Testing the Commitment Theory of Cellular Aging

The finite lifespan of human fibroblasts may be due to the decline and loss of a subpopulation of immortal cells.

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Summary. The commitment theory may explain both the finite lifespan of diploid fibroblasts and the apparent immortality of transformed lines. Potentially immortal cells are assumed on division to generate with some fixed probability cells committed to senesce after a specific number of divisions. During the period between commitment and senescence, cells are assumed to maintain normal growth so that the uncommitted cells are diluted by committed ones and may ultimately be lost in subculturing. A number of predictions of this model are described and experiments strongly supporting the theory are reported. We conclude that the limited growth of diploid fibroblasts is, in effect, an artifact of normal culturing procedures.

It is well known that human diploid fibroblasts cannot be subcultured indefinitely (*1*-*3*). A long period of normal growth is invariably followed by a senescent phase and, subsequently, death of the whole population; fibroblasts have therefore often been used as a model system for the study of the intrinsic process of cellular aging. Unlike rodent cells, cultured diploid human cells never undergo spontaneous transformation to a permanent cell line. It follows that the whole population in some way becomes committed to cell death.

It has sometimes been suggested that the finite lifespan of human fibroblasts is due to a built-in genetic program and that their cessation of division is due to "terminal differentiation" (*4*, *5*). If senescence is the result of a program that specifies the total number of cell divisions, it might be expected that, at any one time, all the cells in a population would be of roughly the same age, that is, at similar points along the programmed pathway from primary culture (phase I) through the period of steady growth (phase II) to senescence (phase III).

This is clearly not the case, since it has been shown that individual cells taken from a population of fibroblasts are extremely variable in their doubling potential (*4*, *6*). Nevertheless this heterogeneity is not sufficient to allow the emergence of potentially immortal cells. Another important feature of human fibroblast aging is that there is considerable variation from experiment to experiment in the actual lifespan of parallel cultures of any one strain, as measured in passages or population doublings (*3*, *7*).

To explain these observations we previously proposed a commitment theory of fibroblast aging (*8*, *9*). Starting with a population of uncommitted cells, which are potentially immortal, we assumed that there was a given probability that cell division will give rise to fibroblasts that are irreversibly committed to senescence and death. These cells initially multiply normally, but after a given number of cell divisions, which we call the incubation period, all the descendants of the original committed cell die out. We showed that if the probability of commitment is reasonably high and the incubation period sufficiently long, then the number of uncommitted cells in the population will progressively decline to < 1 in 10^6, at which time, with normal subculturing regimes, they will inevitably be lost from the population. At this point, all the remaining cells are committed to senescence, and the population is therefore a mortal one although different cells within it have very different growth potentials. We also showed that a reduction in the probability of commitment or the length of the incubation period could produce an immortal steady state culture, consisting of a small subpopulation of uncommitted cells, a majority of committed cells, and a constant fraction of nonviable cells. We suggested that transformed or permanent lines of mammalian cells may be in such a steady state.

If the theory is correct, then the number of cells in a culture becomes an important factor in determining the longevity of fibroblasts, since the population size is directly related to the probability of losing the last uncommitted cells from the culture. Varying the population size thus provides a means of testing the validity of the theory. Our previous model was a deterministic one and did not take into account the stochastic process of progressive dilution out of a very small number (for example, 1 in 10^5) of uncommitted cells. We have now developed a stochastic model and used it to predict by computer simulation the consequence of drastically reducing population size at different passage levels. We have carried out experiments of this type to test the theory, and these have given results that are in agreement with the computed predictions. In our view, it is hard to explain these observations in any way other than the one we have suggested.

Structure of Fibroblast Populations

Cells can be divided into three categories: uncommitted cells, committed cells, and dead cells. Any difference between dead cells and cells that have ceased to be able to divide is immaterial to discussion of population growth. (We assume that all such cells are passively transmitted, rather than lost, during routine subculture since it is known that many chemical or physical agents that kill cells do not prevent their attachment to solid surfaces. However, this assumption is not essential to the general conclusions we reach.) During the division of an uncommitted cell, we assume a probability P for each daughter cell that

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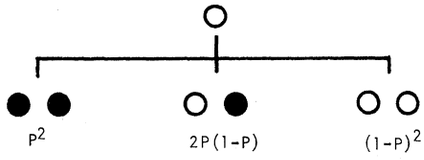
it becomes committed and a probability $1 - P$ that it remains uncommitted. We thus have three possible outcomes to the division of an uncommitted cell:



Commitment is assumed to be irreversible so that division of a committed cell always produces two committed cells. We assume that the path from commitment to death is the same for every cell and define the incubation period M as the number of cell divisions that elapse between commitment and death. (In defining M in this way we assume the cell cycle time to be constant, which is certainly an oversimplification. Variation in cell cycle time about a mean value has no radical effect on the general structure of the model.)

The committed cells can then be divided into M distinct subclasses, the first of which contains newly committed cells, the second of which contains cells that have divided exactly once since commitment, and so on. The last subclass contains cells that have divided exactly $M - 1$ times since commitment and that will die after their next division.

