CELL GENERATION TIMES: ANCESTRAL AND INTERNAL CONTROLS

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1. Introduction

In order to develop adequate models for the kinetics of growth of cell populations, it is necessary to know the generation time distribution for the individual cells and the degree to which the generation times of related individuals are associated. In essence, the generation time of a cell is that period between successive cell divisions, that is, the period between the birth of the cell by fission of its parent and the later instant at which its own fission occurs. In practice, the generation times of cells are measured by recording the passage of some particular state during or near fission as the individual cells are observed at successive times. This state must be chosen with some caution since any variability in its passage will cause a corresponding loss of resolution in the measured generation time distribution and will lead to an unduly negative correlation between successive generation times.

Early models proposed to describe generation time distributions, by Rahn [1] and by Kendall [2], assumed that generation times of cells were independent of one another. We now know that the generation times of closely related bacterial cells are not independent since the generation times of sister bacterial cells are consistently observed to be positively correlated (Powell [3], [4], Kubitschek [5], Schaechter, Williamson, Hood, and Koch [6], Powell and Errington [7]), and associations continue to exist for at least three generations [7], [8]. Similar associations may be anticipated for other cells because of the similarity of their generation time distributions to those of bacteria [5], [9].

2. Correlations between the generation times of bacterial cells

2.1. *Lateral correlations.* Powell measured generation times for six species of bacteria that were grown under almost constant conditions [3]. His criterion for cell division (or more precisely, termination of a generation) was cell separation. He observed a significant positive correlation between the generation times of sister cells in all six species. Later, in a study of four of these species [4] he found in each a positive correlation between first cousins (that is, cells

Studies performed under the auspices of the A.E.C.

with a grandmother as their most recent common ancestor) and, in a more extensive study with Errington [7] in which more than 800 generation times were measured for each of three species grown on two different media, observed a positive correlation for second cousins (that is, cells with a greatgrandmother as their most recent common ancestor). These results provide evidence for lateral correlations between the cells in any given generation, for a period of at least three generations after their descendence from a common ancestor. In these experiments, however, there were no corresponding significant longitudinal correlations between mothers and daughters or between grandmothers and granddaughters. In addition, it should be pointed out that the distributions of generation times were skewed so that it was necessary to correct the product moment correlation coefficients according to a formula developed by Powell [4].

Although there appear to be no other reports of significant correlations

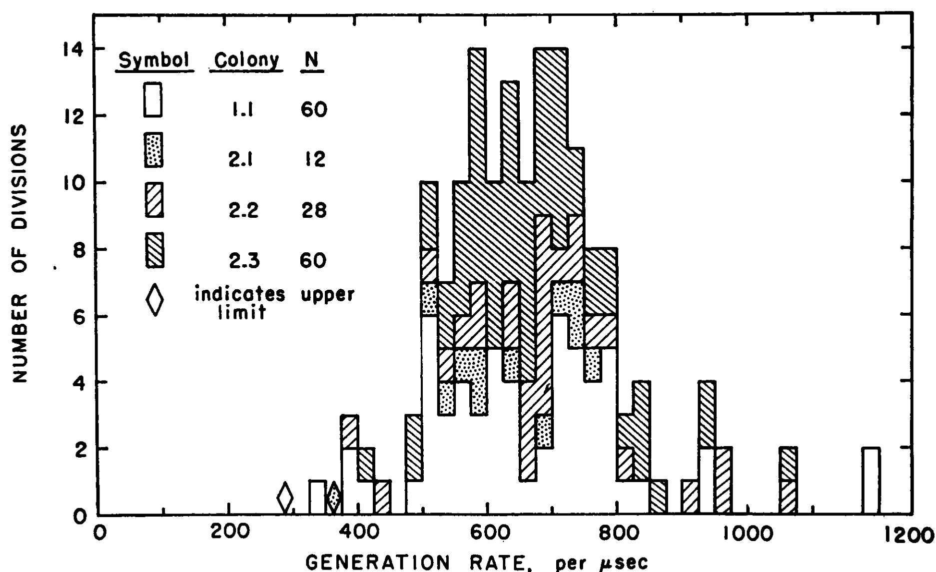

FIGURE 1

The generation rate distribution for *E. coli* B/r.
The number of observations N is given for each microcolony.
This distribution does not include potentially biased data:
data from the first generation are excluded (see figure 10) since
the cells in some microcolonies might not yet have achieved balanced growth;
data are also excluded for all generations in which two or more cells
failed to divide by the end of the photographic record.
For two microcolonies only a single cell failed to divide in the final
generation used. If these single cells had divided immediately after
recording ended, they would have given the maximum rates indicated by ◇;
their true values are almost certainly smaller.

between first or second cousins, the conclusion that there are associations between the generation times of cells for some three generations is confirmed by my own studies [8]. This agreement is of special interest because I used a different criterion for termination and obtained a different frequency function for the distribution of generation times; the criterion for termination was the completion of the septum dividing the cell into two compartments. Cells of *Escherichia coli*, strain B/r, were grown on nutrient agar squares at 37°C on the stage of a phase microscope and photographed at a magnification of 1000, at six or ten second intervals. The time at which a septum was completed could be determined within a resolution of about two per cent. The data were obtained

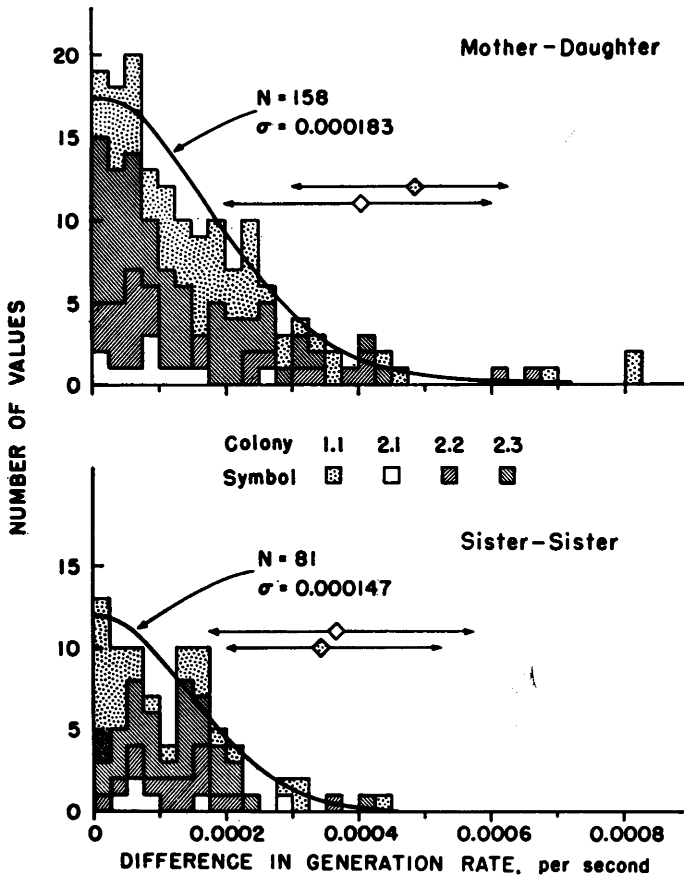

FIGURE 2

The distributions of the differences between the generations rates of mother-daughter and sister-sister cell pairs.
Absolute values of the differences are plotted and fitted with the positive halves of normal distributions centered at zero; σ is standard deviation; N is number of pairs of differences.

from only four microcolonies; fewer than 200 values were obtained, and gave a skewed distribution of generation times. However, it was found that the reciprocals of the generation times, termed *generation rates* for convenience, have a distribution that is approximately normal, as shown in figure 1. The correlation between the generation rates of sister cells was positive and significant; the value of the product moment correlation coefficient was 0.45. For sister cells the standard deviation σ of the differences in generation rates was reduced to about 80 per cent of the values found for unrelated cells or between mothers and daughters, figure 2. There was no significant correlation between the generation times of mother cells and their daughters except when daughter cells had unusually short generation times. Then the generation times of their mothers were significantly longer than the mean, figure 3.

