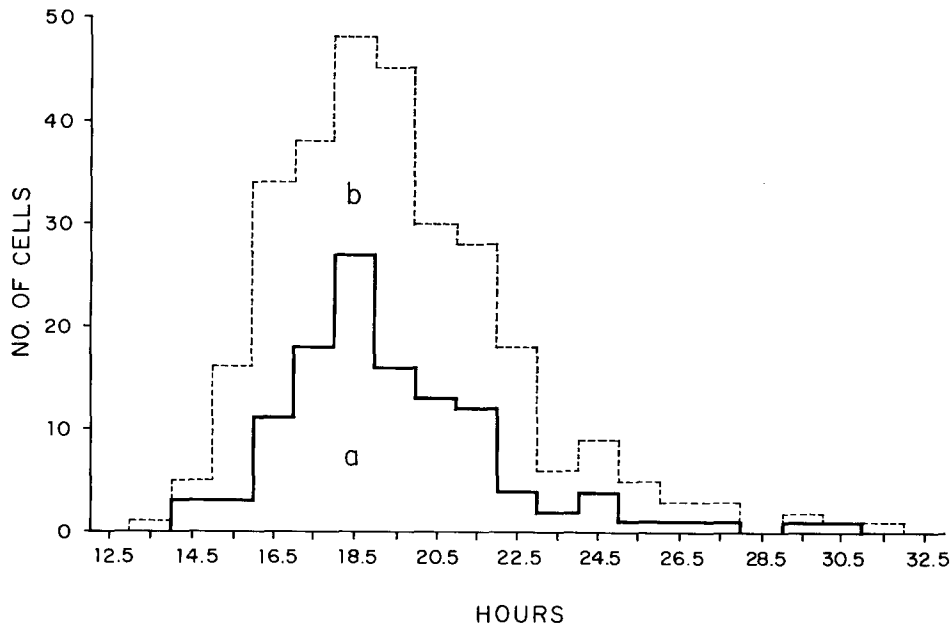


FIGURE 1 Frequency distributions of generation times derived from time lapse films of human amnion cells. Histogram *a* contains only data from film no. 92. Histogram *b* contains data grouped from all five films shown in Table I.

enough time to divide. This problem can be avoided by including in the data only those cell generations where the interdivision times of all cells are observed (13, 18). Powell (18) pointed out that this method is inefficient; and in the present studies on mammalian cells, in which relevant observations can be made for only a few generations, the method would be particularly inefficient. To avoid this bias, the following procedure was adopted. A time point was found, in each film, before which every observable cell born (with the exception of one cell in film no. 85 (2)) still had enough time to divide, and only generation times of cells born prior to this point were included. As a result, no cell was included if born within one mean generation time plus a minimum of 2.33 standard deviations from the end of a film (Table I).

The distribution of generation times shown in histogram *a*, Fig. 1, represents the generation times of the 118 cells taken from film no. 92. The mean generation time for this film was 19.4 hours with a modal value in the 18-19 hour class. Histogram *b* of Fig. 1 is the distribution of a total of 292 intermitotic times obtained from all five films. This distribution has a mean of 19.5 hours and a modal value in the same range as that for film no. 92. If cells from film no. 92 are excluded from the total,

the over-all mean remains the same and, although the modal value shifts one class upward, the shape of the distribution changes very little (see below). Although film no. 92 contributes 40.4 per cent of the measurements in the total population, its time distribution is very similar to the total generation time distribution, and therefore introduces no bias.

THE GENERATION RATE: Kubitschek (12) has shown that the generation time distributions reported in the literature for many kinds of cells are skewed to the right, and that distributions of the reciprocals of the generation times, "generation rates," approach normality.

In Fig. 2 the cumulative distribution of generation rates was plotted on probit paper for the over-all distribution, for the over-all distribution minus film no. 92, and for film no. 92 alone. In each case, the points give a reasonably good fit to a straight line, suggesting that the generation rates of our cells are also normally distributed. The coefficient of variation, as determined from the line drawn by eye to fit the over-all distribution, is 13.6 per cent. A similar plot of the generation time distribution does not give as good a fit to a straight line (see curve A, Fig. 8).

