# Auto-catalysed progression of aneuploidy explains the Hayflick limit of cultured cells, carcinogen-induced tumours in mice, and the age distribution of human cancer

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Evidence continues to accumulate that aneuploidy, an imbalance in the number of chromosomes, is responsible for the characteristic phenotypes of cancer, including the abnormal cellular size and morphology of cancer cells, the appearance of tumour-associated antigens, as well as the high levels of membrane-bound and secreted proteins responsible for invasiveness and loss of contact inhibition. Aneuploidy has also been demonstrated to be the self-perpetuating source of the karyotypic instability of cancer cells. Here it is shown that the auto-catalysed progression of aneuploidy explains the kinetics of the finite lifetime of diploid cells in culture, the time course of the appearance of papillomas and carcinomas in benzo[*a*]pyrene-treated mice, and the age-dependence of human cancers. Modelling studies indicate that the ease of spontaneous transformation of mouse cells in culture may be due to a chaotic progression of aneuploidy. Conversely, the strong preference towards senescence and resistance to transformation of human cells in culture may be the result of a non-chaotic progression of aneuploidy. Finally, a method is proposed for quantifying the aneuploidogenic potencies of carcinogens.

Key words: carcinogenesis, dynamics, metabolic control analysis.

## INTRODUCTION

Recently, we have provided experimental and theoretical evidence [1–4] supporting Theodor Boveri's proposal that aneuploidy, an imbalance in the number of chromosomes, is the underlying cause of cancer [5]. Aneuploidy provides a simple and coherent explanation for the fundamental phenotypes of cancer cells, independent of gene mutation. Accordingly, the abnormal cellular size and morphology of cancer cells [6], the appearance of tumour-associated antigens [7,8], the high levels of membrane-bound and secreted proteins [9] responsible for invasiveness and loss of contact inhibition, and even the daunting genetic instability [10,11] that enables cancer cells to evade chemotherapy are all the natural consequence of the massive over- and under-expression of proteins due to aneuploidy [1–3].

Using Metabolic Control Analysis we were able to demonstrate how the fraction of the genome undergoing differential expression, rather than the magnitude of the differential expression, controls phenotypic transformation [3]. In order to transform the robust normal phenotype into cancer the expression of thousands of normal gene products must be increased by an average of 2-fold [3]. The results showed that alterations in a handful of 'gatekeeper' or 'caretaker' genes [12,13] are insufficient for the generation of cancer-specific phenotypes, since their numbers are too few to alter the normal phenotype. Indeed, not one mutant cellular gene, nor even a group of mutants, has ever been shown to transform a normal human or animal cell into a cancer cell [14–18].

We were also able to show that aneuploidy is the self-perpetuating source of the genetic instability of cancer cells [1,3,4]. Aneuploid cells are trapped in an endless cycle of rearranging the genome to produce the most economical production of translation products at the expense of chromosomal balance. The mitotic division of an aneuploid cell is an experiment in evolution that results in the random shuffling and redistribution of the genome. The generations of offspring of aneuploid cells are unlikely to ever have identical genetic compositions [3,19,20]. It is this intrinsic genetic instability of aneuploid cells that fuels the progression to malignancy of cancer cells.

Since a loss of gene dose is more deleterious than a gain [3,21–24], hyperploid cells have a survival advantage over their hypoploid siblings. The survival advantage of hyperploid cells, coupled with the inherent genetic instability of aneuploid cells, fuels the auto-catalysed progression to DNA indices between 1.5 and 2 that are found in most malignant cancers [25].

Here I analyse the time course of the auto-catalysed progression of aneuploidy from its known experimental and unknown spontaneous origins. The results show that the finite *in vitro* lifetime of human diploid cell strains [26,27], the kinetics of tumour formation in benzo[*a*]pyrene-treated mice [28,29] and the age distribution of human cancers [30] can all be explained by the auto-catalysed progression of aneuploidy during cell division.

## THE HAYFLICK LIMIT OF MICE AND MEN

Due to the work of Hayflick [26,27], the finite lifetime of diploid cells in culture has become commonly known as the Hayflick limit. After a period of active multiplication, generally less than one year (approx. 50 cell divisions), primary human fetal cells in culture demonstrate an increased generation time, gradual cessation of mitotic activity, accumulation of cellular debris and, ultimately, total degeneration [27]. Only during the degenerative phase (phase III) in cell culture do primary cells lose contact inhibition and become obviously aneuploid [26,27,31]. In contrast to primary, diploid cells derived from an animal or humans, cell lines (immortal cells) are a heterogeneous mix of heteroploid cells.

According to Levan and Biesele [19], the very first mitoses of mouse cells *in vitro* show chromosomal irregularities. A zero level of numerical and structural chromosomal abnormalities '… is possible only with cells *in situ*, and that as soon as they are

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$$D + \phi \xrightarrow{k} \phi$$

**Scheme 1 Kinetic model of an auto-catalysed process**

explanted they start mutating … The genetic diversity thus induced in the tissue culture will increase steadily as new aberrations are continuously released by the mitotic mutation process. This situation prevails either until the tissue culture fades out or until some truly superior cells happen to appear …' [19].

In the spirit of Levan and Biesele I propose that the auto-catalysed progression of aneuploidy explains the observed time course of the Hayflick limit, including differences in spontaneous rates of transformation. The simplest kinetic model of an auto-catalysed process is shown in Scheme 1. It is important to point out that Scheme 1 represents an auto-catalysed process, not an auto-catalysed chemical reaction, which would be written as $A + B \rightarrow 2B$ [32]. $D$ and $\phi$ do not represent chemical species undergoing a chemical reaction and, therefore, do not imply stoichiometry. $D$ is the diploid fraction and $\phi$ is the aneuploid fraction of an aneuploid cell. $D$ and $\phi$ can also represent the average diploid and aneuploid fractions, respectively, of a population of cells.

Following an event that produces the initial aneuploidy (i.e. produces $\phi > 0$), the values of $D$ and $\phi$ tend to change with each mitotic division. We have previously shown that the aneuploid fraction, $\phi$, is equivalent to the flux control coefficient of Metabolic Control Analysis and is a measure of the extent to which a given aneuploid segment of the genome controls phenotypic transformation (see Appendix A of [3]).

