

## A Mortalization Theory for the Control of Cell Proliferation and for the Origin of Immortal Cell Lines

SYDNEY SHALL AND WILFRED D. STEIN

Biochemistry Laboratory, Biology Building,  
University of Sussex, Brighton, Sussex BN1 9QG, England

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An increasing rate of cellular mortalization sets a limit to the proliferation of fibroblast populations. Failure of the mortalization mechanism leads to the formation of immortal clones of cells. A possible molecular model for the mortalization process is described.

### 1. The Control of Cell Proliferation

A widely-used model for the study of cellular proliferation is the *in vitro* culture of fibroblasts (Hayflick & Moorehead, 1961). This paper advances a simple hypothesis for the control of fibroblast cell proliferation in cell culture. We describe a molecular model consistent with this hypothesis. We discuss the relevance to cancer of this kinetic theory and the molecular model.

Reproduction is the characteristic property of living cells. In multicellular animals, however, cells in different adult tissues show varying reproductive behaviour. Thus, neurones and skeletal muscle never reproduce; liver and uterus reproduce at a low rate but may be stimulated to short periods of intense proliferative activity; the lining of the gut, the skin and the haemopoietic tissues are in a constant state of cellular proliferation, although the tissue as a whole is in a steady-state. Clearly, some fine biological control mechanisms are responsible for cellular reproduction. Cancer may be, in part, a dysfunction of the molecular mechanisms that control cellular reproduction.

Fibroblast secondary cell strains characteristically have a finite reproductive lifespan (Hayflick & Moorehead, 1961). Human fibroblasts in secondary culture achieve about 50 doublings (Hayflick, 1965; Martin *et al.*, 1974), mouse fibroblasts about 10 to 20 doublings (Todaro & Green, 1963; Lan, 1971). After this time the culture seems to stop reproductive activity or is overrun by induced or spontaneous mutants which give rise to immortal cell lines.

We explain the finite lifespan of animal cells by the concept of mortalization. This we define as the process by which an individual cell permanently loses the capacity for further reproduction. We assume that each newborn cell has the choice either to divide or never to divide again, with a definite probability for each of these stochastic events. We postulate that the probability of mortalization ( $P_m$ ) in secondary fibroblast cultures increases with time.

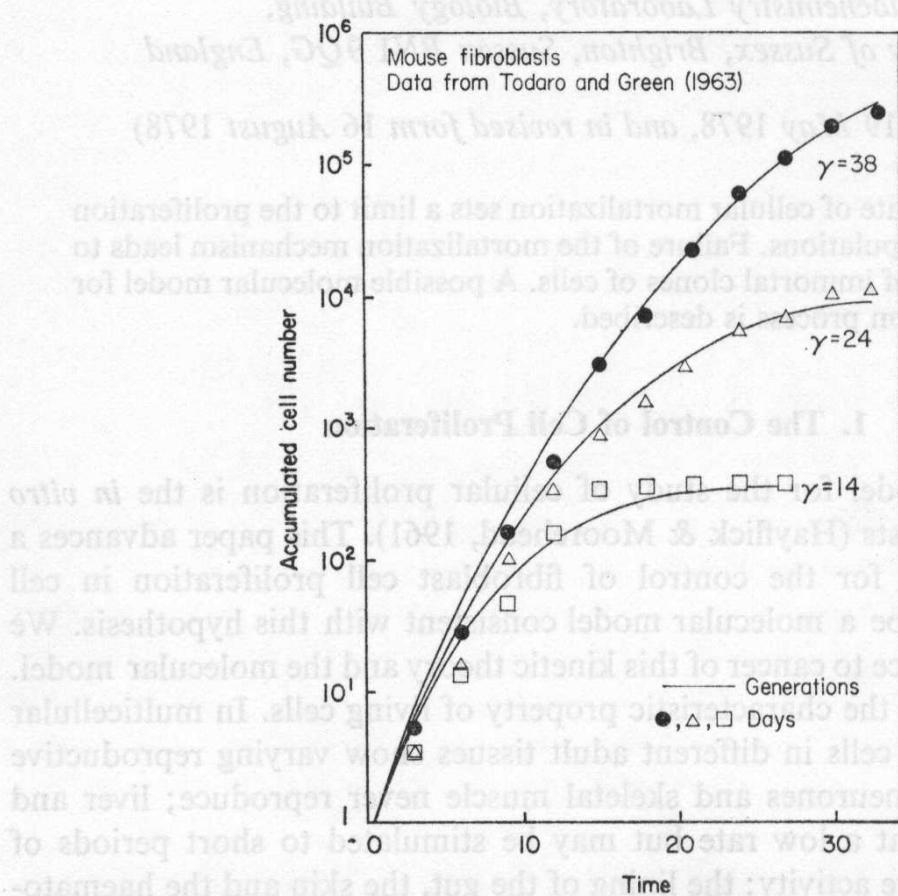


FIG. 1. Theoretical curves for the cumulative cell number as a function of number of generations, where the probability of mortalization ( $P_m$ ) increases with generation number.  $P_m$  is given by  $t/(y+t)$  where  $t$  is number of generations and  $y$  is a constant, taken at 14, 24 and 38 generations for the three curves depicted. The points are experimental values of the cumulative cell number as a function of time in days for mouse embryo secondary cultures, taken from the work of Todaro & Green (1963). The theoretical curves were calculated using the formula that the number of newly-born cells at time  $t$  is given by