OVER-ALL AGE DISTRIBUTION: A further indication that the kinetics of our populations fit

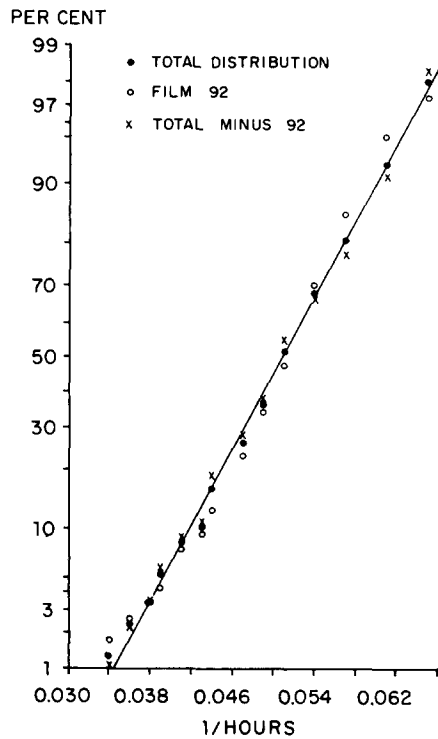


FIGURE 2 The cumulative distribution of generation rates derived by time lapse cinemicrography. Closed circles, total distribution for all five films; open circles, distribution for film no. 92; crosses, total distribution excluding data from film no. 92. Line is drawn by eye to fit total distribution.

those which occur in microorganisms is the age distribution (Fig. 3). Age is defined as the time since the previous division of a cell. In Fig. 3 the solid smooth line is the theoretical age distribution derived by many investigators (2, 9, 10, 17, 19, 25, 29) for exponentially growing populations in which all cells are assumed to have identical generation times, and the dashed line is an approximation of a theoretical age distribution calculated by Powell (19) which takes into account the effects of the dispersion of generation times. He showed that this second line was in agreement with distributions he actually observed. The histogram in Fig. 3 is the age distribution (age here defined as the time since the beginning of anaphase) at the time of fixation of all cells from the five films of Table I. The age distribution thus obtained from our cell populations resembles that expected, on the basis of Powell's work, despite an apparent excess of older cells.

Parts of the Cycle

G1: Immediately before fixation, all cultures were exposed to T-H³ and autoradiographs prepared. The cells which appeared in the films were also identified in the autoradiographs, so that it was possible to determine both the age of each cell at the time of fixation, and whether or not it was synthesizing DNA just before fixation.

Data concerning the percentage of cells synthesizing DNA as a function of time since their previ-

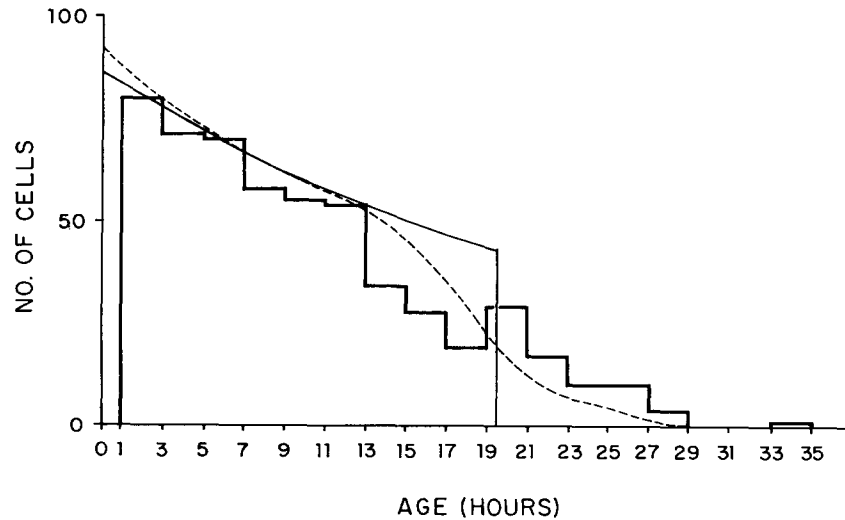


FIGURE 3 Age distributions of exponentially growing cells. Solid smooth line, theoretical age distribution assuming that all cells have identical generation times. Dashed line, an approximation of the theoretical age distribution derived by Powell (19) which takes into account the characteristic dispersion of generation times. Histogram, age distribution of human amnion cells at the completion of our experiments. No cells born within an hour of the end of photography are included in the data.