The rate equation for the increase in the aneuploid fraction, $\phi$, in Scheme 1 is given by eqn. (1). Since $\phi$ appears on both sides of Scheme 1, the growth of the aneuploid fraction, $\phi$, is auto-catalysed. In other words, the greater the level of aneuploidy, the faster the growth of the aneuploid fraction $\phi$. The constant $k$ in Scheme 1 is a measure of the growth-rate of the aneuploid fraction $\phi$.

$$\frac{d\phi}{dt} = k\phi D \tag{1}$$

Since the sum of the diploid fraction, $D$, and the aneuploid fraction, $\phi$, always equals 1, the diploid fraction can be expressed in terms of $\phi$, i.e. $D = 1-\phi$. Making this substution for $D$ in eqn. (1) gives eqn. (2).

$$\frac{d\phi}{dt} = k\phi(1-\phi) \tag{2}$$

Integrating eqn. (2) yields eqn. (3), which gives the aneuploid fraction, $\phi$, as a function of time.

$$\phi_t = \frac{1}{e^{-kt}\left(\frac{1}{\phi_0}-1\right)+1} \tag{3}$$

The constant $\phi_0$ is the initial aneuploid fraction of an individual cell, or the average for a population of cells, at time zero, the time when aneuploidy is initiated (e.g. by a carcinogen or cell culturing). The rate constant $k$ has units cell-cycle$^{-1}$ when $t$ is in cell-cycles. Since $\phi_t$ ranges from 0 to 1 (i.e. 0–100%), eqn. (3) gives the time course for the progression of aneuploidy, and thus the time course of any phenotypic change that depends on it.

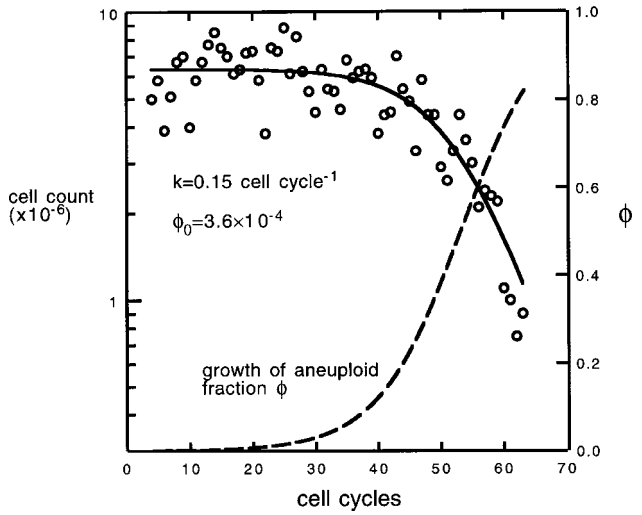


**Figure 1 Hayflick limit due to auto-catalysed growth of aneuploidy**

Hayflick limit of 63 cell divisions for the human cell strain WI-44. The solid line is the best-fit curve of eqn. (4) to the serial passaging data from Figure 3 of Hayflick [27]. The broken line represents the auto-catalysed progression of the aneuploid fraction, $\phi$, for the same data using eqn. (3).

With the growth of aneuploidy there is a corresponding reduction in the diploid fraction, and hence a reduction in the number of dividing cells since aneuploid cells are less viable than diploid cells [3,21–24]. If we assume that cell proliferation is due primarily to diploid cells, then the number of non-transformed dividing cells in culture at time $t$ is proportional to the diploid fraction $D_t$. Using the relationship $D_t = 1-\phi_t$ and the value of $\phi_t$ in eqn. (3), one can derive eqn. (4), which shows that the number of dividing cells ($N_t$) remaining during serial passaging is equal to the number of cells at time zero ($N_0$) times the diploid fraction at time $t$ [i.e. $N_t = N_0(1-\phi_t)$].

$$N_t = N_0\left(1-\frac{1}{e^{-kt}\left(\frac{1}{\phi_0}-1\right)+1}\right) \tag{4}$$

Figure 1 (solid line) shows that eqn. (4) fits the data from Hayflick's Figure 3 for the serial passaging of the human cell strain WI-44 [27]. The sharp decline in the cell count at around 43 passages (beginning of the degenerative phase III) is mirrored by the steep growth in the aneuploid fraction, $\phi$ (Figure 1, broken line).

The calculated value $\phi_0 = 0.0004$ (Figure 1) indicates that the average aneuploid fraction at the beginning of the exponential phase (phase II) of cell culture was 0.04%. However, this initial aneuploid fraction is only an estimate since it does not take into account that some aneuploid cells will be viable and divide, albeit at a reduced level. The initial aneuploidy in the WI-44 cells was almost certainly caused by the culturing process itself, especially the mechanical and enzymic treatments used to promote proliferation *in vitro* [19,26,27].

In contrast to cultured cells, the extra copy of chromosome 21 in Down's syndrome individuals is present at fertilization. Trisomy of chromosome 21 represents an aneuploid fraction $\phi = 0.018$ for each cell [3,22,33], which is substantially larger than the initial average value in Figure 1 for normal human explants. As a consequence, one would predict that the Hayflick limit of Down's syndrome cells in culture should be significantly shorter

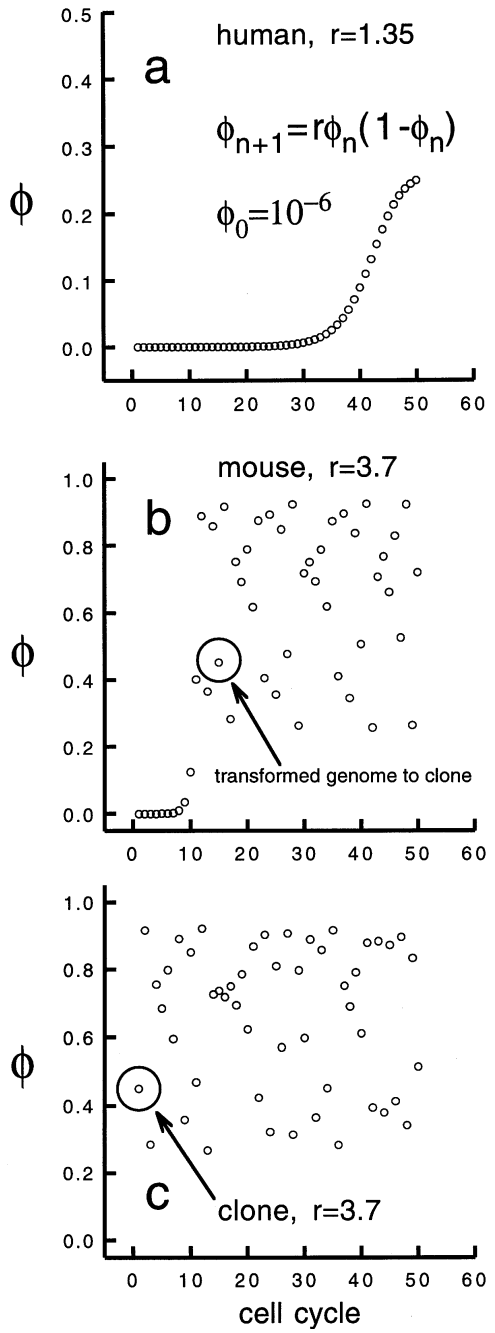


**Figure 2 Chaotic growth of aneuploidy drives transformation**

The logistic equation, eqn. (5), was used to model the auto-catalysed progression of aneuploidy for primary human (**a**) and mouse (**b**) fetal cells in culture. The control parameter $r = 1.35$ and the initial average aneuploid fraction $\phi_0 = 10^{-6}$ (see text for derivation) were used in eqn. (5) to model the 50 cell cycle Hayflick limit for primary human fetal cells (**a**). The circles represent the average aneuploid fraction, $\phi$, for the population of cells at each cell cycle. In panel (**b**) the same $\phi_0$ was used for the mouse fetal cells (see text). Although values of $r > 1.5$ are completely unrealistic for modelling the Hayflick limit of human cells, we could use values of the control parameter greater than 3.57 to model mouse cells. A control parameter greater than 3.57 for the logistic equation [eqn. (5)] produces chaotic growth patterns [39]. Therefore, the value $r = 3.7$ produces a chaotic progression of the aneuploid fraction, $\phi$, for all cell divisions beyond approx. 12 cycles (**b**). While the aneuploid human cells will probably die out before they can be transformed into an immortal cell line, because so little genome space is being explored, the chaotic redistribution of the mouse genome provides a greater opportunity for the cells to hit upon a genetic combination that leads to transformation and immortalization. (**c**) Shows that when the transforming genome in panel (**b**) (arrow) is cloned, its intrinsic karyotypic instability immediately leads to a heterogeneous population of heteroploid offspring.