The total population thus consists of $M + 2$ classes, one of uncommitted cells, M of committed cells, and one of dead cells. The population structure is determined by the number of cells in each class, with transitions from one class to another occurring at each cell division.

Since the number of cells under consideration is usually large (10^5 to 10^7), it is reasonable to neglect random fluctuations in the numbers of cells becoming committed and to use the resulting deterministic form of the model for a general study of the expected population growth (*9*).

The process of commitment cannot result in an immediate reduction in cell growth rate since cellular selection would then maintain the population indefinitely. We therefore assume for the present that all live cells divide at the same rate. (This assumption is probably unjustified, since there is evidence that cells divide more slowly as they approach death, but we make it to simplify the mathematical treatment. Slowing down the growth rate toward the end of the incubation period does not significantly alter the conclusions that we reach.)

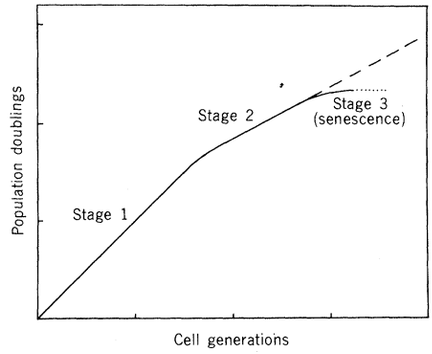


Fig. 1. The commitment theory predicts that an initially uncommitted cell population will double in size with each cell generation (stage 1) until, at the end of the incubation period, the first cells die. The growth rate then falls by an amount determined by P, the probability of commitment. The population subsequently grows at a steady but reduced rate (stage 2). Depending on whether or not the uncommitted cells were lost by dilution before the end of stage 1, the population will either become senescent (stage 3) and die after a finite period in stage 2, or will continue stage 2 growth indefinitely. The scale of cell generations on the abscissa represents the number of cumulative cell divisions by the viable cells in the population.

If we assume that a cell population is initially fully uncommitted and if we allow it to grow without restraint, we expect it to double in size with each successive cell division until, at the $(M + 1)$th division, cell deaths occur as the first committed cells reach the end of the incubation period. Inevitably the growth rate falls. What happens to the population then depends on the value of P. If $P > .5$, each uncommitted cell produces an average of less than one uncommitted daughter so that the uncommitted cells decline to extinction and the population dies out rapidly. If $P < .5$, the cell population growth rate falls to a level determined solely by the value of P, and the population thereafter maintains this reduced but steady growth rate with a stable distribution over the classes. We term the first period of growth, prior to the first cell deaths, stage 1, and the subsequent growth at reduced rate, stage 2 (Fig. 1).

However, in the normal laboratory context populations do not grow without restraint. Populations of size N cells ($\simeq 10^6$) are repeatedly split (for example, 1:2) and the subcultures are allowed to grow until they again contain approximately N cells each. (Each 1:2 split is referred to as a passage, and this is approximately the same as one population doubling.) Clearly the total number of subcultures soon becomes prohibitively large if all are kept; and it is usual to discard all but a few, these being assumed to be representative of the potential total population. The essential feature of this procedure is that at any time no single unit of the population contains more than N cells. During stage 1 growth the accumulation of committed cells dilutes the uncommitted cells even though the potential total number of uncommitted cells may be increasing (for $P < .5$). There may thus come a point where the number of uncommitted cells is so diluted that there is a high probability that individual subcultures contain no uncommitted cells, and are therefore mortal. Alternatively, if a reasonable number of uncommitted cells remains in the individual subcultures at the transition from stage 1 to stage 2, the adoption of a stable distribution of cells over the classes means that no further dilution of the uncommitted cells occurs. Thus, under these circumstances, cultures may continue to grow indefinitely.

For this reason, both the population size N and the incubation period M play important parts in determining the mortality or immortality of the population. For a given value of P, if N is too small or M is too large, the uncommitted cells are likely to be lost during stage 1 and the populations are then certain to be mortal. When this happens, the growth rate will still show the transition from stage 1 to stage 2 growth as before and will remain steady for a number of cell divisions in stage 2. However, as the last cells to become committed approach death, the growth rate will again begin to fall, this time steadily to extinction. We term this final stage of growth stage 3. The transition from stage 2 to stage 3 is not a sharply defined event but corresponds roughly with the appearance of visible senescence in the culture.

The general predictions of the model are thus as follows. For $P > .5$, cultures will grow for a time at a steady rate and will then show a rapid decline followed by death. For $P < .5$, cultures will grow at a steady rate until the first cell deaths occur when they change to a reduced, but again steady, growth rate. Depending on the combination of values of P, M, and N the cultures will then either senesce and die or will grow on indefinitely (Fig. 1). The predicted sudden change in growth rate at the transition from stage 1 to stage 2 is specific to the model and it is interesting that such an effect has been observed by us and by others (*10*). Indeed a growth experiment performed with MRC-5 fibroblasts (*11*) some years ago gives a close fit to the model's prediction (*9*).

Stochastic Predictions

Statistical variation in lifespan is an inherent feature of fibroblast growth experiments. By incorporating the elements of the random processes embodied in our model into a stochastic structure, we have been able to simulate the observed distribution of lifespans and to predict the outcomes of experiments designed to test the validity of the model.