$$\frac{2^t}{\prod_{k=1}^t (1+k/y)}$$

and accumulating in each generation the number of such newly-born cells that will never divide again, given by  $t/(y+t)$  times the number of newly-born cells. The cells were plated out at the following initial numbers per 5 cm dishes: squares,  $1 \times 10^5$ ; triangles  $3 \times 10^5$ ; circles,  $6 \times 10^5$ .

A convenient form for the dependence of  $P_m$  on time is

(Later, we will consider the molecular model for mortalization consistent with this formulation).  $t$  is time in units of generations.  $\gamma$  is that time, number of generations, at which the probability of mortalization reaches 0.5.  $P_m$  has the desired characteristic of increasing with time from zero to unity. Thus,  $\gamma$  determines the number of generations required to reach any defined probability of mortalization, and hence the final limiting population reached by a particular culture. In Fig. 1 we depict typical population growth curves for  $\gamma$  values of 14, 24 and 38.

How well do these simulated curves correspond to actual experimental data? Figure 1 shows experimental data of Todaro & Green (1963) for growth of mouse fibroblast secondary cultures at three different cell plating densities. Clearly the agreement is quantitatively satisfactory especially at later times. Figure 2 shows our experiments similar to those of Todaro and

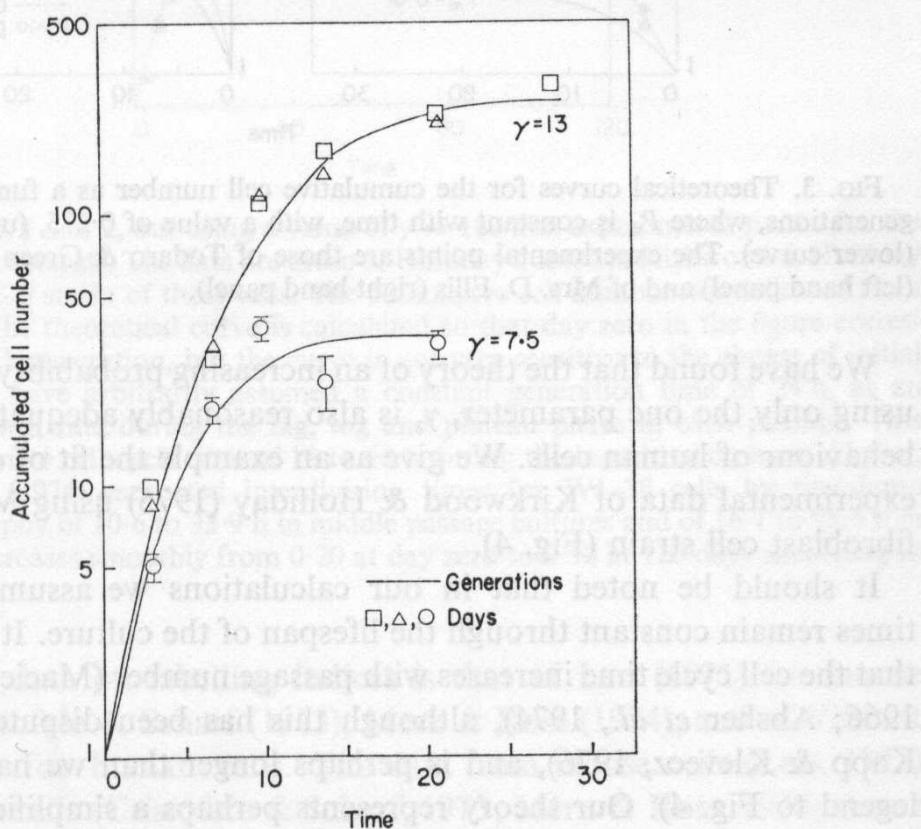


FIG. 2. As in Fig. 1, but using values of  $\gamma = 7.5$  and 13. The experimental points are cumulative cell numbers of mouse embryo secondary cell cultures grown in our laboratory by Mrs. D. Ellis, using mice of the strain CD-1(ICR) BR. These give consistently lower cumulative cell numbers than do the Swiss mice used by Todaro & Green (1963). The lower curve shows mean cell number  $\pm$  one standard error bar, for four cultures grown in parallel. The upper curve depicts the behaviour of two individual cultures.

Green but using a different strain of mouse embryo. Again, an appropriate value of  $\gamma$  satisfactorily describes the population kinetics (Fig. 2). It is necessary to assume that the probability of mortalization does increase with time. If one takes constant values of  $P_m$ , then one either obtains too few cells (if  $P_m \geq 0.5$ ) or with  $P_m < 0.5$  the population increases without limit (Fig. 3).