2.2. *Variance of the sum of successive generation times.* A recent reexamination [8] of the data from my experiments was stimulated by Koch's finding that the

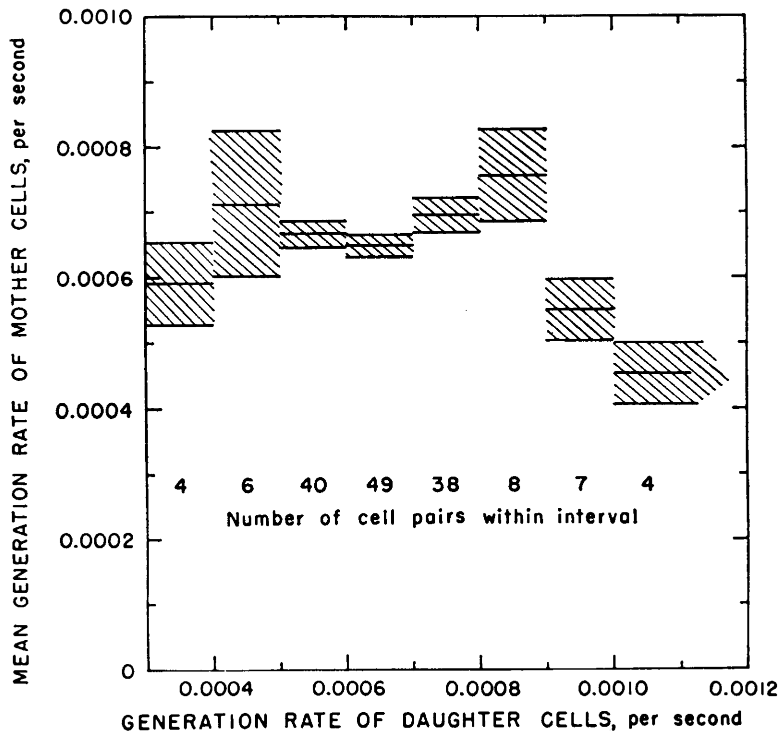

FIGURE 3

Grouped values of mother cell generation rates as a function of the generation rates of their daughters. The eleven daughter cells in the two most rapidly dividing groups have come from mother cells with a mean generation rate that is significantly less than the mean for all cells.

variance of the sum of successive generation times is far more constant than would be expected if generation times were independent [10]. Koch's approach permits an estimate of the extent to which generation times of cells are correlated with those of their ancestors. To see this, let $T_1, T_2, \cdots, T_n$ represent the successive generation times in a progeny line of n generations, and define the sum

$$S_n \equiv T_1 + T_2 + \cdots + T_n. \tag{2.1}$$

Then, if these generation times are independent,

$$\text{Var } S_n = \text{Var } T_1 + \text{Var } T_2 + \cdots + \text{Var } T_n. \tag{2.2}$$

We can ask what the behavior of Var S_n would be for either of two extremes, independence of generation times or complete dependence. However, we shall be interested only in cultures for which the distributions of generation times does not vary from generation to generation. In order to obtain cultures of this kind it is necessary to maintain balanced growth. In balanced growth every extensive property of the culture increases at the same rate. For such cultures the distributions of cell number, mass, and composition remains unchanged in successive generations.

At the first extreme, if generation times are independent, or if they are merely uncorrelated, then

$$\text{Var } T_1 = \text{Var } T_2 = \cdots = \text{Var } T_n = \text{Var } T, \tag{2.3}$$

where Var T is the constant variance contributed by any generation. In this case,

$$\text{Var } S_n = n \text{ Var } T. \tag{2.4}$$

This linear dependence of the variance on n is shown by line A in figure 4.

At the other extreme, if we consider the particular case where fluctuations in generation time only occur just before or just after cell division and if the fluctuations occurring just after division completely nullify those occurring just before, then Var S_n cannot increase, but must remain constant at the value it would have had for a single complete generation

$$\text{Var } S_n = \text{Var } T. \tag{2.5}$$

This kind of constancy is shown by line B in figure 4.

Values of Var S_n were calculated for those generations in my experiments in which it was possible to obtain measurements of the interdivision times of all cells or all but one; for example, for $n = 2$, all mother-daughter pairs were included from the mother cell in the initial generation to the daughter cells in the final generation. The dependence of Var S_n upon the number of elapsed generations, shown by the experimental points in figure 4, does not agree with either extreme dependence, line A or line B. Instead, the variance increases rather uniformly for the first three generations, then remains relatively constant thereafter. These results show that the generation times of cells in a progeny line

are essentially uncorrelated with those of their immediate ancestors, but they cannot be independent since Var S_n approaches constancy after three generations. Furthermore, these results are in agreement with the absence of longitudinal correlations discussed earlier, and they support the finding of Powell and

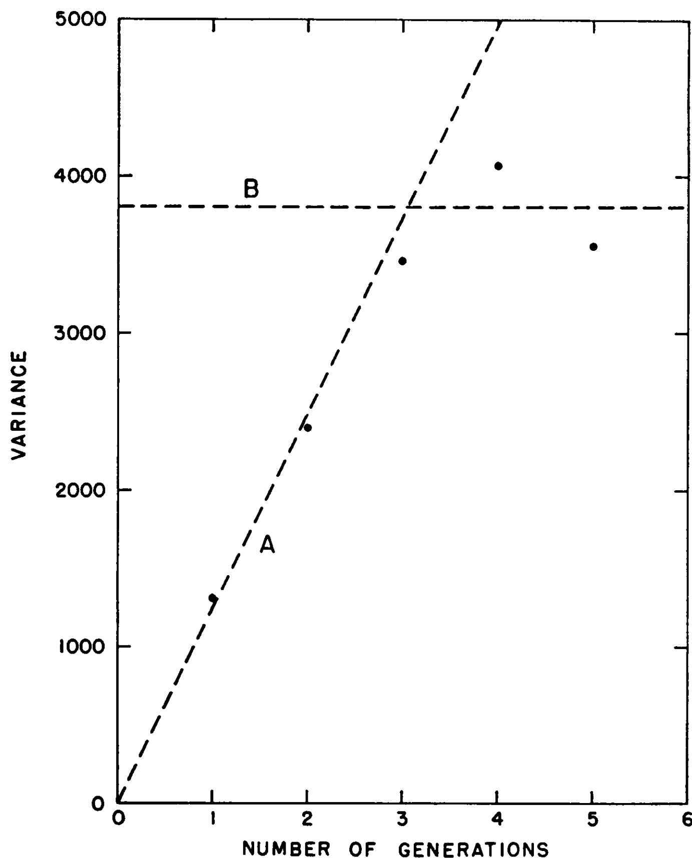

FIGURE 4

The dependence of Var S_n, the variance of the sum of successive generation times, upon the number of generations. Estimates of the errors of the variances [8] are too small to portray conveniently.