than normal cells. A perusal of the literature shows that this prediction is correct. Schneider and Epstein reported in 1972 that, '... Skin fibroblasts derived from patients with Down's syndrome (trisomy-21) have a significantly decreased number of cumulative cell population doublings ... measured from the initial passage to senescence when compared with cultures from karyotypically normal age-matched controls ...' [34]. The Hayflick limit they measured for the Down's syndrome cells was 20% shorter than for normal cells, which represents an approximate 2000-fold reduction in the exponential process of cell doubling.

Recently, Mukherjee and Costello used fluorescence *in situ* hybridization to study the progression of aneuploidy in cultured fibroblasts from patients with three premature aging syndromes: Cockayne, Hutchinson–Gilford, and Werner [35]. '...[T]he interphase aneuploidy levels of all chromosomes under study were significantly higher in cells from the syndromes as compared to those of the normal controls at both earlier and later passages. In general, the interphase aneuploidy levels of each of the chromosomes in both the control and experimental cell cultures increased with *in vitro* proliferation and aging, although to a much lesser extent in the controls ...' [35].

The meticulous studies of Hayflick indicated that, '... the finite lifetime of ... diploid cell strains is an innate characteristic of the cells ...' [26,27]. Furthermore, he argued that, '... Cells which can be cultivated indefinitely *in vitro* (heteroploid cell lines) can only be compared with continuously cultivable cells *in vivo*, i.e., transplantable tumors. Likewise, diploid cells having a finite lifetime *in vitro* can only be compared with normal cells *in vivo*, i.e., normal somatic cells ...' [27]. Hence, the transformation of mortal (diploid) cells in culture into immortal (aneuploid) cell lines 'can be regarded as oncogenesis *in vitro* ...' [27].

Transformation of cells in culture can also be viewed as evolution *in vitro*. Explanted cells are forced to evolve into viable single cell organisms in the laboratory or perish. Most cultured human cells stop dividing after entering phase III (the degenerative phase) and only rarely undergo spontaneous transformation into immortal (aneuploid) cell lines [27]. In contrast, cultured primary rodent cells frequently undergo spontaneous transformation to become immortal cell lines [19,36]. The 70% shorter Hayflick limit (14 cell divisions) may be a clue as to why primary mouse fetal cells spontaneously transform into immortal cell lines much more readily than human cells [36].

The auto-catalysed genetic instability inherent in aneuploid cells, coupled with the chromosomal heterogeneity of cancer cells, suggests that an underlying chaotic process may be involved in the mitotic division of some aneuploid cells [37]. With this possibility in mind, I suspected that primary mouse cells in culture experience a more chaotic progression of aneuploidy than human cells. A chaotic redistribution of the genome would provide a greater opportunity for aneuploid cells to hit upon a genetic combination that would lead to immortalization and transformation.

While eqn. (2) gives the continuous rate of change of the aneuploid fraction, $\phi$, as a function of the instantaneous value of $\phi$, the actual change in DNA content of an aneuploid cell is a discrete process that takes place at each cell division. Alternatively, then, the growth of the aneuploid fraction, $\phi$, of Scheme 1 can be modelled by eqn. (5), a form of the logistic rate equation that is known to reveal certain chaotic processes [37–39].

$$\phi_{(n+1)} = r\phi_n(1-\phi_n) \quad (5)$$

The right side of eqn. (5) is formally identical with eqn. (2). However, the left side of eqn. (5) replaces the instantaneous change in $\phi$ with a discrete value of the aneuploid fraction for each cell division. Eqn. (5) shows that the average aneuploid