The model contains three main sources of random variation. The first governs the outcome of the division of an uncommitted cell. The second represents the selection of the cells that are to form the subculture. The third determines the "choice" of those cells which are to divide more than once during stages 2 and 3 (when dead cells are present) in order to restore the population size to N. The mathematical nature of these three stochastic elements and the details of the model are to be described (*12*).

Prediction according to the stochastic version of the model was achieved by computer simulation since its structure was too complex for full mathematical analysis. The computer programs were based on two subroutines. The first, called SPLIT, performed the subcultures and the second, called GROWUP, allowed the subcultures to grow back to the specified size. Both subroutines utilized a common function that generated pseudorandom outcomes to the various stochastic elements of the model. Thus any culture experiment could be simulated with comparative ease by an appropriate sequence of calls to the subroutines. All simulations were run on the Hewlett-Packard 3000 computer at the National Institute for Medical Research, London.

Estimation of Parameters

The model is defined in terms of three parameters, N (population size), P (probability of commitment), and M (incubation period). In order to test its validity, it was necessary to estimate values for these parameters and show that they would give satisfactory predictions of experimental results.

Population size, N, is determined by the size of containers used for growing cells. We routinely use 150-milliliter glass Bow bottles: a confluent monolayer contains 1.5×10^6 to 3.0×10^6 cells. We assume N to be equal to 2×10^6.

The probability of commitment, P, may be estimated in two fairly direct ways. The first is to use the observed reduction in growth rate at the transition from stage 1 to stage 2 since this depends only on P. The growth experiment mentioned above gave an estimate of P equal to .275. The second, discussed below and in more detail elsewhere (*12*) is that the model predicts that the variation in lifespan of mass populations should decrease as P increases. As we shall see, comparison of the observed and predicted distributions of lifespan supported the estimate $P \simeq .275$.

The incubation period, M, is more difficult to determine since, within fairly broad limits, the predictions of the model are relatively insensitive to changes in M. The major problem is that we do not have any means at present of estimating the true "age" of our cultures in terms of the numbers of population doublings from the time when the population was fully uncommitted, since some of these will have occurred in vivo.

In fact, for this reason, we shall find it necessary in later comparisons and discussion to express different points in the culture lifespan in terms of population doublings or passages "before normal death" (BND). In the growth experiment referred to above, the transition from stage 1 to stage 2 apparently occurred after 42 population doublings in culture. Thus we may place a lower limit of 42 on M. It may further be shown (*12*) that for $P = .275$, $N = 2 \times 10^6$ we must have $M \geq 55$ if the chance of uncommitted cells surviving into stage 2 is to be negligible. Since increasing M increases the number of cell classes and, thus, the computer time necessary to calculate predictions from the model, we chose to work with the value $M = 55$. We discuss this point below and show that, most probably, M lies in the range 55 to 60.

Verification of Predictions

Lifespans of mass cultures. It has been apparent for some years that a given fibroblast strain such as MRC-5, WI-38, or WI-26 (all derived from fetal lung tissues), does not achieve the same number of population doublings (PD's) in replicate longevity experiments. Our ex-