Errington that associations exist between related cells over a period of three generations [7].

2.3. *Longitudinal correlations.* Since the distribution of generation rates for the bacterial cells in my study was approximately normal, it was possible to use a simple sign test for the presence of correlations between cells in progeny lines. The deviation of the generation rate from the mean was recorded for each cell

as positive (+) or negative (−). Deviations of related pairs were compared to see if signs were the same (+,+ or −,−) or alternate (+,− or −,+); the frequency of alternate signs was compared to that expected by chance, and the significance of the result was assessed by t test. Further comparisons were made between the deviations of single cells (of arbitrary generation g) and the sums of the deviations of two or more cells in successive generations in the same progeny line (such as granddaughter and greatgranddaughter in generations $g + 2$ and $g + 3$). The results presented in table I show that there are significant negative correlations between the generation rates of single cells and the mean deviation of some pairs or triplets of successive cells in their ancestral and progeny lines: significant correlations exist with the means of greatgrandmothers and grandmothers, with the means of all three preceding generations, with the means of triplets of successive cells in the three following generations, and with the means of granddaughters and greatgranddaughters in the same line (that is, with the means of $g - 3$, $g - 2$, of $g - 3$, $g - 2$, $g - 1$, with $g + 1, g + 2, g + 3$, and with $g + 2, g + 3$).

TABLE I

CORRELATION BETWEEN GENERATION RATES IN ANCESTRAL AND PROGENY LINES

Generation					
Ancestral	Progeny	N	A	t	P
g	$g+1$	160	81	.159	NS
g	$g+2$	144	76	.667	NS
g	$g+3$	112	61	.941	NS
g	$g+4$	64	28	1.000	NS
g	$g+1, g+2$	144	74	.334	NS
g	$g+2, g+3$	112	72	3.015	.01
g	$g+3, g+4$	64	31	.250	NS
g	$g+1, g+2, g+3$	112	67	2.072	.05
g	$g+2, g+3, g+4$	64	35	.750	NS
g	$g+1, g+2, g+3, g+4$	64	31	.250	NS
$g-1, g-2$	g	144	83	1.834	NS
$g-2, g-3$	g	112	71	2.825	.01
$g-3, g-4$	g	64	32	0	NS
$g-1, g-2, g-3$	g	112	73	3.203	.01
$g-2, g-3, g-4$	g	64	34	.501	NS
$g-1, g-2, g-3, g-4$	g	64	32	0	NS
$g, g+1$	$g+2, g+3$	112	74	3.390	.01
$g, g+1$	$g+3, g+4$	64	41	2.250	.05
$g, g+1$	$g+2, g+3, g+4$	64	38	1.501	NS
$g, g+1, g+2$	$g+3, g+4$	64	44	3.000	.01

Deviations from the mean cell generation rate were compared for each cell of an arbitrary generation g and its progeny in later generations $g + i$, or its ancestors in earlier generations $g - i$, where $i = 1, 2, 3,$ or 4. A comparison

was also made with the average rate for sums of generations in ancestral or progeny lines. In the table, N is the total number of pairs of values, and A is the number of pairs of values with deviations of alternate sign. These values were compared by t test. The probability P that the observed frequency of alternate signs would have occurred by chance is given in the final column; NS indicates that the difference was not significant.

It is instructive to plot the experimental values for the frequencies of alternate signs, figure 5. This figure shows that the frequencies of alternate signs increase to a maximum value between 2 and 2.5 generations.

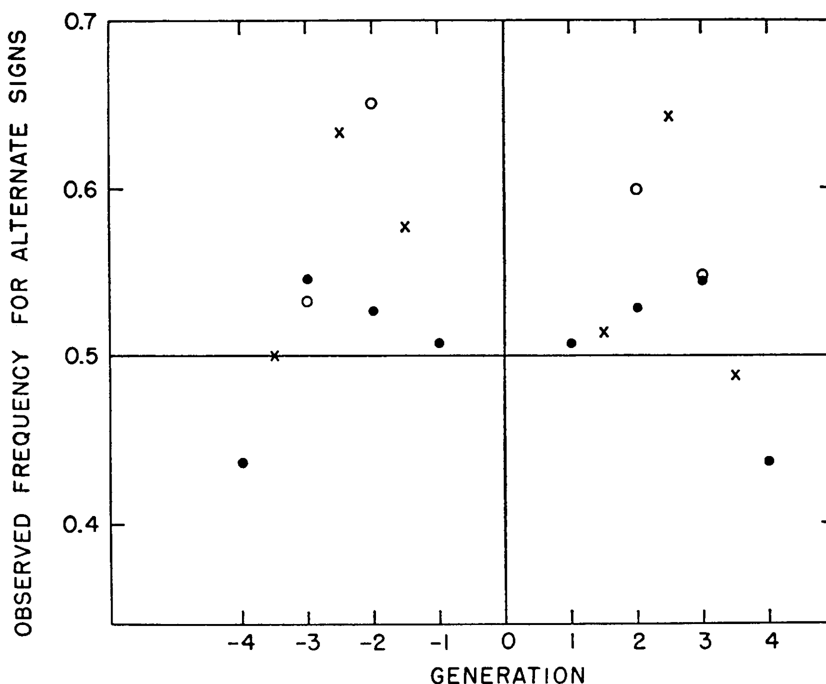

FIGURE 5

Observed frequencies
for pairs of generation time values with alternate signs.
The deviation of the generation rate of the reference cell
(generation 0) from the mean generation rate was compared with
the deviation of related single cells in ancestral or progeny lines,
and with the mean value of successive pairs or triplets in these lines.
Frequencies of alternate deviations are plotted
at the average generation number.
Filled circles indicate pairs of single cells;
cross indicates reference cell and two successive generations;
open circles indicate reference cell and three successive generations.

These correlations are in agreement with the variance analysis, and again confirm earlier results for the absence of significant longitudinal correlations between single pairs of related cells in a progeny line. In addition, they extend those results, demonstrating that significant correlations arise when average generation rates over two or three generations are considered.

Finally, in these studies it has also been possible to relate the generation times to cell size [8]. From the original photographs, it was possible to measure the relative lengths of these rod shaped bacteria just after formation of the septum in the parental cell ("birth") and their lengths in turn at the instant of formation of their own septa. The distributions of these lengths are shown in figure 6. As generation rates are increased, average birth lengths first decrease to a minimum value, and then increase again. Final cell lengths appear to go through a greater change and to reach a minimum value at a greater generation