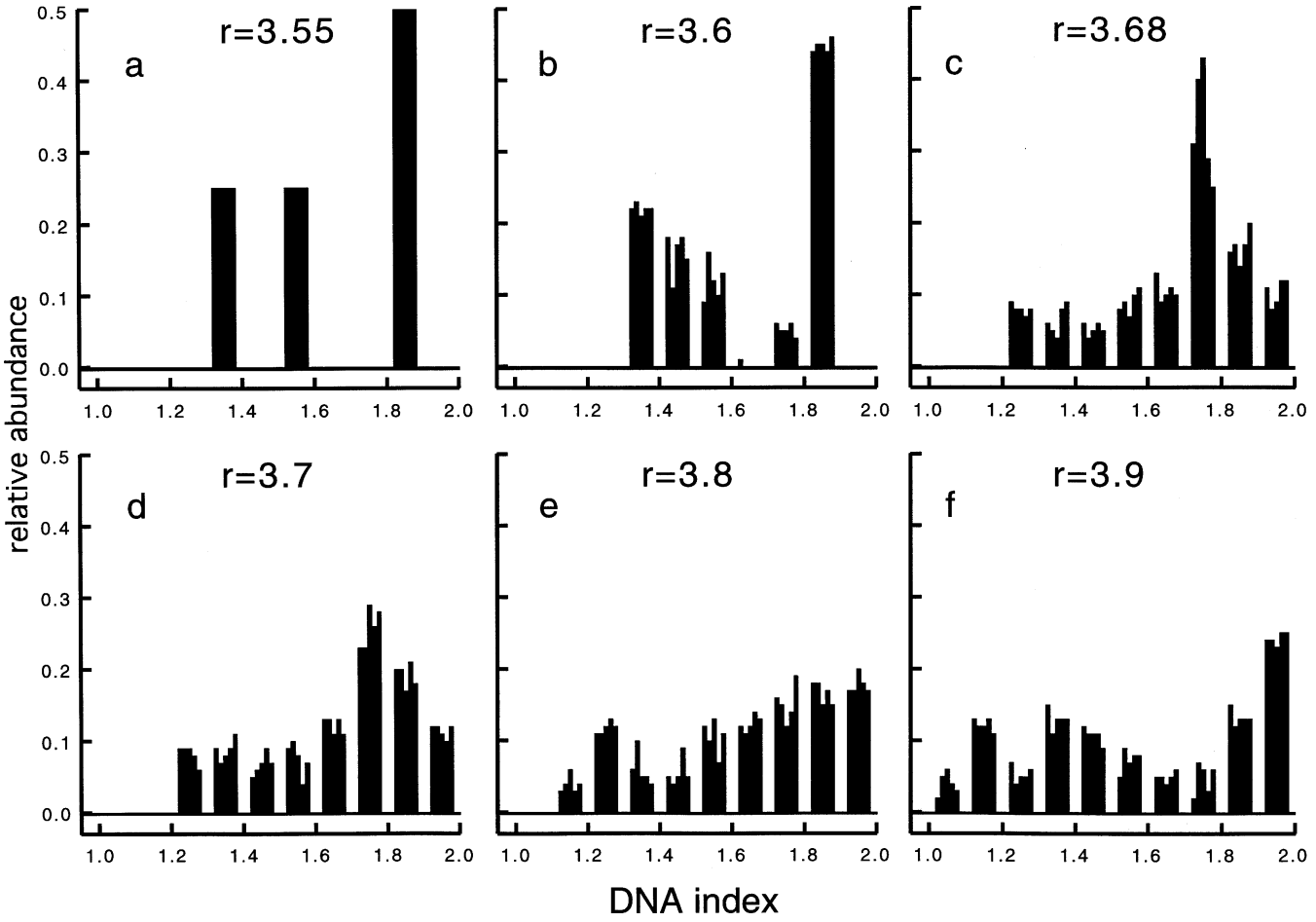


**Figure 3 DNA index of cancer cells is due to chaotic growth of aneuploidy**

Using the relationship DNA index $= 1 + \phi$ (see text for details) and the value $n = 1000$ cell cycles, eqn. (5) was used to model the distribution of DNA indices for various values of the control parameter $r$. In order to capture the long-term average behaviour in the distribution of DNA indices, we used only cell divisions 501–1000. The DNA indices were divided into 10 equal sampling bins centred at DNA index = 1.05, 1.15, 1.25, etc. There were five sampling windows comprising 100 cell divisions each (e.g. 501–600, 601–700, etc.). For each of the five sampling windows, the number of times a value of DNA index fell into a bin was counted. (**a**) A simple distribution of DNA indices for a non-chaotic value of $r$. (**b**–**f**) Show that in the chaotic region there is an increasing number of cells with a wider spectrum of DNA indices. (**c** and **d**) Show the DNA indices clustering around a value of 1.75, which compares with DNA indices of 1.7 for cervical cancer [44], 1.7–1.8 for breast cancer [45,46] and 1.6 for liver cancer [47].

fraction of a population of cells at the $n+1$ cell division is determined by the average level of aneuploidy at the $n$th cell division. The control parameter, $r$, in eqn. (5) is unitless and is different from the rate constant, $k$, in eqns. (2) and (3). Since $\phi_i$ ranges from 0 to 1 (i.e. 0–100%), eqn. (5) models the discrete growth, including chaotic, of any phenotypic change that depends on the progression of aneuploidy.

Since the non-disjunction frequency of mammalian cells *in situ* is estimated to be $10^{-4}$ to $10^{-5}$ per chromosome [40], and since there are 23 and 22 chromosome pairs in normal human and mouse cells, respectively, then the initial aneuploid fraction, $\phi_0$, for the explanted cells is no larger than approximately $10^{-6}$ [i.e. $10^{-4}/(22–23) \approx 10^{-6}$]. Therefore $\phi_0 = 10^{-6}$ was used in eqn. (5) to model the auto-catalysed progression of aneuploidy for human and mouse primary fetal cells in culture (Figure 2).

Several values of the control parameter, $r$, were tested before finding the value of 1.35 (Figure 2a) that reproduces the 35 cell-divisions of phase II and the sigmoidal growth of the aneuploid fraction, $\phi$, of Figure 1 for human cells. Values of the control parameter, $r$, greater than 1.5 were completely unrealistic since they resulted in phase III starting at approx. 10 cell divisions for the human cells.

In contrast to the human cells, the control parameter, $r$, had to be increased to at least a value of 2.5 in order to model the Hayflick limit of approx. 14 cell-cycles for primary mouse fetal cells. But what is more interesting, a value of the control parameter greater than 3.57 worked just as well. A value of $r >$ 3.57 for the logistic equation [eqn. (5)] results in chaotic growth patterns [39]. Thus the value $r = 3.7$ in Figure 2b produces a chaotic progression of the aneuploid fraction, $\phi$, for all cell divisions beyond approx. 12 cycles (Figure 2b).

Figures 2(a) and 2(b) fail to take into account the fact that almost all of the aneuploid cells would lose the gene-shuffling lottery and not become transformed into an immortalized cell line. However, if a transforming genome did happen to appear, for example the hypothetical one circled in Figure 2(b), and was cloned, it would generate from the very first cell division a heterogeneous population of heteroploid offspring as shown in Figure 2(c). Figure 2(c) models exactly the well-known karyotypic instability of cloned transformed cells [1,27,41]. As Hayflick and

Moorhead have said, '–The use of cloning as a means of reducing ... variability in heteroploid cell lines is unfortunately limited by the rapid re-emergence of a range of chromosomal types among the progeny of a clone ...'[26]. In fact, '... each subpopulation can regenerate the entire range of subpopulations ...' of a heteroploid population of cells [42].

The results of Figure 2 show that it is possible to model the hypothesis that the transformation-prone mouse cells can exhibit a substantially more chaotic pattern of aneuploidy than the transformation-resistant primary human cells in culture. If the results of Figure 2 reflect reality, an interesting question presents itself. What are the biochemical, genetic, or other factors responsible for the dramatically different values of the control parameter, $r$, that lead to the non-chaotic growth of aneuploidy in human cells on the one hand, and a chaotic progression of aneuploidy in mouse cells on the other? At present we don't know. What is known is that carcinogens initiate aneuploidy and also greatly accelerate its growth [1,2].

## THE DNA INDEX OF CANCER CELLS

As discussed above, the DNA index of a population of aneuploid cells tends to increase with each cell division until reaching steady-state values between 1.5 and 2 typical of late-stage cancer [43]. Recently, we have shown that the DNA index $= 1-\phi+\pi\phi$, where $\pi$ is the segmental ploidy factor (e.g. $\pi = 1.5$ for trisomy) [3]. While there is a limitless number of $\pi$ values, we have previously shown that $\pi = 2$ is the most economical, hence likely, value of the ploidy factor in cellular transformation [3]. Making this substitution for $\pi$ gives a DNA index $= 1+\phi$. Exploiting this simple relationship between the DNA index and the aneuploid fraction, $\phi$, eqn. (5) can be used to explore the chaotic growth of the DNA index of dividing cells.

Figure 3 shows a series of histograms for the relative abundance of aneuploid cells compared with DNA index for different values of the control parameter $r$. The most striking feature of Figure 3 is the clustering of cells around certain values of DNA index and the pronounced bias for most cells having hypertriploid to hypotetraploid DNA indices reminiscent of human cancer [43]. Particularly noteworthy is the clustering of cells around a DNA index of 1.75 in Figures 3(c) and 3(d). This value compares with DNA indices of 1.7 for cervical cancer [44], 1.7–1.8 for breast cancer [45,46] and 1.6 for liver cancer [47].