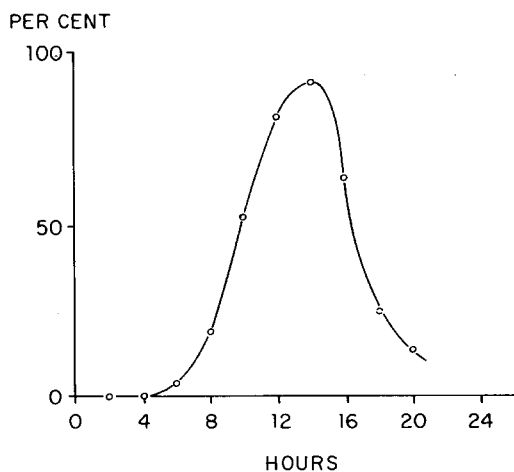


FIGURE 4 Distribution of the percentage of cells in various age classes which incorporate tritiated thymidine during a short labeling period. Data are derived from the experiment of film no. 92.

ous division may be used to measure the A + T + G1 period. (Because anaphase and telophase are short relative to the length of G1, for convenience and with the necessary reservations we shall hereafter consider them to be part of G1). Since G1 appears to be the most variable part of the cycle (see 26 for references) and since variations exist between some of the individual cultures, only data obtained from the experiment of film no. 92 are included in Fig. 4, which is a plot of the percentage of labeled cells as a function of cell age. In this experiment, we were able to obtain both labeling data and mitotic histories on 140 cells. The shape of this curve is governed by a number of factors. The slope of the ascending part reflects variations in the time required for individual cells to get through G1 and into S. The minimal time for G1 (based on the youngest cell) to acquire label is 6.85 hours. The 50 per cent level, which may be considered a median G1 for the population, is 9.8 hours. The fact that the highest point on the curve, at 14 hours, is less than 100 per cent indicates that some cells completed synthesizing DNA before others started. The curve descends beyond the peak because fewer cells are entering S and an increasing percentage are completing S. If we subtract 2.2 hours for G2 + P and 0.3 hour for metaphase (see below) from the mean intermitotic time of this population, 19.4 hours, we should expect about half the cells to be able to complete S by 16.9 hours after their previous division, and therefore should expect the declining line to cross the 50 per cent level at about this time. Considering the

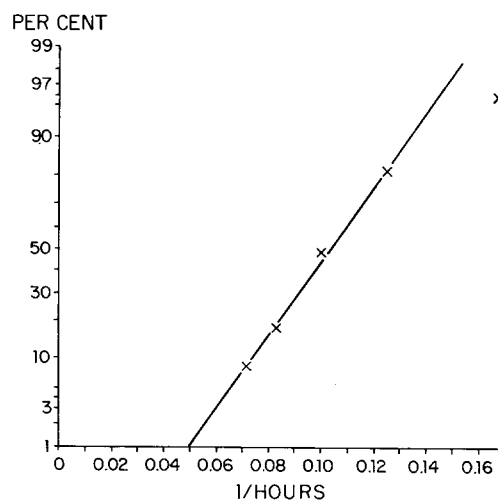


FIGURE 5 Cumulative distribution of the G1 "rates" derived by taking the reciprocals of G1 times as determined from the ascending line in Fig. 4.

fact that the points for the older age groups represent small populations (Fig. 3), there is a good fit between expected and observed results.

Up to the point where the line is affected by cells leaving S, the ascending curve in Fig. 4 represents a cumulative distribution of G1 times. When this is plotted on probit paper, the resultant curve (*b*, Fig. 8) roughly parallels the generation time distribution but is not quite so steep. The reciprocals of the G1 times, the G1 "rates," are plotted in Fig. 5. One point is off the line because one cell (out of 24) whose age, 6.85 hours, was close to the limit of its age class (5.0–6.99 hours) was labeled. The other points give a fairly good fit to a straight line, suggesting that the data are consistent with the possibility that, as in the case of the total cycle, the G1 rates may be normally distributed although the times are skewed.