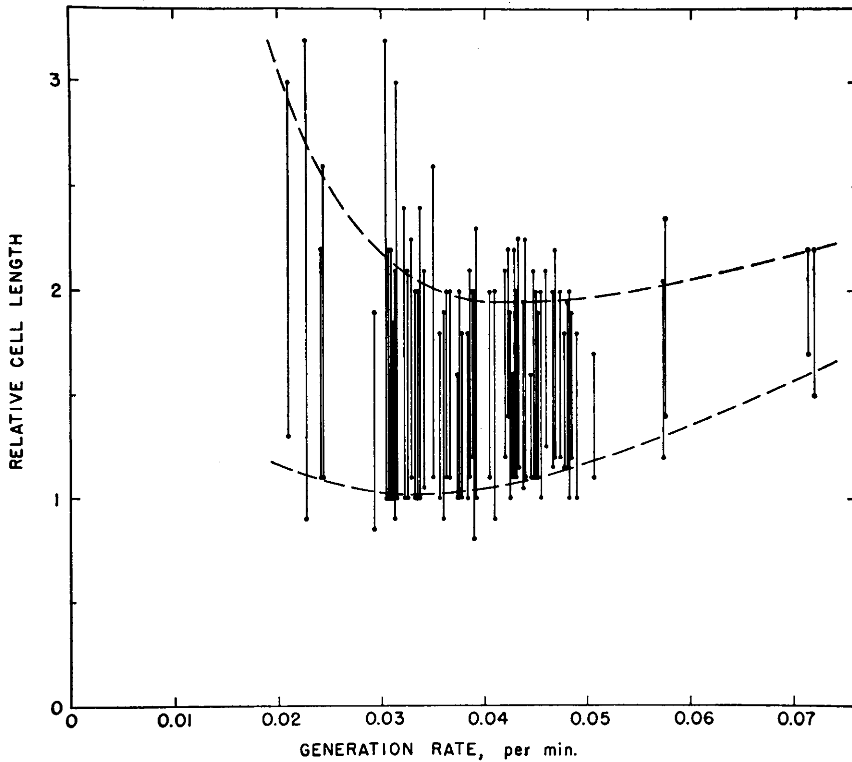

FIGURE 6

Distributions of relative lengths of cells at birth and final lengths at division as a function of generation rate. Lengths of each cell at birth and at division are connected with a vertical line. The two curved lines are visual approximations.

rate. Thus, for almost all birth lengths there are two different average values of generation rate, and the cells in each of the two classes grow to different final average lengths at division. Similarly, for many final cell lengths there are two different classes of length at birth. It follows that the generation rates of cells cannot be determined solely by their lengths at birth or by their final lengths. The latter possibility was assumed in a deterministic model for the kinetics of bacterial division suggested by Koch and Schaechter [11].

The results of figure 6 again imply that the generation rates of daughter cells are strongly dependent upon those of their mothers, despite the absence of significant correlations in earlier tests. Cells with the smallest generation rates (longest generation times) reach the greatest final lengths, and their progeny are so large at birth that they fall into the class of birth lengths associated only with the most rapid generation rates. This relationship provides a more detailed accounting for the negative correlation shown earlier in figure 3. Furthermore, this kind of dependence of the generation rates of daughter cells upon those of their mothers must occur for all such pairs: the terminal length of a mother cell establishes the permissible ranges of birth lengths of its daughters, and thereby, the permissible ranges of their generation times. Since these ranges are large and since there are two classes of generation time for most birth lengths, these relationships are usually concealed in tests of correlation.

The results of figure 6 require the presence of at least two nongenetic factors controlling generation rates in daughter cells, transmitted from mother to daughter and tending to be compensatory [8]. One of these might account for the increase in generation rate as birth lengths are increased. The other factor would then have to account for the decrease in generation rate with increase in birth length that occurs at the smallest generation rates.

2.4. *Summary.* To summarize this section, there is a great deal of evidence that the generation times of bacterial cells depend upon those of their ancestors, despite the absence of observable correlations between the generation times of individual cells. The range of permissible generation times of daughter cells is established by the lengths of their mothers through the transmission of factors that tend to compensate for each other. These factors give rise to the appearance of two classes of generation time for most cell lengths at birth, tending to obscure this dependence in simple correlation tests. Nevertheless, significant positive lateral correlations have been observed between sisters, between first cousins, and between second cousins, and significant negative longitudinal correlations have been observed between single cells and the mean values for several ancestral or progeny generations. These correlations are supported by a variance analysis of the time required for several successive cell divisions. Altogether they demonstrate the association of generation times over a period of three generations.

3. On the generality of the truncated normal distribution of generation rates

3.1. *Evidence supporting the distribution.* The observation that the generation rate distribution was approximately normal led to an examination of generation time distributions published for other kinds of cells [5]. Generation rate distributions were constructed for the yeast *Saccharomyces cerevisiae* from the data of Burns [12], for the protozoan *Tetrahymena geleii* HS from the data of Prescott [13], for mammalian cells in culture, HeLa, from the data of Hsu

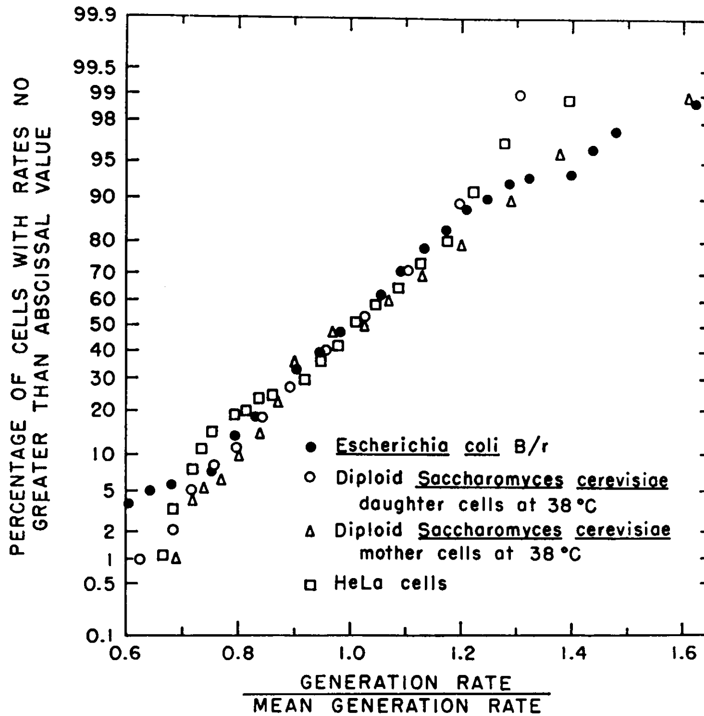

FIGURE 7

Cumulative distributions of cell generation rates when control is relaxed.

[14], and in a later communication [15] for another protozoan, *Euglena gracilis*, from the data of Cook and Cook [16]. The criterion of termination was different for each cell type: the initial appearance of a daughter bud was scored for yeast, cytoplasmic fission for the protozoa, and mitotic anaphase for HeLa cells. Despite these different criteria, each distribution of generation rate is an approximate agreement with a normal distribution. The agreement is evident even when the data consists of no more than 160 values, as in the cumulative distributions shown in figure 7, and the agreement is improved for larger numbers,

figure 8 and figure 9. In addition, Sisken and Morasca [17] have recently observed that the generation rates of human amnion cells appear to be normally distributed in culture.