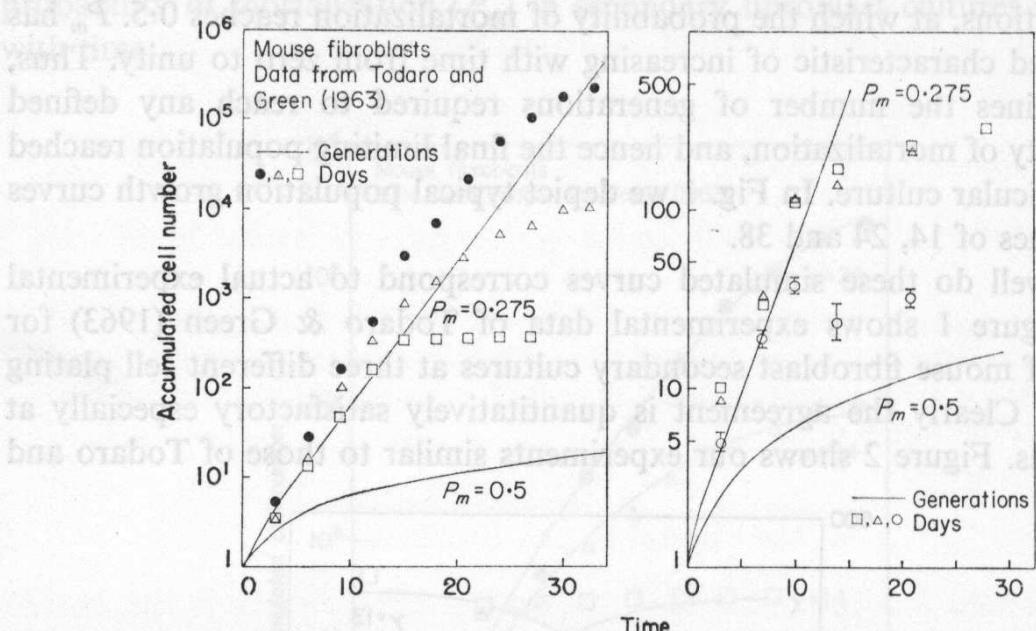


FIG. 3. Theoretical curves for the cumulative cell number as a function of number of generations, where  $P_m$  is constant with time, with a value of 0.275, (upper curve) or 0.50, (lower curve). The experimental points are those of Todaro & Green (1963) as in Fig. 1, (left hand panel) and of Mrs. D. Ellis (right hand panel).

We have found that the theory of an increasing probability of mortalization using only the one parameter,  $\gamma$ , is also reasonably adequate to describe the behaviour of human cells. We give as an example the fit of our theory to the experimental data of Kirkwood & Holliday (1975) using MRC 5, a human fibroblast cell strain (Fig. 4).

It should be noted that in our calculations we assume that cell cycle times remain constant through the lifespan of the culture. It seems, however, that the cell cycle time increases with passage number (Macieira-Coelho *et al.*, 1966; Absher *et al.*, 1974), although this has been disputed (Smith, 1977; Kapp & Klevecz, 1976), and is perhaps longer than we have assumed (see legend to Fig. 4). Our theory represents perhaps a simplified, first approximation to what is actually happening.

Cells which are not yet mortalized are able to synthesize DNA. The fraction of such cells can be estimated by measuring the labelling index. This is the fraction of cells which incorporates radioactive thymidine into DNA during a long incubation.