G2 + P: Measurement of G2 + P is generally obtained from a curve such as that in Fig. 6 showing the percentage of metaphases which are labeled as a function of time since a short exposure of the cells to T-H³. The time at which the line crosses the 50 per cent level is the median G2 + P and is usually considered the average time required for cells to get from S to metaphase. The points for the curve shown in Fig. 6 are derived from four experiments. The most rapid cells require 1.0 to 1.5 hours to get from the end of S to metaphase, and by 4 hours nearly all the cells in metaphase had been in S when the cells were exposed to label. The 50 per cent level is reached at about 2.2 hours. Stanners and Till (29) showed that by taking the derivative

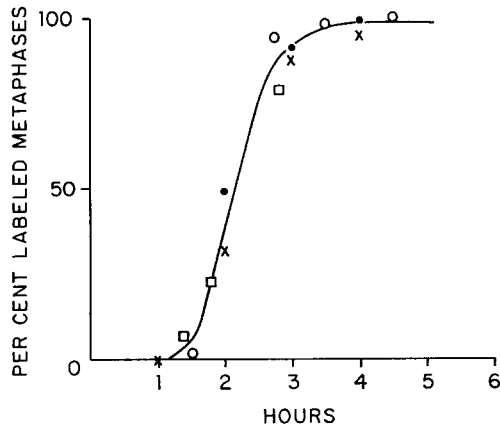


FIGURE 6 The percentage of labeled metaphases as a function of time since cells were exposed for a brief period to tritiated thymidine. The four different symbols indicate data derived from four different experiments.

of the curve expressing the percentage of metaphases which are labeled, a distribution showing the relative frequencies of G2 + P times is obtained. Since the curve we thus obtained was skewed slightly to the right, resembling the generation time distribution, the cumulative frequency of the reciprocal of the G2 + P times was plotted on probit paper, where the points give a reasonably good fit to a straight line (Fig. 7). In analogy with the situation with G1 and the complete mitotic cycle, in these experiments, the rates at which cells proceeded from the end of S to metaphase appear normally distributed whereas their times appear to be skewed.

S: Taking the width of the curve in Fig. 4 at the 50 per cent level as an indication of the average length of S, the mean time required for cells to synthesize DNA is 6.8 hours. Another way to measure S is to subtract the median times for G1, G2 + P, and M from the median generation time (see Fig. 8); this gives a value of $18.4 - (9.8 + 2.2 + 0.3) = 6.1$ hours.

A third method is based upon the percentage of cells which take up T-H³ during a short labeling period. According to this procedure, the percentage of cells in S should equal the percentage of the total area under a theoretical age distribution curve (smooth curve, Fig. 3) which lies above that part of the cycle taken up by S. James (9) shows that the difference between the true and theoretical age distributions does not greatly affect the calculation. Since the percentage of cells in S and the

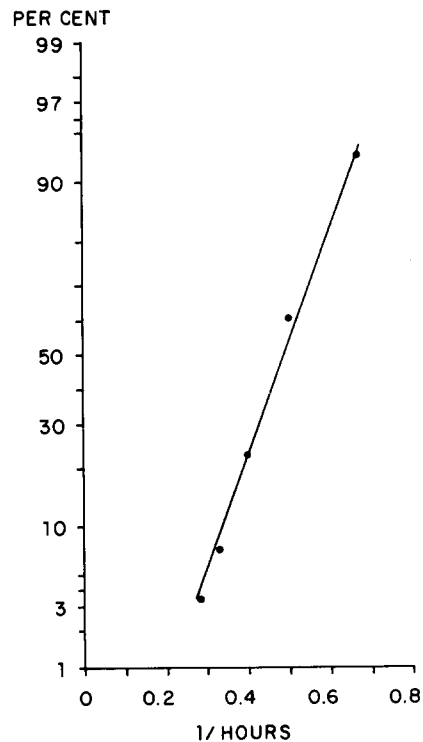


FIGURE 7 Cumulative distribution of G2 + P "rates" derived by taking the reciprocals of the G2 + P times as determined from the line in Fig. 6.

generation time are known, the total area which represents S can be determined. Since G2 + P and M are also known, the upper time limit for S can be estimated. By properly locating the known area under the curve and seeing how far to the left it extends, the length of G1 can be read off the abscissa. Analyzed in this way, our data yield a G1 of 9.9 hours and an S of 7.3 hours.