In the more extensive experiments the agreement with the normal distribution of generation rates is so good that rather a large number of observations are required to give a good estimate of the deviation from normality. The data for

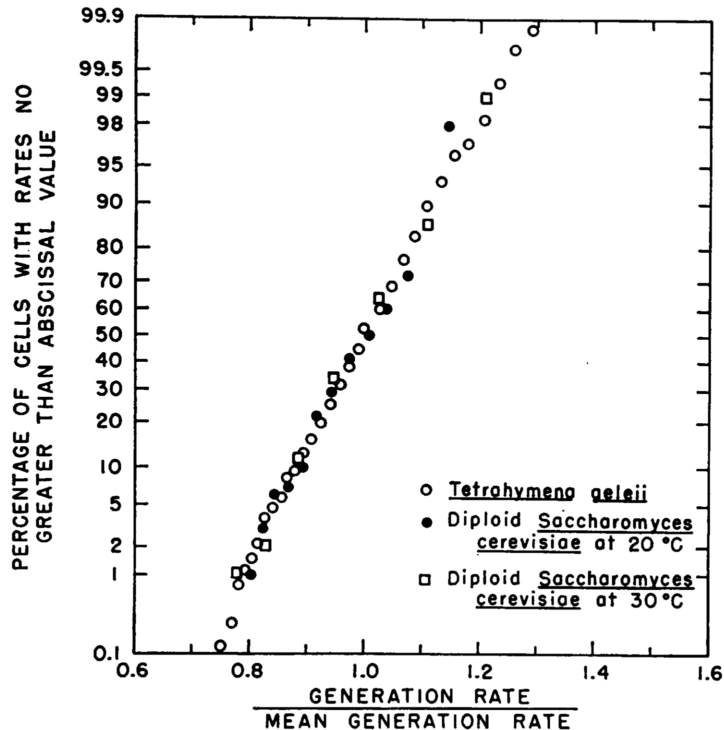

FIGURE 8

Cumulative distributions of cell generation rates when control is stringent.

figure 9 led to no significant departure from normality for generation rates as small as three standard deviations below the mean and as large as two standard deviations above the mean [9]. This unusual agreement was unexpected because growth and division of cells are limited by a variety of processes and consequently a maximum generation rate must exist, and might have been expected to occur at smaller values.

These results lead to the possibility that generation rate distributions are similarly normal for all or almost all cells dividing by simple regular fission. This point is of some importance because it implies that cell division might then

be controlled in a generally similar fashion, and if true, it would then be desirable to construct a general model.

3.2. *Evidence against the normal distribution.* Some published generation time distributions do not yield even approximately normal distributions of generation rate. We need not consider reports prior to Powell's initial study [3] since the earlier work either was not sufficiently systematic, or there are

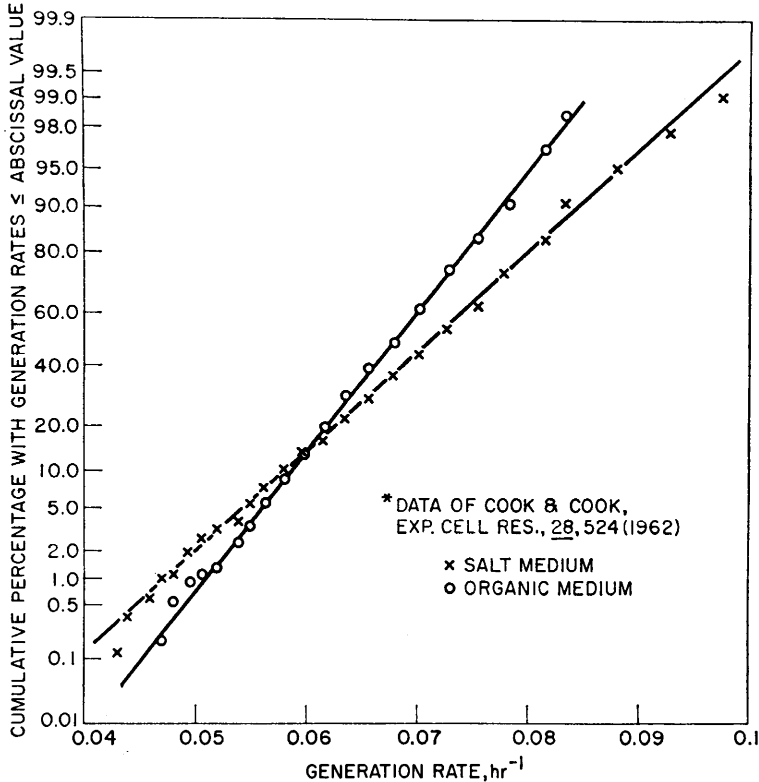

FIGURE 9

Cumulative distributions of cell generation rates for *Euglena* in two different growth media.

ambiguities in the description of the measurements, and furthermore, the concept of balanced growth was not enunciated nor can we ascertain that it was intuitively understood. Some of the experiments done since can be discarded because they fail to satisfy one or more of the conditions that are required to obtain unbiased distributions.

In order to obtain valid generation time, or rate, distributions three conditions must be met [3], [4], [5], [9].

(1) Whatever the criterion chosen for the termination of a generation, it must permit good resolution: the terminal instant must be clearly distinguishable and have but small variability in the sequence of events composing the cell division cycle.

(2) Cells must be in balanced growth. (That is, every extensive cellular property, such as mass, number, and composition, must increase at the same rate. Statistical fluctuations would occur for small numbers of cells, but these would be negligible if the culture were indefinitely large.) This requirement ensures that the generation time distribution remains unchanged from generation to generation.

(3) The distribution must not be calculated from data so selected that the distribution is biased. For example, selection ("cutoff" bias) would occur against long generation time values if every value in a complete photographic record was used to construct the distribution, since only cells with successively shorter generation times can be recorded as the end of the record ("cutoff") approaches. Although corrections for bias of this kind are available [3], or the bias can be made negligible by properly discarding appropriate values [9], it is far more satisfying to avoid this bias by deciding beforehand to include data for all cells for only a fixed number of generations, as suggested by Powell [3].

These conditions must also be satisfied, of course, when the population under study contains inviable cells or subpopulations known to be under other kinds of selection. In addition, it is necessary in these cases to establish the constancy of this selection, generation by generation. There are as yet no reliable studies on populations of these kinds. In one study that included nonviable bacteria [18], the frequency of nonviable cells did not remain constant at the level found in early generations, and in addition, mean generation times decreased with successive divisions, failing to satisfy the second condition of balanced growth.

Techniques for culturing mammalian cells have been developed only relatively recently, and it is frequently quite difficult to maintain constant growth conditions over a period of many generations. Even when precautions are taken to minimize variability in sera and other growth factors, some cells are strongly influenced by their local environment, which changes rapidly with the growth of the population. It is especially important to provide evidence for balanced growth in studies of these cells. No such evidence was reported for the two generations in Froese's study of HeLa cells [19], although cell division rates were more rapid with human serum (28 values) than with fetal calf serum (about 200 values). The differences between the three experiments using fetal calf serum strongly suggest that growth conditions were not well controlled; in one of the experiments cells were so unusually motile that some 50 per cent of the divisions could not be followed.

In a similar study of a strain of rat sarcoma cells [20], the constancy of the mean generation time was tested statistically for each clone and each passing generation. Except for one experiment, the means did not differ significantly

among the seven experiments comprising some 200 values. The data were also examined for possible cutoff bias, but since none could be detected, almost all of the data were used. In itself, this inability to obtain evidence for this bias is disturbing, since such bias must exist. (Actually, the reduced variance in the partial data for the fifth generation is evidence for this bias.) More serious, however, was the fact that the data were not complete even to the fourth generation (see, for example, their figure 4), presumably because of cell migration. To make absence of bias convincing, these authors would need to establish that the unobtainable data were not in a selected subpopulation.