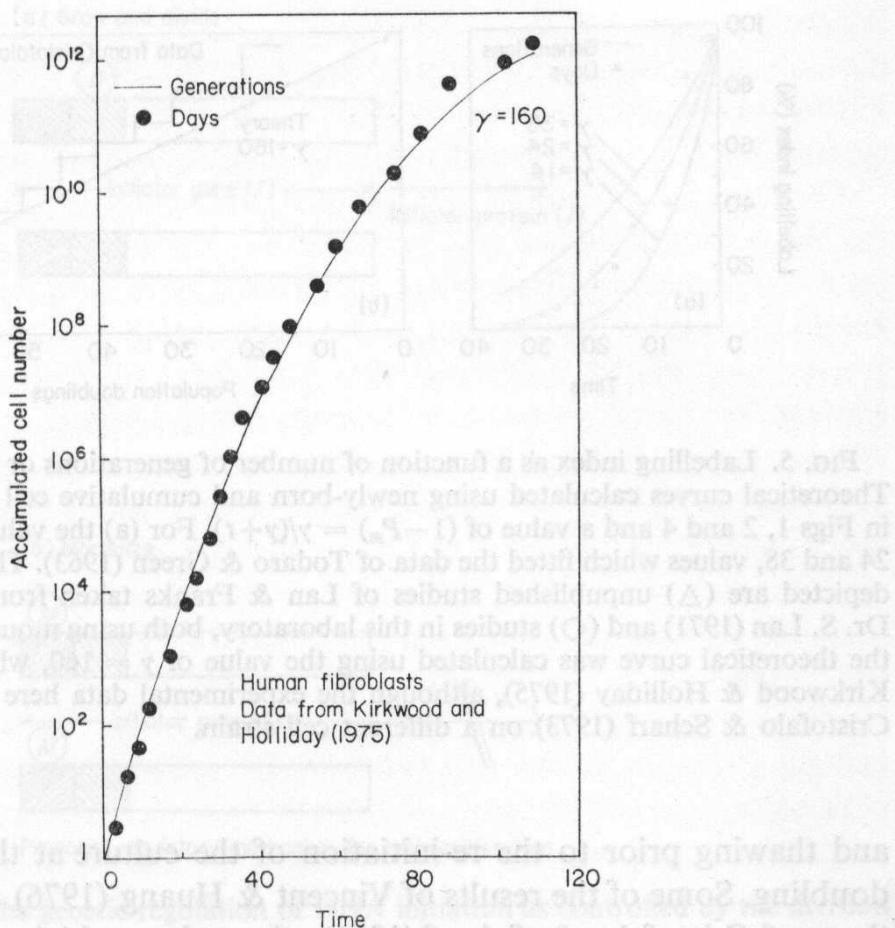


FIG. 4. As in Figs 1 and 2, but using a value of  $\gamma = 160$  and experimental points for the growth of human fibroblasts. The data are those of Holliday quoted in Kirkwood & Holliday (1975) on the MRC-5 strain of these cells. The cumulative cell number was followed from the 24th passage. The theoretical curve is calculated so that day zero in the figure corresponds to the fortieth generation, but the curve is not very sensitive to the choice of initial starting point. We have arbitrarily assumed a constant generation time of 24 h, as an average of the growth rate during the lag, log and plateau phase in each passage. This implies an approximate cell cycle time of 12 to 16 h during the logarithmic phase. Absher, Absher & Barnes (1974) estimated interdivision times for W1-38 cells by time-lapse cinemicro photography of 10.6 to 33.9 h in middle passage cultures and of 16.1 to 98.4 h in late cultures.  $P_m$  increases smoothly from 0.20 at day zero to 0.50 at 120 days according to equation (1).

The available data on labelling indices is that of Lan (1971) for mouse cells and of Cristofalo & Scharf (1973), Merz & Ross (1974), and of Vincent & Huang (1976) for human cells. The data from mouse cells (Lan, 1971) and from human cells (Cristofalo & Scharf, 1973; Merz & Ross, 1974) shows a reasonable correspondence with our theory using  $\gamma$  values derived from Figs 1 and 4 [Fig. 5(a) and (b)]. We note especially the changing value of the labelling index as the cultures mature. The small discrepancy in Fig. 5(b) between the theory and the data of Cristofalo & Scharf (1973) may have been partly due to the possible loss of non-proliferating cells during freezing

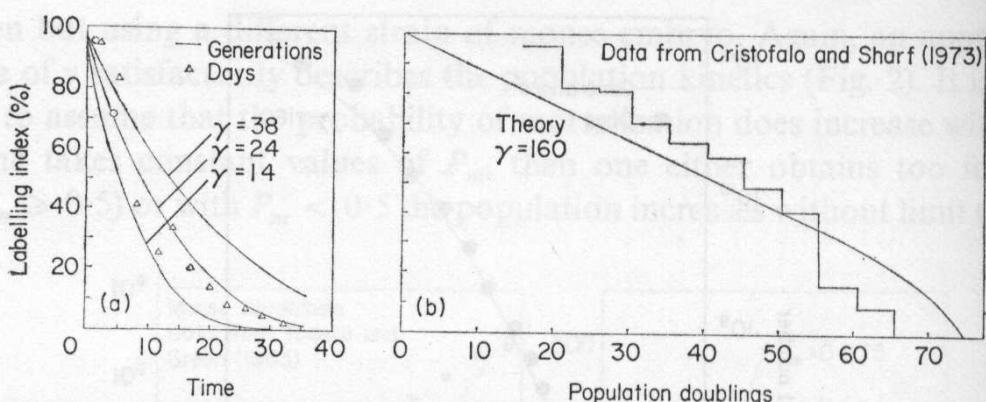


FIG. 5. Labelling index as a function of number of generations or population doublings. Theoretical curves calculated using newly-born and cumulative cell number as calculated in Figs 1, 2 and 4 and a value of  $(1 - P_m) = \gamma / (\gamma + t)$ . For (a) the values of  $\gamma$  chosen are 14, 24 and 38, values which fitted the data of Todaro & Green (1963). The labelling index data depicted are ( $\Delta$ ) unpublished studies of Lan & Franks taken from the Ph.D. thesis of Dr. S. Lan (1971) and ( $\circ$ ) studies in this laboratory, both using mouse fibroblasts. For (b), the theoretical curve was calculated using the value of  $\gamma = 160$ , which fitted the data of Kirkwood & Holliday (1975), although the experimental data here are those taken from Cristofalo & Scharf (1973) on a different cell strain.

and thawing prior to the re-initiation of the culture at the 18th population doubling. Some of the results of Vincent & Huang (1976) are in conflict with those of Cristofalo & Scharf (1973); these data which are in conflict are not consistent with our theory. We discuss this in more detail below.