Relationships between Parts of the Cycle

The distribution curves of times or "rates" for the total mitotic cycle, G1, and G2 + P appear to have similar shapes. Since the cumulative distributions of G1 and generation times are so much alike, and G1 takes up so much of the cycle, the cells with long G1's must, in general, be those with long generation times. Fig. 8 shows that the range of values between the 5 and 90 per cent levels for G1 is 0.9 hours greater than the range between the same levels for the total cycle. This means that G1 has more than enough variation to account for all the variation in generation times and suggests that the rest of the cycle (S + G2 + P + M) tends to

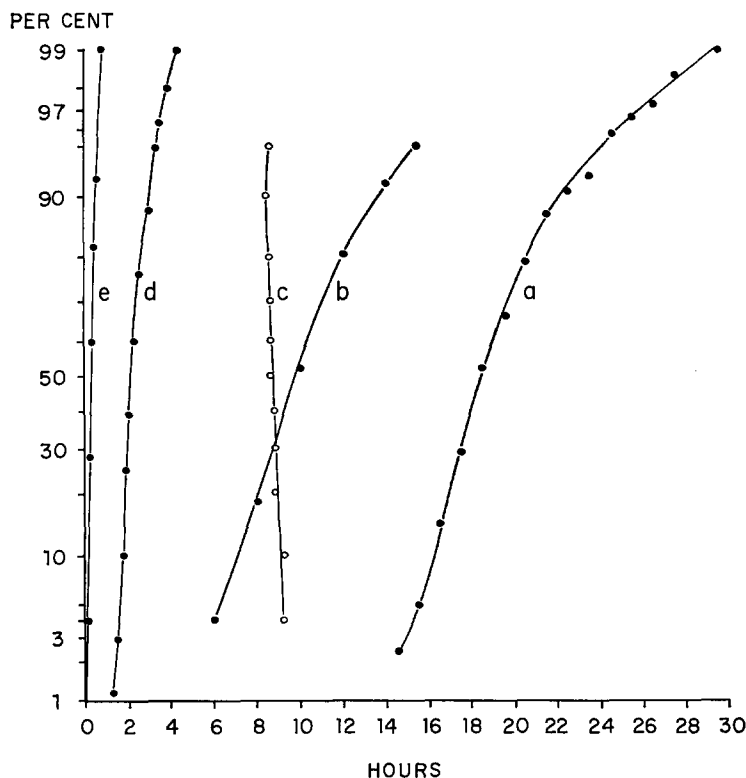


FIGURE 8 Cumulative distributions for the various time parameters of the mitotic cycle. Curve *a*, cumulative distribution of generation times. Curve *b*, cumulative distribution of G1 times. Curve *c*, cumulative distribution for the sum of S + G2 + P + M derived by direct subtraction of the G1 distribution from the generation time distribution; see text for explanation of the anomalous behavior of this curve. Curve *d*, cumulative distribution of G2 + P times. Curve *e*, cumulative distribution of meta-phase times.

become shorter as G1 gets longer. The actual distribution of S + G2 + P + M obtained by subtraction is shown in Fig. 8, curve *c*, to be a nearly vertical line with a coefficient of variation of 2.3 per cent. This line shows the anomalous behavior of leaning to the left because G1 is slightly more variable than the total cycle.

The cumulative distributions for G2 + P and M are also shown in Fig. 8. The G2 + P distribution between the 5 per cent and 90 per cent levels ranges from 1.6 to 3.0 hours with a coefficient of variation of 18.2 per cent. In this case, the range for G2 + P (1.4 hours) is greater than the range for the sum of S + G2 + P + M (0.9 hours).

When the distributions for S + G2 + P + M and for G2 + P are known, information concerning the distribution of S can be obtained. Since G2 + P is more variable than the sum of S + G2 + P + M, the relationships between the lengths of

S and G2 + P in individual cells are apparently not random. Therefore, two kinds of relationships between S and G2 + P can be postulated:

A. The first is that cells with a long G2 + P are those which have the slightly longer S + G2 + P + M. The appropriate subtraction of G2 + P from S + G2 + P + M leads to a distribution for the sum of S + M (Fig. 9, curve *a*) which has a coefficient of variation of 4.6 per cent. Since M is short and only slightly variable in terms of actual time, this line indicates that S is fairly constant with, perhaps, a slight tendency to be longer when G1 is long. Under this hypothesis, G2 + P tends to be short when G1 is long.