The strongest evidence against a normal distribution of generation rates would appear to come from the studies of the bacterium *E. coli* by Schaechter, Williamson, Hood, and Koch [6] and the more extensive studies by Powell [3], [4] and Powell and Errington [7]. Schaechter, Williamson, Hood, and Koch observed a normal distribution of generation times, while Powell's experiments led to a distribution skewed toward longer generation times (Pearson Type III, or similar). My own studies led to a distribution of generation times even more highly skewed toward longer generation times [5]. Furthermore, in all of these studies there was no evidence that growth was unbalanced, nor did it seem that the data were collected in a manner leading to cutoff bias. Thus, these studies would seem to have led to three different distributions of generation time for the same bacterial species.

However, it should be noted that the criterion of termination in these other experiments was different from mine. They measured cell wall separation, the production of two essentially mechanically independent daughters (termed *o*-fission by Powell [4]), whereas in my studies the criterion was cytoplasmic separation, the development of a septum isolating the contents of a parental cell into two daughter units that remained tightly attached to each other (Powell's *p*-fission). Powell anticipated the possibility of different distributions when he expected only a rough correspondence between these two generation times [4].

Recent results of my own support Powell's expectation. Cultures of *E. coli* 15 THU$^-$ (requiring thymine, histidine, and uracil), kindly supplied by S. S. Cohen, were grown in a minimal medium (M9 salts, 2 μg/ml thymidine, 20 μg/ml histidine, 20 μg/ml uracil, and 0.1 per cent glucose) and transferred daily to fresh medium for several weeks. In the final culture, exponential growth was permitted for only about six generations, and the cells were then observed by phase microscopy. Few, if any, long filamentous forms were seen. However, there were many pairs of joined cells that appeared to be about twice the usual cell length. These pairs usually were constricted at their attachment, with diameters frequently reduced from only slightly to about half the usual cell diameter. Of 1872 cells examined, at least 15 per cent were pairs of this kind. As a control, cells of a similar strain, B/r/T1,try$^-$, from a continuous (chemostat) culture were also examined. In this culture not more than 1.5 per cent were

joined pairs. Since constriction is concomitant with septum formation or soon follows it, these observations show that cell wall separation was unusually delayed in the 15 THU^- strain.

The observation that cell separation can be unusually delayed provides a possible explanation for the three different kinds of generation time distributions observed for *E. coli*. If the criterion of termination is cell wall separation, then cells that are unusually tardy in separating after their septa are formed will give unusually large values for their generation times. However, if this unusual delay is absent at the next or later divisions, then two or more progeny cells will appear to have unusually short generation times. Thus, an unusually variable interval between septum formation and cell wall separation would lead predominantly to an apparent increase in the frequency of short generation times (and also to an increased value of the coefficient of variation CV for generation rates). The distortion of the distribution would increase with both the magnitude of this delay and the frequency with which it occurs. For these reasons, cell separation would seem to be a poor criterion of termination, failing to satisfy the first condition of providing good resolution. Septum formation appears to afford greater resolution.

If these conclusions are correct, the studies of Powell, of Powell and Errington, and of Schaechter, Williamson, Hood, and Koch present no strong evidence against the generality of the truncated normal distribution of generation rates.

3.3. *Estimation of mean generation rates.* In order to obtain a valid genera-

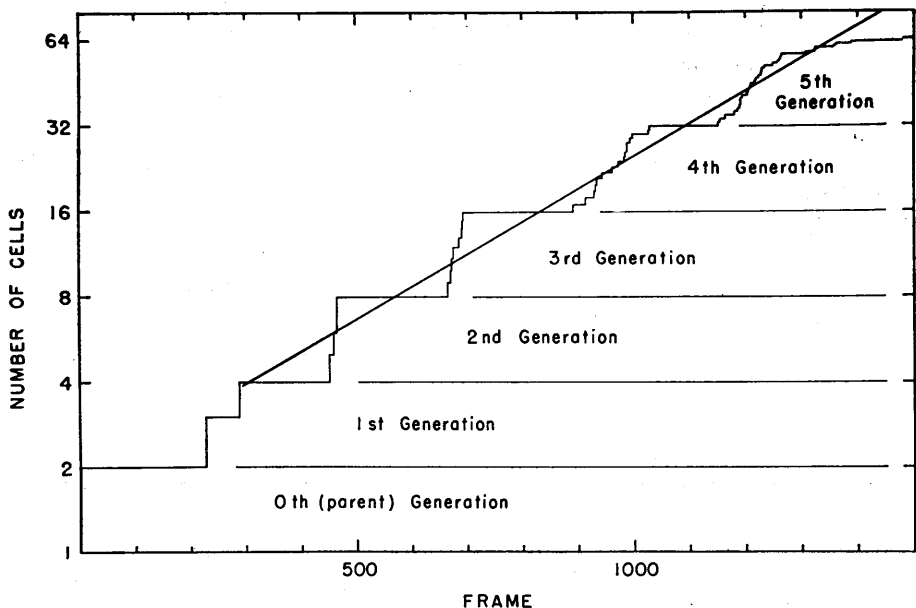

FIGURE 10

Growth curve from a single cell of *E. coli* B/r.

tion time (or rate) distribution, the culture must be in balanced growth with reproducible mean rates of cell division. Mean generation times are not very accurate when they are computed from clonal histories comprised of 50 to 60 cell divisions, and minor deviations from balanced growth can escape detection, leading nevertheless to biased distributions. A more accurate procedure is to plot the growth curve for each microcolony arising from a single cell, as in figure 10, and to make a visual estimate of the line best fitting these data. These estimates have a variability that is three to four times smaller than the mean generation rate computed from the average of individual values (see, for example, table II in [5]). This increased accuracy can be attributed to the added information of the order in which cell divisions occurred, contained implicitly in the growth curve [5]. It would be valuable to have a mathematical procedure for fitting the best straight line to data with cyclic fluctuations of this kind; a major problem is that such fitting could not be allowed to depend unduly strongly upon the part of the cycle at which data collecting was discontinued.

3.4. *The advantage of the generation rate distribution.* Generation rate distributions have at least one theoretical advantage over generation time distributions when inviable cells are present. Even one such cell would cause the mean generation time to become infinite. However, such a cell would add only a single value to the zero class of a generation rate distribution, and the mean generation rate could be correspondingly reduced, usually only slightly.

4. Discrete distributions of generation rate

4.1. *Evidence for two groups.* When the coefficient of variation CV was calculated for the generation rate distributions of cells growing in complex (organic) media, a striking relationship emerged for these cultures growing at or nearly at their maximum growth rates: only two classes of values of CV were observed [9]. That is, the *widths* of these two distributions did not appear to be distributed at random, but fell into two groups. The presence of the two groups is easily seen by comparing the slopes of the cumulative distributions in figures 7 and 8, since the values of CV are proportional to these slopes. Considering each figure separately, the slopes are not significantly different from one another. However, the slopes in figure 8 are all about twice as great as those in figure 7. The broader distributions in figure 7 have values of CV of about 20 per cent. We may interpret these distributions as reflecting an intrinsic control by each cell, and we may say that the control of generation rate is *relaxed* for the cultures in figure 7. Correspondingly, the narrower distributions in figure 8 have values of CV of about 10 per cent, and for these control is *stringent.* The distribution for *Euglena* grown in an organic medium (figure 9) also has a value of about 10 per cent for CV, and therefore these cells were also under stringent control.