We now describe a simple molecular model which gives rise to the kinetic behaviour discussed above (Fig. 6). We assume that an initiator protein ( $I$ ) is responsible for the transition of a cell from the non-reproductive to the reproductive part of the cell cycle; that means to DNA synthesis and thence to cell division. We suppose that this initiator protein ( $I$ ) is coded for by an initiator gene ( $i$ ) which has a regulatory sequence with two binding sites; one for a growth and division protein ( $D$ ) and one for a mortalization protein ( $M$ ). When the division protein ( $D$ ) binds at its binding site the initiator gene is activated to produce the initiator protein ( $I$ ) mRNA. However, when a mortalization protein ( $M$ ) binds at its binding site, we suppose that it modifies or blocks the  $D$  binding site so that  $D$  can never again bind. In consequence, this cell will never again synthesize initiator protein ( $I$ ) and will never again divide, that is, the cell is mortalized. That protein which binds first,  $D$  or  $M$ , commits the cell either to divide or never to divide again. In each generation, those cells which have not yet mortalized have a given probability ( $P_m$ ) of being mortalized, or of dividing yet again ( $1 - P_m$ ). In each cycle in a dividing cell the  $D$  protein is subsequently displaced from its binding site in readiness for the next cycle. We assume that  $P_m$  is given by

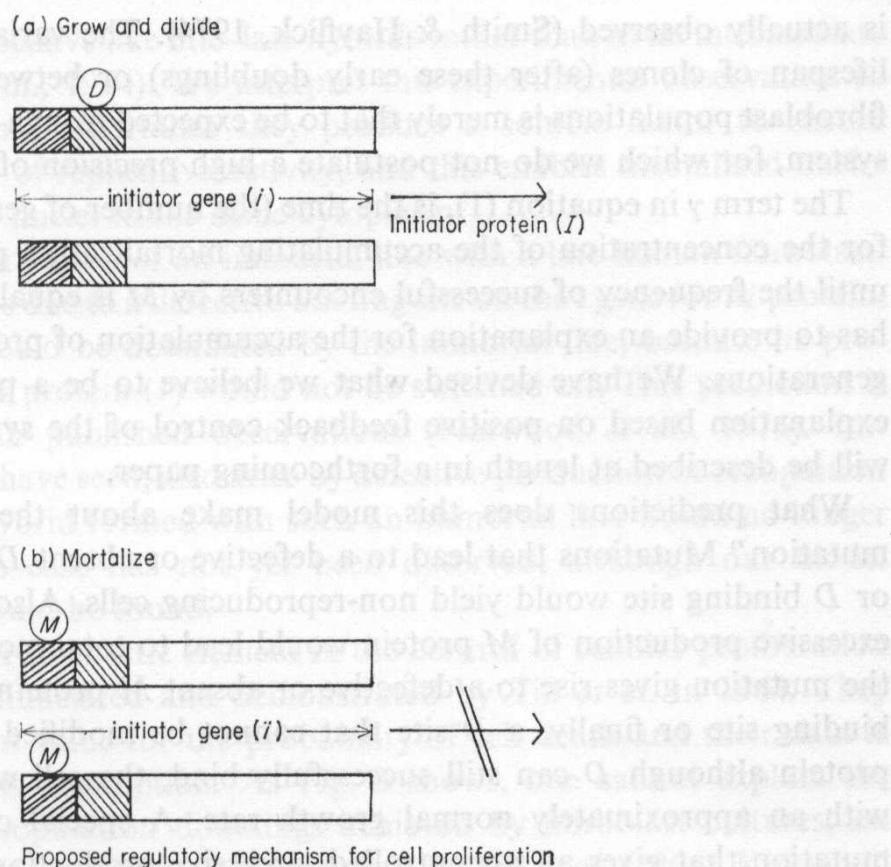


FIG. 6. Model for the genetic regulation of DNA initiation as controlled by the division and mortalization proteins.

the relative frequency of successful encounters between the regulatory sequence and either *M* or *D*. Thus,  $P_m$  can be set equal to

$$\frac{[M]}{[M]+[D]}.$$

We have described a particular, simple molecular model which would generate the observed kinetic behaviour. Other molecular models may be consistent with our kinetic theory. Perhaps the *M* site is on a separate gene whose product it is that modifies the *D* binding site.

The model we have described is essentially stochastic in nature, because it depends on the relative frequencies of successful encounters of the two proteins (*M* and *D*) with their respective regulatory sequences. A consequence of the stochastic nature of the model is that even at low concentrations of the protein *M* there will be occasions when it interacts with the binding site before *D* does so. In such a case the cell is mortalized at a low *M* value. Thus, in cloning experiments even at low passage numbers, there will be a definite number of cells which only go through one or a few divisions. This is what

is actually observed (Smith & Hayflick, 1974). The variability between the lifespan of clones (after these early doublings) or between the lifespan of fibroblast populations is merely that to be expected from a complex biological system, for which we do not postulate a high precision of control.