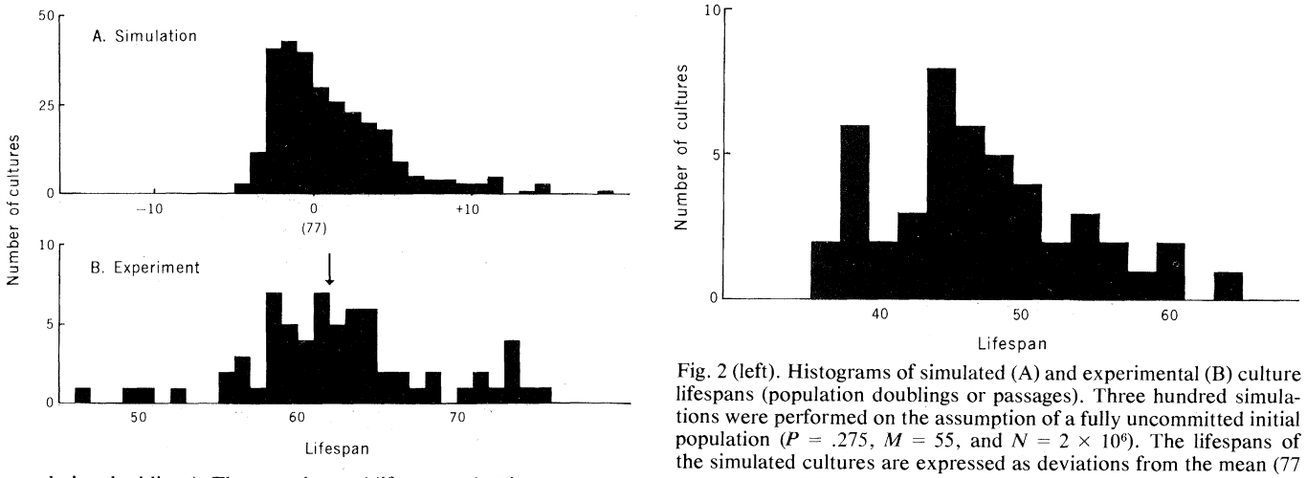


Fig. 2 (left). Histograms of simulated (A) and experimental (B) culture lifespans (population doublings or passages). Three hundred simulations were performed on the assumption of a fully uncommitted initial population ($P = .275$, $M = 55$, and $N = 2 \times 10^6$). The lifespans of the simulated cultures are expressed as deviations from the mean (77 population doublings). The experimental lifespans of 67 independent populations of MRC-5 fibroblasts are expressed in passage numbers, which are very similar to population doublings. The cultures were started from early passage cells and split either 1:2 (one passage) or 1:4 (two passages), according to the procedures described by Thompson and Holliday (*3*). The histogram includes previously published lifespans, including ten cultures grown at 34°C, and unpublished results, including six cultures grown throughout at 33°C. Cultures grown at these temperatures had the same average lifespan as those at 37°C. One culture, which died at passage 33, probably from an experimental mishap, is excluded. The histogram does not include any of the longevity results shown in Fig. 5. The mean of the experimental lifespans is indicated by an arrow. Fig. 3 (right). Distribution of lifespan in passage numbers of 47 cultures of the human diploid fibroblast strain WI-38. These results were obtained in Hayflick's laboratory between 1968 and 1972. Cultures were derived from a total of 28 passage 8 ampuls and grown until senescence and death according to procedures described by Hayflick (*1*).

periments were carried out mainly with MRC-5. This strain was originally characterized by Jacobs *et al.* (*13*) and many passage 8 ampuls are stored in liquid nitrogen. We normally receive MRC-5 cells at passage 10 or 11, and over a 7-year period during which various longevity experiments have been carried out, it is evident that parallel cultures set up from a population derived from one ampul and grown under identical conditions exhibit considerable variation in lifespan. Moreover, the doubling potential of cells derived from different ampuls is also significantly different and cannot be accounted for by possible variation in viable cell number between ampuls. The combined longevity data for 67 populations derived from several different ampuls is shown in Fig. 2B. (It is important to mention that in these experiments the individual populations were set up at an early passage when, according to our model, a significant number of uncommitted cells are present. Thus, the loss of these cells by dilution in any one culture is independent of that in any other culture.)

The mean lifespan is 62 passages and, as can be seen, some experiments give longevities well to either side of the main body of the distribution. A short lifespan may, of course, be the result of unknown culture mishaps, such as inferior batches of serum or medium. Although we routinely test for mycoplasma infection by the method of Russell *et al.* (*14*), very small numbers of particles are not seen and these may sometimes spread rapidly through the population as it becomes senescent and thereby accelerate cell death. It should be noted that the observed variation in the longevity of MRC-5 represents a very real heterogeneity in growth potential. For instance, a difference in lifespan of 10 PD's represents a difference of 2^{10}, that is, a factor of 1000 in growth potential.

In Fig. 2A we represent the lifespan distribution of 300 simulated cultures. The scatter is similar to the experimental results except that the distribution of lifespan is skewed and there are no very short-lived cultures. Since the predicted variance in lifespan is strongly dependent on P (*12*) we can limit our estimate of P to a range of approximately 0.2 to 0.3, and thus the value $P = .275$ may be fairly accurate. We have also received from L. Hayflick information about the longevity of WI-38 cells reconstituted at different times from passage 8 ampuls. The distribution in lifespan of 47 cultures is shown in Fig. 3. The results are very similar to those shown in Fig. 2, A and B, except that the mean lifespan is much shorter than MRC-5, and the scatter is somewhat greater.

Effect of bottlenecks. Culture size, N, has a critical effect on lifespan since it determines the time at which the uncommitted cells are lost by dilution. Increasing N increases the expected lifespan until a size is reached where virtual immortality is possible, although N may need to be so large that this may not be seen in laboratory cultures. Conversely, decreasing N decreases lifespan. We can thus see that, if we can increase or decrease N by a sufficient amount, we may