This grouping of values of CV is lost when cells are not grown near or at their maximum rates of division. When *Euglena* was grown in a salts minimal

medium (figure 9) the value for CV was increased to 16.0 per cent (SE: 0.4 per cent), intermediate to the two groups [16]. Another intermediate value, 13.6 per cent, was observed by Sisken and Morasca for human amnion cells cultured in a salts medium supplemented with amino acids, vitamins, horse serum and antibiotics [17]. Furthermore, in each of the three species of bacteria that Powell and Errington studied [7], growth in a chemically defined medium gave markedly larger values for CV than did growth in a complex organic medium.

I have suggested that these findings might be explained in the following way [9]. The generation time of each cell is determined by the time required to complete a large number of reactions, any one of which might be made rate limiting. Some of these reactions can be omitted if these cells are exposed to an exogenous supply of compounds that they would otherwise be required to synthesize. The elimination of these reactions would reduce the time necessary for cellular replication. In addition, the elimination of these reactions would also eliminate the fluctuations arising from them, thereby decreasing the value for CV.

4.2. *Alternate states of control of cell division.* Since only a relatively small number of studies of generation rate distributions have been made for cultures grown in complex media, there was the possibility that the grouping of values of CV was accidental, and therefore of no further significance. Fortunately, it was possible to test for the occurrence of discrete groups by a different approach, suggested by the results of Burns' studies [12]. For diploid *Saccharomyces*, generation rates were under stringent control at 20°C and 30°C (figure 8) with a value of CV of about 10 per cent, but control was relaxed at 38°C (figure 7) with a value of about 20 per cent. Burns had also observed a loss of control manifested by the failure of mother and daughter cells to bud simultaneously at 38°C, although they do so at the two lower temperatures. Further characteristics of these cells and related experiments are described by Tobias [21].

This loss of control led us to inquire about the dependence of CV at intermediate temperatures [22]. If the observed grouping of values were accidental, then we expected to obtain intermediate values of CV as the temperature was raised from 30°C to 38°C. On the other hand, if discrete states of control of cell division exist, then we expected to observe an abrupt transition from stringent to relaxed control at some intermediate temperature. When the experiment was performed, neither expectation was fulfilled, but the results did confirm the existence for two discrete states of control of cell division in diploid *Saccharomyces*.

We obtained generation rate distributions for this strain of yeast at a number of different temperatures between 27°C and 35°C, using Burns' criterion of budding for termination. Our experiments were not as accurate as those of Burns, since we did not separate cells by micromanipulation as he did. Instead, because we anticipated the need for measuring a much larger number of generation times (2052 values were obtained), we decided to forego micromanipula-

tion and accept any consequent increase in CV that might occur from unbalanced growth arising through variations in local growth conditions in the growing microcolonies. As a result, it will be seen that our values of CV are consistently about a third higher than those he obtained.

At temperatures below 32.5°C, the values of CV were essentially the same in each experiment (figure 11). Above this temperature, however, we obtained

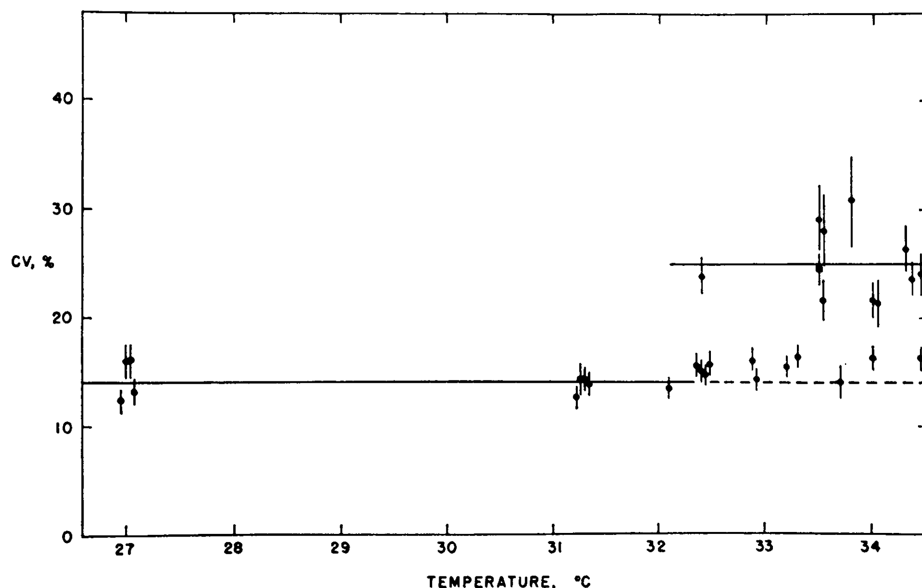

FIGURE 11

Values of CV observed in daily experiments with *Saccharomyces*.
The standard errors of CV are represented
by the vertical bars above and below the circles.
The square at 33.5°C indicates the value that was observed
when the temperature of the culture was deliberately increased at 0.5°C
for a period of 20 minutes at the beginning of the recording period.

an unexpected result: although some values of CV were about the same as in the earlier experiments, many values were about twice as large. That is, the region from 32.5 to 34.5°C is a *transition region* in which the cells in these cultures can be either under stringent or relaxed control at any temperature in this region. In figure 11, it can also be seen that the frequency of large values of CV increases with temperature in the transition region.

Since multiple values of CV implied some inadvertent failure to maintain completely constant conditions, we reexamined the experimental records and found that large values of CV were correlated with rather small inadvertent decreases in temperature, about 0.5 to 1°C, which occurred at the beginning of

the recording period in some of these experiments. This correlation suggested that the control of the interdivision period is unstable in the transition region, and that a short, slight temperature shift in *either* direction might trigger the onset of relaxed control in some cells. An instability of this kind would also explain the increase in frequency of large values of CV with increasing temperature in the transition region: the instability would be expected to increase with temperature. To test this possibility, at the beginning of a final experiment the temperature was *increased* 0.5°C above the steady state temperature for a period of 20 minutes. A large value was again observed for CV; it is represented by the square in figure 11.

Although the standard errors of the values in the transition region were small enough to make it highly unlikely that we had sampled from a single population in these experiments, the variability of the larger values of CV suggested that not all parental cells were triggered to relaxed control. The data had been recorded as clonal histories of cell pairs (cells cannot be separated until budding begins [12]) allowed to divide for two generations, making possible a determination of the value of CV for the twelve generation rate values obtained for each