Figure 3(a) shows three populations of cells for $r = 3.55$, a value of the control parameter just below the chaotic region. Two groups of cells are near triploid (DNA index = 1.35 and 1.55) and the largest group is hypotetraploid (DNA index = 1.85). Although the cells in Figure 3(a) have DNA indices typical of cancer, they are unlikely to lead to a transformed, immortalized cell line because so little genome space is being explored. In other words, it is unlikely that dividing cells would happen upon viable transformants in the limited range of DNA indices presented in Figure 3(a).

A chaotic strategy of gene shuffling, on the other hand, is much more likely to lead to successful cancer cells. For example, Figures 3b–3f shows that for $r > 3.57$ (the chaotic region) an increasing number of cells are exploring a wider spectrum of DNA indices, thus increasing their chances of hitting on an aneuploid combination of genes leading to immortalization and transformation.

## TUMOUR FORMATION IN CARCINOGEN-TREATED MICE

In a large group of mice, the number of papillomas rises continuously from about five weeks after the first painting with benzo[*a*]pyrene until about a month after painting has been discontinued [28]. Charles and Luce-Clausen have proposed t0hat, '...mutation of some particular gene which is essential to normal differentiation of new skin cells ...' is necessary for the production of papillomas in benzo[*a*]pyrene-treated mice [29]. Furthermore, they suggested that papillomas form only after both copies of the hypothetical gene are mutated to the cancerous form. Based on this double-hit scenario, the authors used statistical arguments to derive a model of benzo[*a*]pyrene-induced papilloma formation reproduced here in eqn. (6).

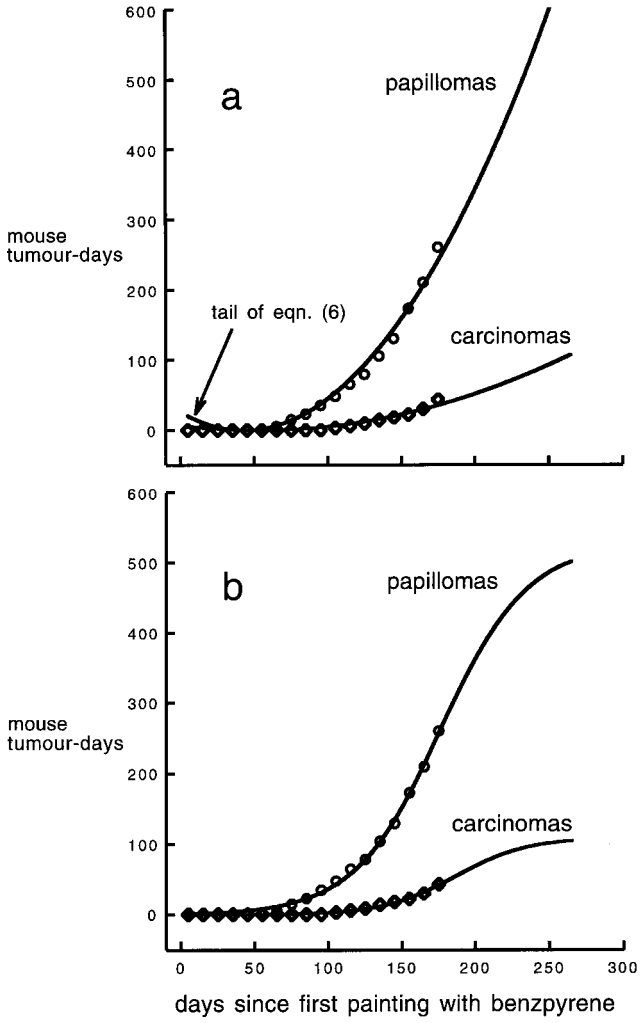


**Figure 4 Two models of tumour production in benzo[*a*]pyrene-painted mice**

Except for the upward tail of eqn. (6) (arrow) near the origin in (**a**), both models, the mutant-gene [eqn. (6)] (**a**) and the auto-catalysed progression of aneuploidy [eqn. (7)] (**b**), fit the experimental data of Morton et al. reasonably well [28]. However, the two models are conceptually quite different. The most notable difference is the shape of the growth curves. The gene mutation models are all parabolic upwards, hence the upward tail for eqn. (6) in (**a**), while the auto-catalysed progression of aneuploidy model is sigmoidal (**b**).

$$n = N\left(\frac{k(t-i)}{c}\right)^2 \qquad (6)$$

The dependent variable, $n$, is the average number of papillomas per mouse produced at time, $t$, after the first painting. The value $N = 4\times10^6$ is the average number of stratum germinativum cells (skin cells) painted with benzo[*a*]pyrene per mouse, $c = 3.5$ days is