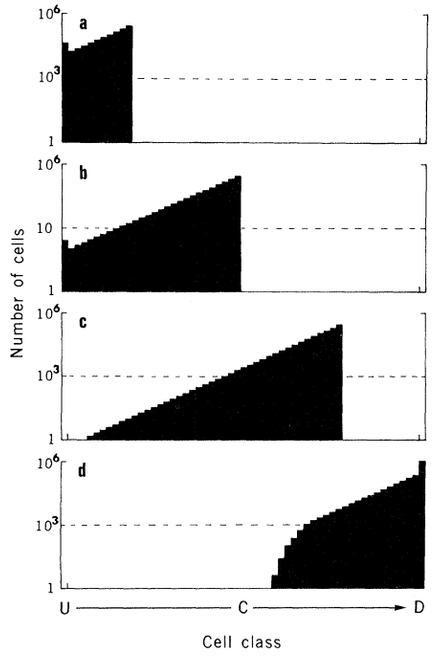


Fig. 4. The expected population profiles, that is, distributions of cells in the classes of the model, at various points during a culture lifespan. Uncommitted cells (*U*) give rise to committed daughter cells, which move steadily to the right through the committed classes (*C*) until at the end of their incubation period, they die (*D*). The broken lines indicate the critical cell number (10^3) below which individual classes of cells may be lost during a 1000-fold reduction in culture size, or bottleneck. (a) After only ten cell generations, the cells in each occupied class are sufficiently numerous to avoid elimination. (b) After 27 cell generations, the uncommitted and recently committed cells are vulnerable to loss, and a reduction in culture lifespan may be expected. Occasional bottlenecks may, however, contain some uncommitted cells and consequently be rejuvenated. (c) After 43 cell generations, the uncommitted cells have been lost by dilution, and bottlenecking will generally eliminate the younger committed cells. Since these have the greatest division potential, a reduction in culture lifespan is expected. This reduction remains constant until the first cell deaths occur since the shape of the profile does not change, the population moving collectively one class to the right with each division. (d) At a late stage in the culture lifespan, cells are accumulating in the dead and final committed classes, and the effect of bottlenecking decreases.

be able to demonstrate a corresponding increase or decrease in lifespan. This, however, presents considerable practical difficulties and so we adopted an alternative approach. Routine cultures were grown normally, but at a certain point in the lifespan were drastically reduced in size, or "bottlenecked," to about 10^3 to 10^4 cells. They were then allowed to grow back up to the normal size N and cultured routinely for the remainder of their lifespan.

According to the time at which the bottlenecks were taken, we would predict a variety of effects. Early bottlenecks, when the cells are concentrated in large numbers in the uncommitted and early committed classes (Fig. 4a) should show little effect on lifespan other than increasing the variance. Similarly, late bottlenecks, when the cells are concentrated in the dead and late committed classes (Fig. 4d), will also have little effect. However, intermediate bottlenecks will correspond to times when the cells are distributed over many or all of the classes (Fig. 4, b and c), and there will then be relatively few cells in the uncommitted or earlier committed classes. These cells represent a considerable proportion of the cell division potential of the population, and their premature loss, as is likely to happen when the bottleneck is taken, will thus result in a reduction in lifespan. Particularly important are the uncommitted cells, since these are capable of producing further uncommitted cells and thus have a high division potential. Bottlenecks taken when the uncommitted cells are relatively few in number may contain no uncommitted cells, in which case the lifespan is reduced, or may contain some uncommitted cells in which case by chance the proportion of uncommitted cells in the culture may be temporarily increased. In the latter case, the population is slightly "rejuvenated" and may show an increase in lifespan. Computer simulations of the distribution of lifespans of bottleneck cultures are shown in Fig. 5A where these various predictions may be seen.

Preliminary experiments were carried out with a culture of human fibroblasts obtained from abdominal tissue of a normal 10-week-old fetus. The primary culture was established, with cells passaged in the normal way. Cell counts were made at every subculture so that cumulative cell numbers and population doublings could be calculated exactly. In bottleneck experiments, 10^4 cells at various passage levels were inoculated into Falcon plastic wells (15 mm diameter) and then allowed to grow up to the nor-

mal population size of $\sim 2 \times 10^6$. In two experiments shown in Table 1, it can be seen that an early bottleneck had a striking effect on the final lifespan, whereas bottlenecks taken at later times had less or no effect.

These results are in accord with our theoretical expectation. Another preliminary experiment (results not shown) was carried out with an MRC-5 culture at passage 10 and also passage 20. Bottlenecks of 10^3, 2×10^3, or 10^4 cells were taken and allowed to grow up to the normal culture size, and the subsequent lifespan was determined. We found that cultures derived from the smaller bottlenecks have the shorter lifespan, as we would expect.