The term  $\gamma$  in equation (1), is the time (the number of generations) required for the concentration of the accumulating mortalization protein ( $M$ ) to rise until the frequency of successful encounters by  $M$  is equal to that by  $D$ . One has to provide an explanation for the accumulation of protein  $M$  over many generations. We have devised what we believe to be a plausible molecular explanation based on positive feedback control of the synthesis of  $M$ . This will be described at length in a forthcoming paper.

What predictions does this model make about the consequences of mutation? Mutations that lead to a defective or absent  $D$  protein,  $I$  protein or  $D$  binding site would yield non-reproducing cells. Also, the uncontrolled excessive production of  $M$  protein would lead to total mortalization. Where the mutation gives rise to a defective or absent  $M$  protein, or a defective  $M$  binding site or finally, a  $D$  site that cannot be modified or blocked by  $M$  protein although  $D$  can still successfully bind, the cell would be immortal with an approximately normal growth rate. A special case arises from a mutation that gives an uncontrolled, excessive production of  $D$  protein; in this case an immortal cell line which grows extremely rapidly would be produced. These descriptions apply to the homozygous diploid state. Heterozygous mutants defective in  $D$  protein,  $D$  binding site or  $I$  protein would produce small colonies. Heterozygous mutants producing defective  $M$  protein would show longer lifespans and larger colonies. Heterozygotes with a defective binding site for  $M$  would be immortal. Hence, a single mutation in such an  $M$  binding site could lead to immortality. The relatively high rate at which permanent cell lines arise from secondary mouse embryos is consistent with the need for only one mutation. The absence of such spontaneous human permanent cell lines may be explained by postulating the presence of several equally effective  $M$  binding sites in the  $i$  gene, rather like the situation in the  $N$  and  $t\sigma f$  genes in phage  $\lambda$ . More than one mutation would then be required for the formation of an immortal line.

We now consider the result of fusing pairs of cells. First, we consider the fusion of cells from early and late secondary cultures. On our model the essential difference between early and late cultures is that the late cultures have accumulated a considerable amount of  $M$  protein. On fusion the late cells will donate part of the accumulated store of  $M$  protein to the early cell, making it behave like a much older cell. Fusion of early and late cells should lead to an equal sharing of any soluble mortalizing factors, and thus to behaviour intermediate between early and late cultures. Early-late human

fibroblast hybrids behave like late-late hybrids rather than in an intermediate way (Norwood *et al.*, 1974). We interpret this experimental observation to mean that when cells mortalize they produce a soluble factor to ensure complete cessation of replicative activity, and this enables mortalized nuclei to switch off other nuclei in the same cytoplasm.

We next consider fusion of an immortal line with a late human fibroblast. If the immortality is due to a defective binding site on the *i* gene for *M* protein, then the hybrid would be dominated by the immortal line, because its production of initiator protein (*I*) would not be switched off. This prediction is in accordance with published observations (Norwood *et al.*, 1975). Immortality may, we have seen, also arise by defective production or recognition of *M* protein. A hybrid formed with such an immortal line would no longer be immortal. This case has not yet been observed, although our model predicts that it should be found.

The concept of a stochastic element in the control of cellular proliferation was first clearly enunciated and demonstrated by Till *et al.* in 1964. They assumed a constant value for the probability of cell death and their data is consistent with this assumption. As Fig. 3 shows, one cannot explain the large number of population doublings achieved by fibroblast cultures, on the basis of a constant value of  $P_m$  as assumed by Till *et al.* (1964). Kirkwood & Holliday (1975) have, however, attempted to resolve this difficulty by introducing the concept of an incubation period between the stochastic commitment and the deterministic execution of cell death. This postulate has the required property of giving large cell numbers if one assumes an appropriately large incubation period. Kirkwood and Holliday retain the postulate of a constant  $P_m$ ; their model accounts adequately for their data on the proliferation of human fibroblasts.

However, in some of the human fibroblast cultures (Cristofalo & Scharf, 1973; Merz & Ross, 1974; Vincent & Huang, 1976), and in the mouse cultures (Todaro & Green, 1963; Lan, 1971) (Fig. 5) the labelling index declines steadily from the first passage. This is precisely what our kinetic theory predicts and is significantly different from the Kirkwood and Holliday theory which predicts that until the end of the incubation period the labelling index will be 100%; however, some of the data of Vincent & Huang (1976) support this latter model.