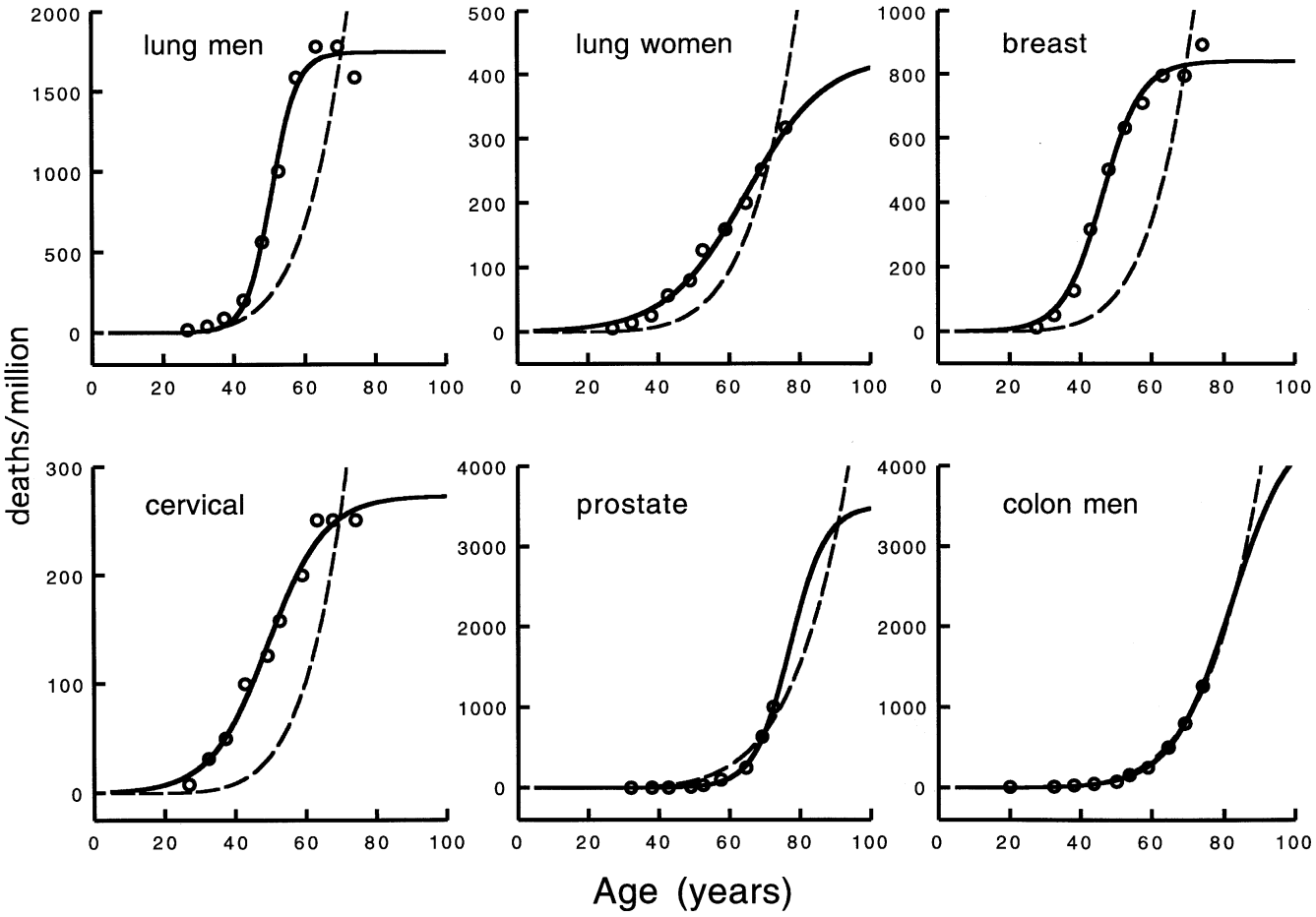


**Figure 5 Auto-catalysed growth of aneuploidy explains age distribution of human cancers**

The superiority of the sigmoidal curve of eqn. (7) for the auto-catalysed progression of aneuploidy is best demonstrated by comparing it with eqn. (8) for the multi-hit version of the gene mutation theory of human carcinogenesis. Eqn. (7) gives a good fit (solid lines) to the number of deaths per million people for six typical cancers as a function of age [30]. The broken lines show the best-fit curves to the same data for the seven successive mutation model [eqn. (8)]. The only good fit for eqn. (8) is with colon cancer deaths in men.

the interval between paintings, the calculated value $i = 32$ days is the average number of days between the moment a skin cell possesses both copies of the mutated hypothetical proto-cancer gene and the subsequent time when the tissue formed by that cell becomes recognizable as a papilloma, and $k$ is the mutation rate constant.

Figure 4(a) shows the best-fit curves of eqn. (6) to the data of Morton et al. for benzo[*a*]pyrene-induced papillomas and carcinomas in black mice [28]. The calculated mutation rate constants, $k$, are $2 \times 10^{-4}$ and $9 \times 10^{-5}$ for the papilloma and carcinoma data, respectively. This compares with a mutation rate constant of $3 \times 10^{-5}$ determined by Charles and Luce-Clausen for the same data [29].

If, on the other hand, the production of tumours is due to the auto-catalysed progression of aneuploidy [eqn. (3)] as a result of benzo[*a*]pyrene treatment, then the number ($N_t$) of papillomas or carcinomas at time $t$ is equal to the plateau number of tumours ($N_\infty$) times the average aneuploid fraction $\phi_t$ at time $t$ [eqn. (7)].

$$N_t = N_\infty \phi_t \qquad (7)$$

Figure 4(b) shows the best-fit curves of eqn. (7) to the data of Morton et al. [28]. Except for the upward tail of eqn. (6) near the origin in Figure 4(a) (arrow), both models, the mutant-gene [eqn. (6)] and the auto-catalysed progression of aneuploidy [eqn. (7)], fit the experimental data of Morton et al. reasonably well. However, the two models are conceptually quite different. The most notable difference is the shape of the growth curves. The gene mutation models are all parabolic upwards [hence the upward tail for eqn. (6) in Figure 4a], while the auto-catalysed progression of aneuploidy model is sigmoidal (Figure 4b).

The gene mutation model, in its various forms, predicts an increasing number of mutations and subsequent cancers over time. Eqn. (6), for example, assumes a specific number of mutations beforehand. In the example considered above, both copies of a hypothetical proto-cancer gene have to be mutated to produce papillomas and carcinomas in benzo[*a*]pyrene-painted mice. The aneuploidy model, on the other hand, makes only one assumption that holds for all cases, which is: the auto-catalysed progression of aneuploidy.

## THE AGE DISTRIBUTION OF CANCER IN MAN

The superiority of the sigmoidal curve is best demonstrated by comparing the auto-catalysed progression of aneuploidy with the prevailing multi-hit version of the gene mutation theory of human carcinogenesis. In the 1950s, log–log plots of cancer death-rates compared with age were roughly linear, with slopes of approx. 6 [30]. That meant that cancer death-rates increased

proportionally with the sixth power of age. It wasn't long before the sixth-power dependence was interpreted in light of the gene mutation hypothesis of cancer. It was hypothesized that cancer is the end-result of seven successive mutations [48,49]. However, this hypothesis did not lead to the observed result in all circumstances (Figure 5, broken lines). Aware of this shortcoming, Armitage and Doll warned that the successive cellular changes leading to the development of cancer were not necessarily gene mutations [30]. This is an important consideration since carcinogenic and mutagenic activities do not always go hand-in-hand [1,2,16,50]. This insight was short-lived, however. In deriving eqn. (8) to model the incidence-rate of cancer with age, Armitage and Doll assumed that seven mutations lead to cancer, and that the mutations should be specific, discrete, stable and proceed in a unique order [30].

$$Cancer\ rate_t = kp_1p_2p_3p_4p_5p_6p_7t^6 \quad (8)$$

Eqn. (8) shows that the incidence-rate of cancer at age $t$ (assumed to be proportional to death-rate [30]) will be proportional to the product of the probabilities of the occurrences of each of the seven mutations ($p_i$) and to the sixth power of age, where $k$ is the rate constant. With the exception of colon cancer in men, eqn. (8) is a poor model of the incidence-rate for a number of human cancers (Figure 5, broken lines). In an effort to salvage their model, Armitage and Doll argued that, due to ignorance of the individual mutation probabilities, $p_i$, they had to combine all the probabilities into one constant. According to the authors, this combined probability was the source of the poor fit between eqn. (8) and the data. They speculated, that if only one knew the individual mutation probabilities or could fashion a suitable weighting scheme to derive the appropriate mean probability, then eqn. (8) should fit the real-world data. Unfortunately, the authors were not able to come up with either. I was also unable to derive a parabolic equation that fits the sigmoidal incidence-rate data of Figure 5.