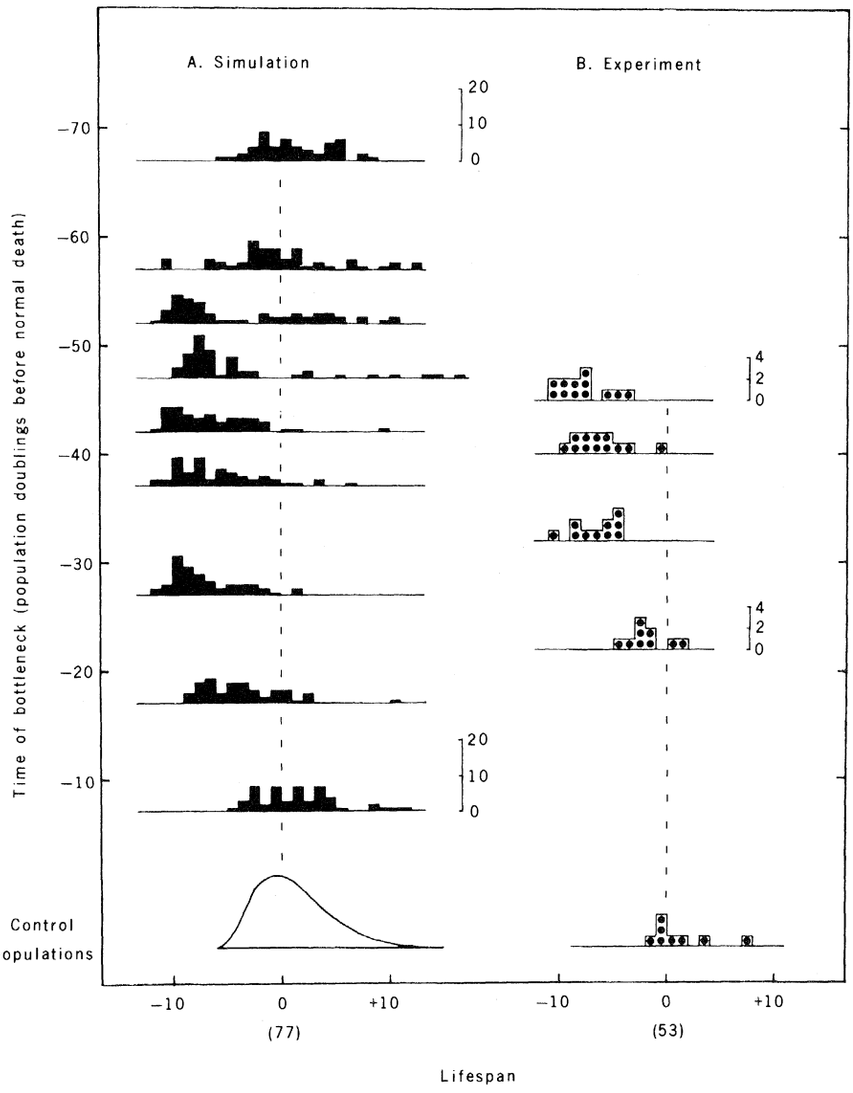


Fig. 5. Histograms of simulated (A) and experimental (B) bottleneck culture lifespans. Each simulated distribution is derived from 50 initially uncommitted cultures whose size was reduced from 2×10^6 to 2×10^3 at the indicated time in the lifespan. These times are denoted as population doublings prior to the mean population doubling level at which control populations died out. The experimental distributions are derived from cultures of MRC-5 cells which experienced a similar 1000-fold reduction in size at various points during their lifespan. To facilitate comparison between simulation and experiment, the same convention has been adopted to describe the times of the bottlenecks. The cells were all derived from one passage 8 ampul and subcultured with a 1:2, 1:4, or 1:8 split ratio (depending on the age of the culture); Eagle basal medium (BME) was used, supplemented with 10 percent fetal bovine serum previously heated at 56°C for 30 minutes, 1 percent nonessential amino acids and antibiotics (penicillin, 100 unit/ml; streptomycin, 100 μg/ml; aureomycin, 10 μg/ml). Confluent cells were harvested with trypsin-versene (*3*) and counted with a Coulter counter prior to subculture to calculate cumulative population doublings. Bottleneck cultures were established at passages 8, 13, 21, and 31 by placing 2×10^3 cells in 15-mm Falcon plastic wells. These cultures were transferred to containers of increasing size until the normal population of $\sim 2 \times 10^6$ cells was reached, and this was then subcultured normally until death. Cell counts during the buildup of the bottleneck populations show an exponential growth curve, and extrapolation back to zero time demonstrates that most if not all of the cells in the bottleneck were viable. In calculating population doublings we assumed that viability was 100 percent.

The above experiments can be criticized since the stochastic nature of the process of dilution and the loss of uncommitted cells will tend to result in considerable variation in longevity from experiment to experiment. The definitive experiment therefore requires the study of many populations, which in practice is hard to achieve. We have carried out one major experiment with MRC-5 in which 8 to 12 bottlenecks of 2×10^3 cells were taken at each of four PD levels. Results are shown in Fig. 5, together with the longevity of control cultures derived from the same original ampul. (This particular ampul consistently produced mass cultures with an average lifespan of about 54 PD's.) It is clear that the average lifespan of the 8, 13, and 21 PD bottlenecks was reduced by approximately eight PD's, which is close to the reduction seen in the simulated experiments. The likelihood of obtaining these results by chance is negligible. The 31 PD bottlenecks had a significantly greater subsequent lifespan. These overall results strongly support our theory, and more particularly, they indicate that our choice of $P = .275$ may be close to reality.

We did not, however, see a bimodal distribution of longevity with passage 8 bottlenecks, which suggests that at this stage the proportion of uncommitted cells is already as little as about 0.005 percent. If this is so, we can estimate when the population might have consisted entirely of uncommitted cells since approximately 30 cell generations would be required to reach this level, if $P = .275$. We have estimated from the growth of primary cultures that small tissue explants contain 10^3 to 10^4 fibroblasts capable of proliferation. The passage 1 population of 2×10^6 cells would therefore have doubled 8 to 11 times in vitro. The proportion of uncommitted cells in the tissue explant would therefore be approximately 1 percent. MRC-5 cells are derived from a 14-week-old fetus weighing approximately 75 grams. If we assume that 0.1 to 1 percent of the cells are fibroblasts, there would have been approximately 10^8 to 10^9 fibroblasts altogether. Our calculations above suggest that these were derived from a population of primordial uncommitted fibroblasts by approximately 15 PD's. Our estimate of the size of this population is therefore 10^3 to 10^4, a reasonable figure, and we calculate that passage 1 fibroblasts have already achieved approximately 20 to 25 in vivo and in vitro population doublings.