B. The second possibility is that cells with a long G2 + P are those which have the slightly shorter S + G2 + P + M. The appropriate subtraction gives a distribution for S + M which has a coefficient of variation of 10.8 per cent (Fig. 9, curve

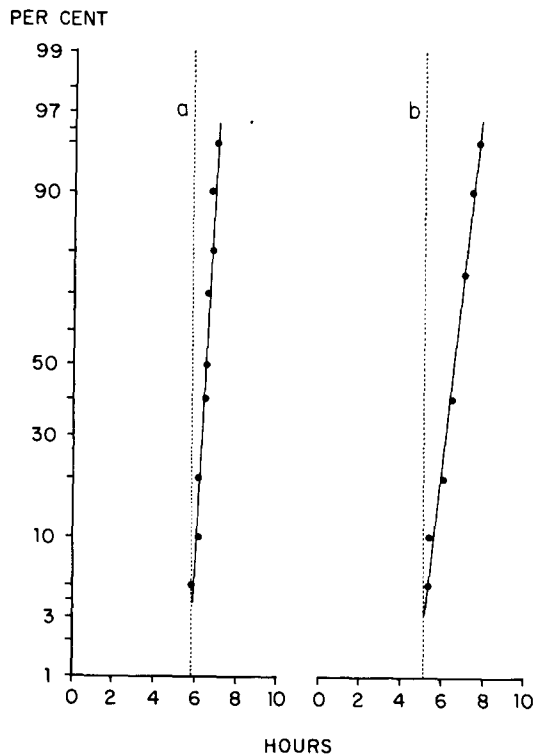


FIGURE 9 Cumulative distributions of the lengths of S. Curve *a*, computed according to hypothesis A in text. Curve *b*, computed according to hypothesis B in text. Dotted lines are vertical reference lines which indicate the shape of the S distribution if all S times were identical.

b). Under this hypothesis, S is inversely related to both G1 and G2 + P, so that when G1 is long, S is short and G2 + P is long, and when G1 is short, S is long and G2 + P is short. According to either hypothesis, a straight line is obtained between the 5 and 95 per cent levels, suggesting that, whatever the degree of variability, the distribution of lengths of S is approximately normal.

Table III contains a summary of the coefficients of variation of the times and rates for the total cycle and its measurable parts. G1 and G2 + P are equally variable in relative terms and S is much less variable.

DISCUSSION

Comparison of the age and generation time distributions presented here and those reported for microorganisms shows that the population kinetics of the two systems are quite similar even though in mammalian cells the scale extends over a longer period of time. However, this correspondence is not complete since the coefficient of variation for our generation rate distribution, 13.6 per cent, is intermediate between those reported by Kubitschek (13). Since the variability in generation times or rates represents the variabilities accumulated

TABLE III
Relative Variations of the Different Parts of the Mitotic Cycle

	Coefficients of variation	
	Rate	Time
	<i>per cent</i>	<i>per cent</i>
Total cycle	13.6	14.4
G2 + P	21.3	18.0
A + T + G1	22.8	21.2
S + G2 + P + M		2.3
S*	3.9	4.6
S†	10.4	10.8

* According to hypothesis A in text.

† According to hypothesis B in text.

from each part of the cycle and is affected by any non-random relationships between individual parts, we tried to obtain distributions for each measurable portion. The data indicate that the distributions of the lengths of the total mitotic cycle and of the G1 and the G2 + P parts of the cycle may all be skewed to the right. The cumulative distributions of the reciprocals of the times, the

rates, when plotted on probit paper, tend to fit straight lines, indicating normal distributions of rates for each of these quantities. On the other hand, the distribution of S times appears normal. This suggests that the factors regulating the length of S are different from those determining the lengths of G1 and of G2 + P. However, not all experiments with bacteria have produced skewed distributions of generation times; and the skewness observed has been explained in various ways (11, 21). Also, Stanners and Till (29) clearly showed that the distribution of times of at least one part of the mitotic cycle of mammalian cells can fluctuate considerably. In two different experiments with the same cells, those authors obtained two kinds of distributions for G2 + P, one normal, the other skewed.

It seems axiomatic that a cell must attain some critical state or carry out some critical event before DNA synthesis can begin. Considerable evidence shows that the time required to arrive at this crucial moment covers a wide range and is responsible for much of the variation observed in intermitotic times (see 26). According to our data, there is more than enough variability in G1 to account for all the intrapopulation variation in intermitotic times. Subtracting the G1 distribution from the generation time distribution, we obtain a line for the sum of S + G2 + P + M which suggests that this sum is nearly constant, tending to be shorter when G1 is prolonged.