Now, Lan (1971) measured the incorporation of labelled thymidine into nuclei of mouse fibroblasts, as a function of passage number after 24 h of incubation with thymidine, and found the steady drop in labelling index with culture age. Similarly, using human fibroblasts and a labelling period of also about one day, Cristofalo & Scharf (1973) reported a steady drop in labelling index with age. This general approach, namely, of choosing a particular

timepoint of the thymidine incorporation as a measure of the labelling index, has been criticized by Macieira-Coelho (1974) on the following ground: As the culture ages, so (as we have seen above) do the cell cycle parameters seem to change towards a greater average cycle time. Thus, the low labelling index found by Lan (1971) and Cristofalo & Scharf (1973) might, on Macieira-Coelho's arguments, merely reflect a failure to give the cells of the culture sufficient time to incorporate label, and thus be a more or less gross under-estimate of the number of proliferating cells present. Thus, Vincent & Huang (1976), in their study of the labelling index as a function of passage number, of a variety of human normal and diseased fibroblast cultures, incubated cells with thymidine for up to six days and measured the plateau value of labelling index reached, and used this value as a measure of cell viability. In direct contrast to the data reported by Cristofalo & Scharf (1973), these latter workers found that the labelling index of a culture of normal human fibroblasts dropped precipitously only after forty population doublings, being steady and high previously.

We believe that a simple measure of the labelling index at some fixed point (either 24 or 48 h or even at a "plateau" value) does not give an unambiguous measure of the parameter desired, namely, the fraction of cells able to divide at least one more time, at the time of the experiment. To appreciate our position requires a brief look at what is indeed happening in a thymidine labelling experiment (see Steel, 1977). At the instant of addition of labelled thymidine, all those cells capable of taking up the label (i.e. all those cells in the process of synthesizing DNA at that instant) will be labelled. The fraction of labelled cells at zero time is thus exactly given by the ratio of the number of cells in the *S*-phase of the cell cycle to the total number of cells present, cycling and non-cycling. (In some of the published studies referred to above, the curves of labelling index with time extrapolate back to zero labelling index at zero time, indicating a failure on the part of the researchers concerned to appreciate this point.) As the time of incubation is prolonged, more cells enter *S*-phase on their way around the cell cycle, until at a time given by  $(T_c - T_s)$ , i.e. the length of one generation time less the length of the *S*-phase, all cells that are capable of labelling will be labelled and the labelling index will reach 100% of such cells. The correct incubation time is therefore not longer than the difference  $T_c - T_s$ . On the available data, this time is not more than 12 h for early passage cells and rises to some 24 h for late passage cells. One might argue that these times are average times and thus to ensure that all cells that can label *will* label, one should increase this time somewhat, but this argument is fraught with difficulty. As time passes, those cells capable of division will divide and thus continually dilute out the non-dividing cells, giving rise to an over-estimate of the fraction of viable cells. If, as in the

experiments of Macieira-Coelho (1974) and those of Vincent & Huang (1976), this time is several, or even many, days, enough time will elapse for, say, five population doublings, or a thirty-two fold increase in the number of viable cells, giving a completely misleading picture of the extent of dividing cells present at the time of initially adding label. To obtain a measure of the fraction of cells capable of division at a particular point in time is not a simple problem, but is crucial to the testing of the theories of cell ageing that we have discussed above. It will be necessary to apply a battery of labelling techniques backed by a full theory for the labelling index as a function of time in order that the desired measurements be obtained. We should then be in a position to resolve the apparent contradiction between the experimental data of Cristofalo & Scharf (1973) and of Vincent & Huang (1976), and hence to test the two opposing theories of cellular senescence, our theory and the Kirkwood-Holliday theory insofar as these make different predictions about how the number of non-dividing cells in a population decreases with passage number of the culture.

The specific feature of our theory which generates our prediction is that at least some cells mortalize from the beginning. If there is, however, an increasing frequency of mortalization then the population will eventually but inexorably cease expanding. We can quantitatively explain the kinetics of the so-called Hayflick phenomenon, that is the finite lifespan of human fibroblasts in culture, with just the single parameter  $\gamma$ , and can also fit adequately much, but not all, of the labelling data. The alternative theory fits the kinetics of the accumulation of cell number, but does not adequately fit the labelling index data in mice and in some of the experiments on human cells. The conflict within the data on human fibroblast cultures needs to be resolved by further experiments before they can be used to discriminate between these rival theories.

The ideas and extensive experimental data of G. M. Martin and colleagues (1977) on the longevity of fibroblasts in culture are in general agreement with our kinetic theory. The elegant "bottleneck" experiment of Holliday *et al.* (1977) in which the longevity of a culture is decreased by drastically reducing the size of the population at an early time, strongly suggests that such early cultures still contain significant numbers of uncommitted cells. In our view these uncommitted cells would not yet have begun the process of mortalization, that is, the accumulation of  $M$ . The transition to the path to mortalization may well occur stochastically. We accept that the "bottleneck" experiment requires the presence of uncommitted cells in an early, secondary fibroblast culture. However, we differ from Holliday *et al.* (1977) in our view of the subsequent molecular events. Our view is that there now begins a gradual accumulation of the mortalization protein  $M$ ; while

Holliday *et al.* (1977) view commitment as a transition to a state of accumulating errors in the biosynthetic machinery of the cell.

These fibroblast cell culture systems have frequently been used as models for the study of senescence. We would like to draw a sharp distinction between reproductive senescence which our theory and model discusses and degenerative senescence, which may be largely post-replicative and about which we do not comment.