We are now in a better position to estimate the value of M, the incubation period. We have reason to believe that the transition from stage 1 to stage 2, that is, the first appearance of dead cells, occurs at about passage 40 for MRC-5. Thus we estimate M to be approximately 60 cell generations.

Variation among clones. Since the proportion of uncommitted cells is extremely small, even in early passage cultures, it would be very hard to obtain clones from uncommitted cells. If they were isolated, they would not give rise to immortal populations although the number of population doublings achieved would often be significantly greater than the average lifespan of mass cultures. Figure 4 illustrates the predicted heterogeneity in doubling potential of individual cells isolated at various points during the lifespan of fibroblast cultures. In each case, there would be considerable scatter in the longevity of clones, with the older populations (Fig. 4d) giving a high proportion of short-lived ones. These effects are well documented by the extensive cloning experiments of Smith and Hayflick (*6*), with WI-38 cells at the 22 to 41 PD levels. With cells at the ninth PD level (roughly equivalent to Fig. 4b), they also observed great heterogeneity in the doubling potential of individual clones. However, there was a subclass of dividing cells (~ 30 percent) that achieved only one to eight cell divisions. We do not predict the existence of such short-lived cells in young populations; and, since it is known that fibroblasts in isolation are inhibited in cell division, we suggest that this subpopulation is not made up of senescent cells.

Mixed cultures. The growth of mixtures of distinguishable but otherwise similar cell populations allows an important test of the model. If the growth rate of the two cell types is the same, the proportion of each should remain constant. However, when we consider the dilution out of uncommitted cells, it is clear that small numbers of these will often result in random drift to an excess of one cell type over the other. The consequence will be that the composition of the final population will often deviate from the proportion maintained throughout most of the lifespan. In 20 simulations of this experiment, starting with a 1:1 mixture, 11 populations diverged to give 80 to 100 percent cells of one type at the time of senescence. The behavior of six typical simulations is shown in Fig. 6.

We were pleased to discover that experiments of this type had already been carried out by Zavala, Fialkow, and Herner (*15*). They established many skin fibroblast cultures from female donors who were heterozygous for glucose-6-phosphate dehydrogenase (G6PD) A and B, which are electrophoretically distinguishable allelic variants. Since G6PD is X linked and only one X chromosome is active, individual cells have either G6PD A or B, and it is well known that this phenotype is completely stable. In most cultures approximately equal numbers of each cell type were present. In a few cases the ratio of A to B diverged steadily through passaging, which indicates that one cell type grew rather more quickly in culture than the other. However, in most cases the ratios of A to B stayed constant for many population doublings, which showed that there was no selection for one cell type. Our specific prediction was borne out since it was frequently observed that at the end of the lifespan either G6PD A or G6PD B cells became predominant. In the populations where there was no evidence of cellular selection, about half showed divergence to 80 to 100 percent of one cell type during the senescent period (*15*). We believe that these final populations are derived from a very small number of uncommitted cells which, by chance, consisted only of G6PD A or B types. This "genetic drift" to uncommitted cells of one phenotype occurred, of course, many cell generations prior to senescence and possibly in the skin tissue itself.

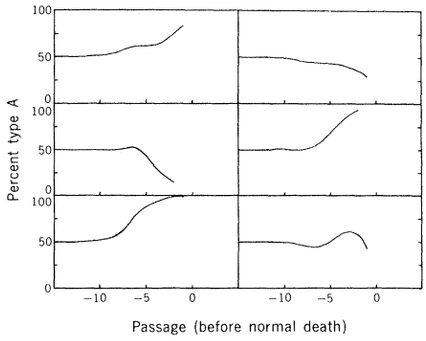


Fig. 6. The predicted behavior of six typical cultures consisting initially of a mixture of equal numbers of uncommitted cells of two distinguishable but otherwise similar types, A and B. For the greater part of the lifespan, the equal proportions of the two cell types are maintained. However, toward the end of the lifespan, the cultures deviate in a variety of ways from this equal distribution. This behavior results from stochastic differences in the losses by dilution of the uncommitted cells of the two types during an earlier part of the lifespan.

Comparable studies have been carried out by Ogden and Micklem (*16*) with mice in which hematopoietic cells were serially transplanted from an initial donor to successive recipients, which had previously been irradiated to destroy their own hematopoietic cells. The T6 chromosome marker, either heterozygous or homozygous, was used to track the fate of donor cells. When mixtures of the two distinguishable cell types were used, the ratio remained constant for three to four serial transplants. But in the last one to two transplant generations, before the cells finally died out, one or the other type came to predominate. This result is essentially the same as that obtained by Zavala *et al.* (*15*) and suggests that the dying out of the cells may be due to a stem line of uncommitted, perhaps immortal, cells being diluted out—a possibility that Ogden and Micklem themselves discuss.