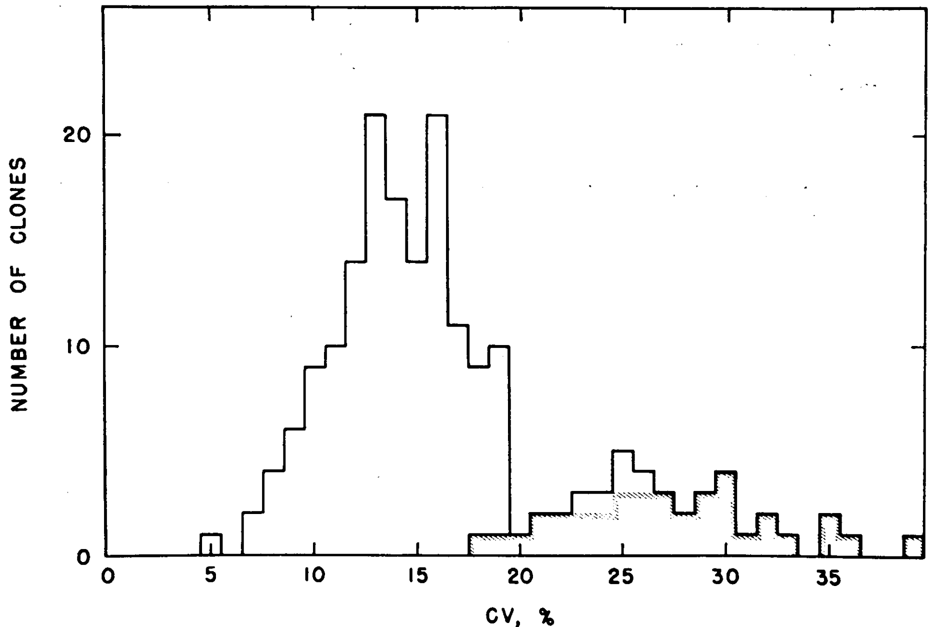

FIGURE 12

The distribution of clonal values of CV.
Clonal values of CV were calculated for the 12 cell generation rates observed for each clone that grew from its parental cell doublet. The shaded line encloses that portion of this bimodal distribution for which the average value of CV is 26.3 per cent.

clone. (Inviable cells were not observed.) The distribution of the clonal values of CV (figure 12) was clearly bimodal, and was fitted by a graphical analysis of the cumulative data. The results of this analysis are also shown in figure 12; the shaded boundary encloses the estimated distribution of those clones with the larger value of CV. The parameters are: stringent control, 154 clones, with a mean value of 13.7 per cent for CV; relaxed control, 37 clones, with a mean value of 26.3 per cent. Slightly better estimates can be calculated for these mean values of CV and their standard errors [22].

Along with these shifts between states of control of the interdivision period, we also observed a corresponding decrease in the mean generation rate of these cultures, figure 13. Our estimate of the average decrease, about 10 per cent, is

FIGURE 13

Cumulative daily generation rates as a function of temperature. Open circles indicate experiments for which the value of CV was less than 20 per cent; filled circles indicate experiments for which the value of CV was greater than 20 per cent; square indicates the experiment for which the temperature was increased for 20 minutes.

necessarily crude, and it is entirely possible that actual decreases in generation rate might be dependent upon the generation rate of the unperturbed culture.

In a further attempt to examine these changes in control, cultures in diploid *Saccharomyces* in exponential (balanced) growth in suspension in an organic medium (malt extract broth, Difco Laboratories) were divided into two parts,

one of which was briefly exposed to an increased temperature. At first, these experiments showed no consistent change in cell division rate. Later it was realized that good results might require triggering all of the parental cells to relaxed control, and therefore, larger temperature shifts might be required. The results of a more successful experiment of this kind are shown in figure 14.

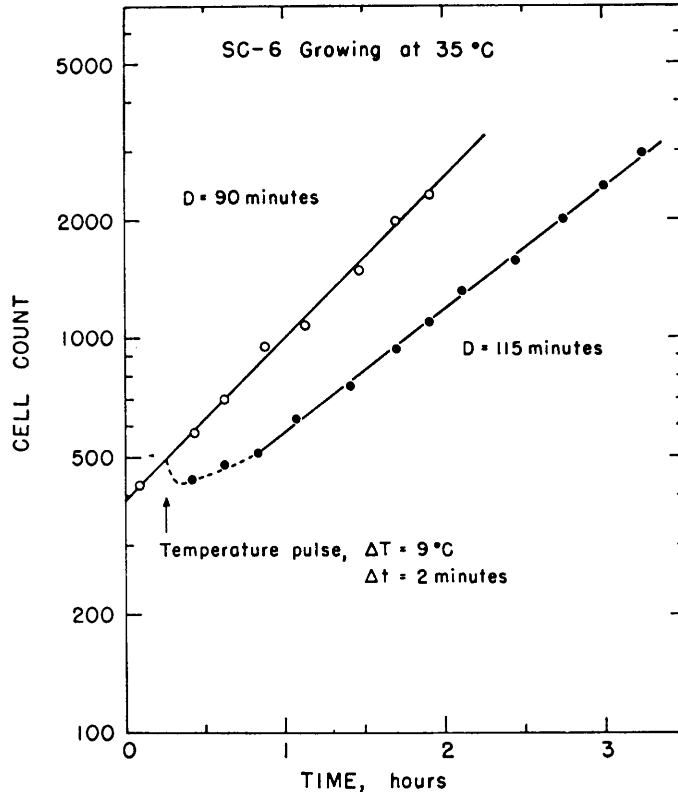

FIGURE 14

Change in cell division rate of a culture of diploid yeast exposed to a temperature increase of 9°C for a period of two minutes.

In this experiment the temperature was raised 9°C for a period of two minutes. Cell division rates were determined using a Coulter electronic cell counter. After the temperature perturbation, the cell division rate was reduced by about 20 per cent in the shifted culture. This reduction in mean generation rate agrees with those from our earlier cell studies.

4.3. *Control "memory systems."* Since the state of control of cell division, relaxed or stringent, depends in these experiments upon the history of the culture, it follows that a "memory system" is involved. Cox [23] described temperature dependent hysteresis effects in macromolecular RNA that should permit the storage of information, and Katchalsky, Oplatka, and Litan [24]

have reviewed the evidence that cells contain systems capable of a "memory" of this kind. Macromolecular complexes would appear to be the most likely candidates for regulating cellular reactions, and thereby affecting cell generation times. Nevertheless, at present these temperature dependent hysteresis effects seem insufficient for a detailed explanation of alternative states of control, since *either* a temporary increase or a temporary decrease can lead to relaxed control [22].

5. Concluding and summarizing remarks

If the results of the experimental studies and the interpretations that I have presented are essentially correct, then we have entered a new phase in our knowledge of the processes controlling generation times. Clearly, generation times are not entirely independent, as was assumed in the earlier models; nor can it be supposed that generation times of progeny continue indefinitely to be significantly dependent upon their remote ancestors. Rather, there are associations between the generation times of cells for an intermediate period, some three generations in bacterial cultures, as demonstrated both by lateral and by longitudinal correlations between related cells.

The dependence of the generation times of daughter cells upon those of their mothers is usually not revealed in correlation tests, but this dependence becomes evident when the sizes of cells at division are considered. These results show that generation times of progeny are influenced by nongenetic factors transmitted from their ancestors, and furthermore, that at least two such factors are necessary to account for the absence of correlation between mother and daughter, with opposite effects upon the generation times of daughter cells.

Moreover, if evidence continues to support the generality of the truncated normal distribution of generation rates for cells growing at or near maximal growth rates, then it is probable that similar kinds of dependences will be found in other kinds of cells. In this case, too, a general model predicting this distribution would be broadly useful.

However, such a model must take into account the cellular transmittal of compensatory factors leading to the correlations observed between related cell pairs, although it might ignore the existence of discrete states of control of the interdivision period. Such a model must also take into account the results of the excellent study by Sisken and Morasca [17], which showed that an early part of the interdivision cycle before onset of DNA synthesis ($G1$) also gave a normal distribution for the reciprocals of the times required for the completion of this period, and furthermore, that this period appeared to be even more variable than the total cycle.

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