However, it turns out that eqn. (7), which is based on eqn. (3) for the auto-catalysed progression of aneuploidy, gives a good fit to all the cancer incidence-rate data of Figure 5 (solid lines). In this case, the number of cancer deaths per million persons ($N_t$) at age $t$ is equal to the plateau number of cancer deaths per million persons ($N_\infty$) times the right side of eqn. (3), which is the average aneuploid fraction, $\phi_t$, for a population of cells. The only good fit for eqn. (8) is with the incidence of colon cancer deaths in men, which may be the source of Kinzler and Vogelstein's proposal that seven gene mutations are responsible for colon cancer [49].

The sigmoidal nature of the mortality-rate data is more obvious in Figure 5 than in the log–log plots used by Armitage and Doll. The data points for lung cancer in men and women, as well as breast and cervical cancer in women, span much more of the sigmoidal region of eqn. (7) than do the data for prostate and colon cancer in men (Figure 5). This difference is due to the much later onset of prostate and colon cancer in men than with the other four examples. The inflection points of the sigmoidal curves are a measure of this difference. The inflection points for lung cancer in men and women, and for breast and cervical cancer in women are approx. 50, 60, 45, and 50 years of age respectively (Figure 5). However, the inflection points for prostate and colon cancer in men occur at much older ages: approx. 75 and 80 years, respectively (Figure 5).

## QUANTIFYING ANEUPLOIDOGENS

The only variables that a carcinogen, acting as an aneuploidogen, can influence are $\phi_0$ and the growth rate constant, $k$, in eqn. (3) or $\phi_1$ and the growth control parameter, $r$, in eqn (5). If the aneuploidogen is applied only once, it seems reasonable to assume that it will affect only $\phi_0$ or $\phi_1$ and not $k$ or $r$. Thus, the effect of a once-only application of an aneuploidogen is to increase the aneuploid fraction at the time it is applied. Under this condition, the growth parameters $k$ and $r$ are intrinsic to the cell and independent of the aneuploidogen. Using eqn. (3), Figure 6 shows how a once-only application of an aneuploidogen affects the progression of the aneuploid fraction, $\phi_t^a$, relative to $\phi_t$ for the untreated normal human cells of Figure 1 (Figure 6a) and for Down's syndrome cells (Figure 6b) respectively.

On the other hand, if an aneuploidogen is present continuously, then it effectively increases the growth parameters $k$ and $r$. Again

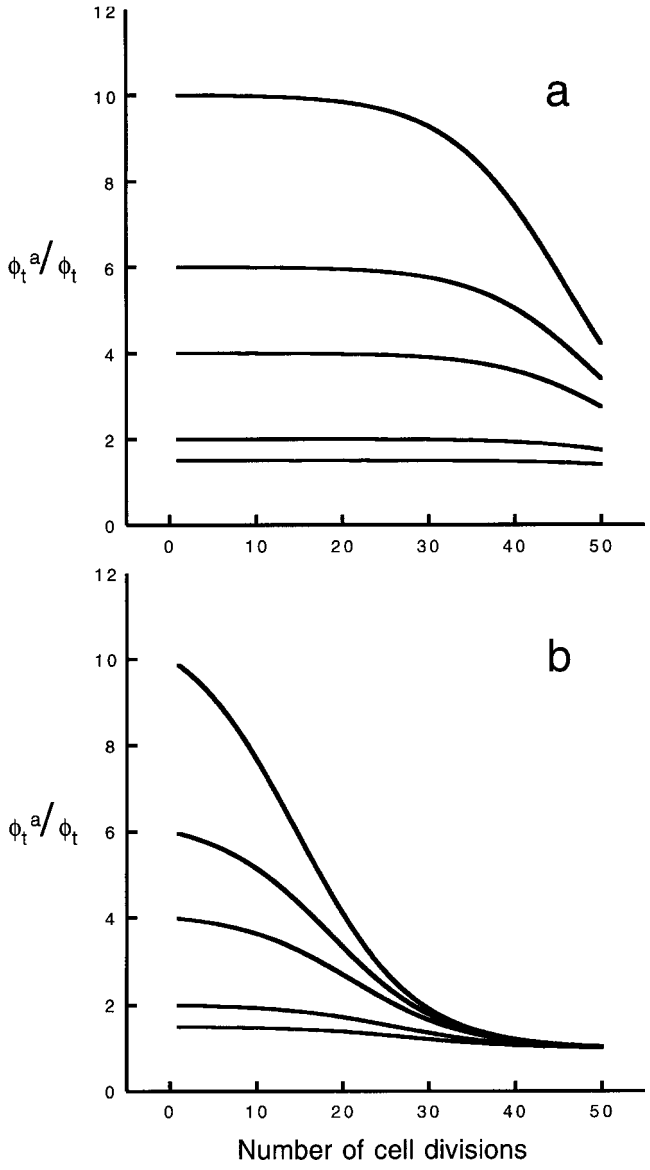


**Figure 6 Once-only application of an aneuploidogen**

A once-only application of an aneuploidogen affects the progression of the aneuploid fraction, $\phi_t^a$, relative to untreated control cells, $\phi_t$, for the normal human cells of Figure 1 (**a**) and for Down's syndrome cells (**b**) respectively ($\alpha = 1.5, 2, 4, 6, 10$, see text). A robust strategy for quantifying aneuploidogenic potencies is a once-only treatment of primary human cells in culture with the test substance followed by periodic measuring of the level of aneuploidy, e.g. at 10, 15 and 20 cell divisions, and recording the average.