Table 1. The longevity of fetal human fibroblasts as a mass culture (~ 2×10^6 cells) and after reduction in population size ("bottlenecks") at different passages. Cells were grown from abdominal wall tissue from a normal 10-week-old fetus. From the rate of growth of the primary culture, it was estimated that the population arose from ~ 10^4 cells. This can be regarded as a passage-0 bottleneck. Later bottlenecks of 10^4 cells were taken at intervals during the lifespan of the mass culture, allowed to grow to 2×10^6 cells, and then subcultured until senescence and death. The cells in experiments 1 and 2 were obtained from two fragments of tissue. Media and methods were the same as those described in the legend to Fig. 2, except that cumulative population doublings were calculated from cell counts at each 1:4 split subculture.

Time of bottleneck (passage)	Population doublings		Total lifespan (PD)	Difference from control
	Before	After		
		Experiment 1		
0 (control)			45	
10	12.5	18	30.5	−14.5
14	17	22.5	39.5	−5.5
20	21.5	22.5	44	−1
26	28.5	14.5	43	−2
32	35.5	7.5	43	−2
38	40.5	3.5	44	−1
		Experiment 2		
0 (control)			40	
10	12.5	17	29.5	−10.5
27	28.5	11.5	40	

Conclusions

The commitment theory of fibroblast aging provides an explanation for the following observations:

1) Individual fibroblasts produce clones with variable growth potential.

2) Parallel populations of one fibroblast strain grown under the same conditions also vary considerably in their longevity.

3) Longevity is related to population size, in particular, a 1000-fold reduction in population size at early passage levels, followed by a 1000-fold increase, results in a reduction of final lifespan of about eight population doublings.

4) When cultures contain equal numbers of two distinguishable types of cell, which have stable phenotypes and the same growth rate, one or other cell type frequently becomes predominant at the end of the lifespan.

We do not believe that any other single explanation for all these observations is available.

Our theory may also help to explain the fundamental difference between mortal diploid cells and immortal heteroploid transformed ones since reduction in the incubation period or the probability of commitment can convert a population with finite growth to one with infinite growth potential. However, it also predicts that diploid fibroblast populations are not intrinsically mortal since, if extremely large numbers of cells could be grown, the uncommitted cells would never be lost and would therefore continually replenish the population. The Hayflick limit may, in a sense, be an artifact that is the inevitable consequence of normal culturing procedures.

Implicit in our model are three simplifying assumptions that could be questioned. These are that P, M, and cell division time are the same for all cells. It is important to know what happens if we relax these and assume instead that the values we have used are simply the means of distributions within the population. Varying M or cell division time does not greatly affect our predictions, the only change being a more gradual transition from stage 1 to stage 2. However, varying P can have significant effects on population behavior. If we suppose either that the range of the distribution is sufficient to allow a small number of cells with very small P values or that the population contains—in addition to the majority of cells with P values close to the mean—a small subpopulation with a heritable small P value, we may expect the following: (i) during stage 1 there will remain a slowly declining number of these particular uncommitted cells (subject, of course, to stochastic variations, and, perhaps, loss); (ii) during stage 2 they will slowly increase in numbers while the overall population growth rate is reduced; (iii) the population will apparently die out in stage 3, but there may remain a few uncommitted cells (with small P value), which will continue to divide, and the population will eventually be regenerated, growing out of the plateau at a rate higher than that of stage 2. This prediction agrees with the observations by Todaro and Green (*17*), who studied the effect of cell density on senescence and transformation in mouse fibroblast cultures. In particular their observations agree with our predictions that culture size should influence the time taken to reach senescence (phase III) and the proportion of cultures showing spontaneous transformation. In interpreting the results of Todaro and Green, we assume that culture vessels of constant size were used, so that varying cell density inevitably changes population size.

So far we have not considered the molecular events which may accompany the process of commitment and the subsequent long incubation period prior to senescence and death. At the outset we explained that the heterogeneity in growth potential of individual cells is incompatible with the setting up of a clock or program in the population as a whole. It is, however, possible that commitment is the setting of such a program, which then runs through the incubation period to cell death. It has not escaped our notice that the value of P we favor, .275, is close to .25, and that mechanisms may occur which result in the segregation of 25 percent programmed cells. For instance, if, in the replication of homologous chromosomes, one daughter chromatid acquires a heritable but recessive epigenetic switch, such as a specific DNA methylation (*18*), then on average one in four cells will inherit a maternal and paternal chromosome containing that switch.

A completely different explanation of commitment is based on the error theory of aging. Orgel suggested that errors in protein synthesis may lead to an irreversible increase of errors and a final lethal error catastrophe (*19*). Later he pointed out that errors may simply reach a steady state (*20*). It is quite possible that such a steady state is unstable, with a given probability (P) that a feedback of errors will begin and eventually lead, many generations later, to senescence and death. Considerable evidence is available which suggests that aging of fibroblasts is accompanied by alterations or defects in enzymes (*21, 22*), genes (*23*), chromosomes (*24*), DNA replication (*22, 25*), and repair (*26*), all of which might be expected if errors in macromolecules are accumulating.

Whatever the basis of commitment, if our theory has validity, it opens up the possibility of selectively isolating uncommitted cells, or cell populations enriched with uncommitted cells. In this way, the population might be propagated indefinitely. This would not only be of intrinsic interest to gerontologists, but it would also facilitate a variety of experimental procedures, not least somatic cell genetics, which are at present impeded by the Hayflick limit.

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