An important corollary of our theory is that the parameter  $\gamma$  may be expected to be sensitive to environmental conditions. It may be valuable to explore this interaction. The data of Todaro & Green (1963) in Fig. 1 show the influence of plating density on the parameter  $\gamma$ . With human fibroblasts the age of the donor (Martin *et al.*, 1977), or the intermittent exposure of cells to fluorescent light (Parshad & Sanford, 1977) affect the longevity of the culture, and hence in our terms, the parameter  $\gamma$ .

Has our theory anything to say about cancer? The appearance of a clinical cancer requires the emergence of an immortal or poorly mortalizing clone of cells; this is a necessary, but certainly not a sufficient condition for the development of cancer. A possible molecular mechanism for the origin of immortal clones of cells follows naturally from our kinetic theory and the molecular model. If the process of mortalization fails in any one of a number of different ways, then an immortal clone will arise, and with further mutations a frank clinical cancer may result.

A failure of the mortalization mechanism could occur in one of two general ways. A primary failure would be an inability to switch on the mortalization mechanism in a stem cell, giving rise to a stem cell tumour. A secondary failure, occurring in reproductively-active mortalizing cells, would be a defect in one or other step of the mortalization mechanism itself. Thus, a failure to produce or correctly recognize *M* protein (Fig. 6) would lead to an immortal cell line. Of these, only the failure to recognize that protein which blocks binding of *D* would produce an immortal cell line when present in the heterozygous state. For this reason, this is likely to be the most common failure. But complementation analysis by cell fusion between tumour cell lines should reveal the full range of classes of defects.

Our theory and model make strong predictions about the kinetics of accumulated cell number, the labelling index and the behaviour of cell hybrids in both normal secondary cell strains and tumour cell lines.

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## REFERENCES

- ABSHER, P. M., ABSHER, R. G. & BARNES, W. D. (1974). *Exp. cell Res.* **88**, 95.  
CRISTOFALO, V. J. & SCHAFER, B. B. (1973). *Exp. cell Res.* **76**, 419.  
HAYFLICK, L. (1965). *Exp. cell Res.* **37**, 614.  
HAYFLICK, L. & MOOREHEAD, P. S. (1961). *Exp. cell Res.* **25**, 585.  
HOLLIDAY, R., HUSCHTSCHA, L. I., TARRANT, G. M. & KIRKWOOD, T. B. L. (1977). *Science* **198**, 366.  
KAPP, L. N. & KLEVECZ, R. R. (1976). *Exp. cell Res.* **101**, 154.  
KIRKWOOD, T. B. L. & HOLLIDAY, R. (1975). *J. theor. Biol.* **53**, 481.  
LAN, S. (1971). Ph.D. Thesis, University of London.  
MACIEIRA-COELHO, A. (1974). *Nature* **248**, 421.  
MACIEIRA-COELHO, A., PONTEN, J. & PHILLIPSON, L. (1966). *Exp. cell Res.* **42**, 672.  
MARTIN, G. M. (1977). *Am. J. Path.* **89**, 513.  
MARTIN, G. M., NORWOOD, T. H. & HOEHN, H. (1977). In *The Molecular Biology of the Mammalian Genetic Apparatus*. (P. Ts'o, ed.) ch. 23, pp. 289–302, Amsterdam: Elsevier.  
MARTIN, G. M., SPRAGUE, C. A., NORWOOD, T. H. & PENDERGRASS, W. R. (1974). *Am. J. Path.* **74**, 137.  
MERZ, G. S. & ROSS, J. D. (1974). *J. cell Physiol.* **74**, 219.  
NORWOOD, T. H., PENDERGRASS, W. R., SPRAGUE, C. A. & MARTIN, G. M. (1974). *Proc. natn. Acad. Sci. U.S.A.* **71**, 2231.  
NORWOOD, T. H., PENDERGRASS, W. R. & MARTIN, G. M. (1975). *J. cell Biol.* **64**, 551.  
PARSHAD, R. & SANFORD, K. K. (1977). *Nature* **268**, 736.  
SMITH, J. A. (1977). *Cell biol. Int. Rep.* **1**, 283.  
SMITH, J. R. & HAYFLICK, L. (1974). *J. cell Biol.* **62**, 418.  
STEEL, G. G. (1977). *Growth Kinetics of Tumours*, Oxford: Oxford University Press.  
TILL, J. E., McCULLOCH, E. A. & SIMINOVITCH, L. (1964). *Proc. natn. Acad. Sci. U.S.A.* **51**, 29.  
TODARO, G. J. & GREEN, H. (1963). *J. cell Biol.* **17**, 299.  
VINCENT, R. A. & HUANG, P. C. (1976). *Exp. cell Res.* **102**, 31.

## 1. Introduction

The electric impedance of the squid (*Loligo pealei*) giant axon was one of the first characteristics to be investigated (Curtis & Cole, 1938) during the early production of this preparation. These incisive measurements indicated an average membrane capacitance of about 5 pF/cm<sup>2</sup> and a time constant of 75–80 ms. In subsequent work, changes in membrane capacitance were measured in squid axon (Cole & Curtis, 1939), and a value