In regard to the relationships between the lengths of successive parts of the cycle, one of the possibilities indicated by our data (see "Relationships between Parts of the Cycle," under Results) is that S is constant or varies but slightly in proportion with the length of G1. This would indicate that the DNA-synthesizing system is highly ordered and integrated, and functions only at a characteristic rate and only in cells which are well prepared by the events of G1. The literature contains a considerable amount of data consistent with this possibility. McDonald (14) has stated that one of the interpretations possible for her data is that the S period for the macronucleus of *Tetrahymena pyriformis* is constant even though G1 and G2 may vary considerably from cell to cell. Also, some data derived from determinations of the percentage of labeled mitotic cells as a function of time since cells were exposed to T-H³ can be interpreted to mean that S approaches constancy within a population of cells. Such data are derived from experiments

like that shown in Fig. 7 except that our experiment was carried out for only a short period of time. When the time of the experiment is extended, the line descends from the maximum to a trough. Quastler and Sherman (24) state that the shape of this descending line depends upon the rate of entry of cells into the S period. If they enter and exit at the same rate, suggesting a fairly constant S, the ascending and descending curves will be symmetrical, which appears to be the case in at least one study (5). More often, however, these curves are asymmetrical (23), a point to be considered below.

As McDonald (14) points out, if S really is constant there must be an inverse relationship between the lengths of G1 and G2 + P. This would indicate that events closely related to DNA synthesis have little to do with determining the time required for cells to get from the end of DNA synthesis to division. This hypothesis suggests that the events involved in DNA synthesis are off on an independent but parallel pathway and that the reactions which occur in G2 + P result from an independent series of events. One could speculate that G2 + P might be shorter in a cell with a long G1 because more time spent in G1 better prepares the cell, in terms of metabolic pools or enzyme content, to carry out the reactions of G2 + P and those leading to it at a faster rate.

The second kind of relationship with which our data are consistent is that when G1 is long, S is short and G2 + P long; or, when G1 is short, S is long and G2 + P is short. This might mean that the initiation of DNA synthesis could occur in cells in different states of preparedness, and implies that the events initiating DNA synthesis are separate from those controlling its rate. A cell which spent a comparatively long time in G1 might be prepared to synthesize DNA at a more rapid rate, whereas a cell which began synthesis early might not be as well prepared and synthesis would take longer.

Considerable direct and indirect evidence in the literature supports this hypothesis of inverse relationships between lengths of successive parts of the cycle. Till *et al.* (30) showed that cultured mouse cells inhibited from entering DNA synthesis by fluorodeoxyuridine and then released were able to get through their next S period more quickly than through subsequent S phases, and more quickly than would have been the case in a random population. Similar observations were made by Davies and Wimber (1) in irradiated *Tradescantia* cells.

Firket (4) also noted a shortening of the period of DNA synthesis and the subsequent G2 in chick fibroblasts which were cooled and rewarmed.

In addition, as mentioned above, many of the curves showing the percentage of labeled mitotic cells as a function of time since a brief exposure of cells to T-H³ are asymmetrical in that the line descends from the plateau at a shallower rate than that at which it ascends. Though this is generally interpreted to indicate variation in the length of S, if the slope of the descending line depends upon the rate at which cells enter the S period (24) an additional interpretation may be made. Harris (6) points out that these asymmetrical curves could mean that the rate at which cells enter DNA synthesis varies more than the rate at which they exit. This could be, therefore, additional evidence for an inverse or compensatory relationship between the lengths of G1 and S.

The inverse relationship between S and G2 + P can be explained in at least two ways: When S is short, G2 + P is long because these cells are not so well prepared to carry out G2 + P functions. Or, the time from the end of G1 to metaphase is fairly constant and independent of S; therefore any change in S causes an apparent inverse change in the time between the end of S and division. An analysis of data published by Monesi (16, Table

VI) indicates that an inverse relationship between S and G2 + P actually occurs during the development of male germ cells in mice. He showed that cells at different stages of development have widely varying S and G2 + P periods. Calculations from his data show, however, that the sums of S + G2 + P remain relatively constant.

Note added in proof: Curves such as the one in Fig. 4 and those constructed by Terasima and Tolmach (their Figs. 5 and 6 in *Exp. Cell Research*, 1963, 30, 344) showing the percentage of labeled cells as a function of cell age should help determine which of the possible relationships between parts of the cycle discussed above is more likely to be correct. Symmetrical curves would indicate that S is relatively constant. Curves skewed to the left would suggest some compensation between G1 and S, whereas curves skewed to the right would indicate that variation in S is independent of variations in G1. Unfortunately, neither Terasima and Tolmach's data (Tolmach, personal communication) nor our own are based on large enough samples to permit determination of symmetries with any degree of confidence.

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