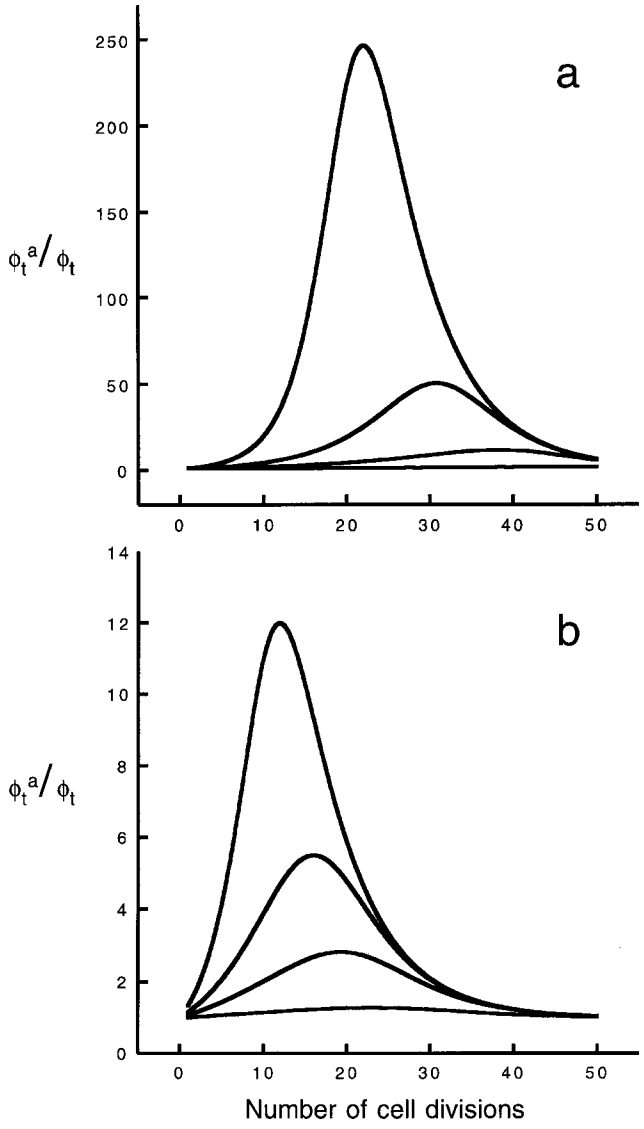


**Figure 7 Continuous application of an aneuploidogen**

The continuous presence of an aneuploidogen effectively increases the growth parameters $k$ [eqn. (3)] and $r$ [eqn. (5)], affecting the progression of the aneuploid fraction, $\phi_t^a$, relative to untreated control cells, $\phi_t$, for the normal human cells of Figure 1 (**a**) and for Down's syndrome cells (**b**) respectively ($k_a = 1.1$, 1.5, 2, 3 times $k$). While the continuous presence of a carcinogen is efficient at generating aneuploidy and transforming cells, a once-only application is a much better strategy for quantifying aneuploidogenic potencies (Figure 6).

using eqn. (3), Figure 7 shows how a continuous application of an aneuploidogen affects the progression of the aneuploid fraction, $\phi_t^a$, relative to $\phi_t$ for the untreated normal human cells of Figure 1 (Figure 7a) and for Down's syndrome cells (Figure 7b) respectively. Figure 7(a) shows that it takes 20–40 cell divisions (depending on the potency or concentration of the drug) for aneuploidogen-treated primary human cells to reach a maximum level of aneuploidy compared with untreated controls. Figure 7(b), as expected, shows that continuously treated Down's syndrome cells should reach a maximum level of aneuploidy sooner than normal cells, i.e. 10–20 cell divisions, depending on the potency or concentration of the drug.

While Figure 7 shows that the continuous presence of a carcinogen is efficient at generating aneuploidy, a once-only application is a much better strategy for quantifying aneuploidogenic potencies (Figure 6). Figure 6(a) shows that compared to untreated human cells in culture, the relative level of aneuploidy, $\alpha$ [eqn. (9)], produced by a once-only application of an aneuploidogen is constant over 25–35 cell divisions.

$$\frac{\phi_t^a}{\phi_t} \approx \frac{\phi_0^a}{\phi_0} = \alpha \tag{9}$$

In contrast, Figure 6(b) shows that for Down's syndrome and other cells, e.g. rodent, which have a high background level of aneuploidy in cell culture, the relative level of carcinogen-induced aneuploidy, $\alpha$, is approximately constant for only 5–15 cell divisions, depending on the potency of the aneuploidogen. Accordingly, I propose that a robust strategy for quantifying aneuploidogenic potencies is a once-only treatment of primary human cells in culture with the test substance followed by periodic measuring of the relative level of aneuploidy, e.g. at 10, 15 and 20 cell divisions, and recording the average.

## DISCUSSION

The fact that euploid cells have a finite lifetime in cell culture has become commonly known as the Hayflick limit [26,27,31]. In the alien environment of cell culture, explanted mammalian diploid cells are forced to reproduce continuously orders of magnitude beyond their *in vivo* existence. Diploid cells *in vitro* respond to the stress of cell culture by altering their genome in order to adapt to life as single-cell organisms or perish. Primary human cells almost never stumble upon the chromosomal alterations needed for unlimited propagation *in vitro* [27].

In contrast with human cells, primary rodent cells forced to endure cell culture often spontaneously hit upon the right combination of aneuploid chromosomes and make the quantum leap to immortalization and thus escape the abyss of the Hayflick limit. The price these cells pay for immortality is perpetual genetic instability due to aneuploidy [1,3]. It may even turn out that genetic instability is necessary for immortality.

In the present study I have proposed that the chaotic growth of aneuploidy is a possible mechanism for the quantum leap to immortalization and transformation. At present it is not known what would lead to the spontaneous chaotic growth of aneuploidy in rodent cells and non-chaotic growth in human cells. It may be that in addition to being aneuploidogens, carcinogens may act to promote the chaotic growth of aneuploidy leading to cancer in humans.

The auto-catalysed progression of aneuploidy during cell division links cancer and the Hayflick limit of cells in culture. The Hayflick limit is due to the production of non-viable aneuploid cells. Transformation and cancer, on the other hand, result from the rare appearance of immortal aneuploid cells. Below I compare the auto-catalysed progression of aneuploidy with the early efforts to model the time course of carcinogenesis.

The early attempts to understand and explain human cancers were largely empirical. For example, in an effort to linearize the strongly non-linear cancer mortality rate data, Armitage and Doll plotted the log of the cancer death rates versus the log of age [30]. Some of the graphs were approximately linear with a slope of 6, to which they and others attributed physiological and biochemical significance. The slope of the log–log plots has been given various interpretations, ranging from indicating that a colony of six or seven cancer cells was a critical size for the propagation of cancer, to that a cancer cell was the end result of seven successive gene mutations. The seven successive gene mutation interpretation has survived to the present [49].

According to the somatic gene mutation hypothesis there are two classes of genes that when mutated lead to cancer. (1) The mutated oncogenes are proposed to actively cause cancer by destabilizing the genome and disrupting hypothetical regulatory networks that maintain homoeostasis. (2) The mutated tumour suppressor genes are proposed to actively protect against and nullify the deleterious effects of mutant oncogenes. In other words, there is a surveillance system of protective genes that actively maintains the euploid state and prevents aneuploidy and cancer. The tumour suppressor gene hypothesis advances the Victorian-like pessimism that an 'unsupervised' euploid cell will follow its natural tendency towards corruption and spontaneously become a cancer cell in the absence of these ever-vigilant guardians of the genome.

A major problem with the somatic gene mutation hypothesis is that to date there is no functional evidence for such genes causing either cancer or, conversely, protecting a cell against aneuploidy and transformation. Furthermore, the somatic gene mutation hypothesis is powerless to explain even the simplest and most glaring facts of carcinogenesis [1–4]. For example, the gene mutation hypothesis cannot explain why a once-only treatment with carcinogens results in tumours only months to years later, long after the inducing carcinogen has reacted with the components of the cell [4,6,51]. The auto-catalysed progression of aneuploidy, on the other hand, readily explains the long latent periods between exposure to a carcinogen and the appearance of cancer (Figures 4 and 5).

The inadequacy of the gene mutation hypothesis to account for phenotypic transformation may be more easily seen with the aid of a metaphor. If the genome is a biological dictionary then the life of a cell is a Shakespearean drama. The most efficient means of rewriting a cell's script, then, would be the wholesale shifting and shuffling of the genes, which aneuploidy accomplishes admirably. Continuing the metaphor, if one were to alter or delete a word here and there in *Hamlet*, for example, such 'point mutations' would be invisible to the vast majority of theatre-goers. The same is true for a multicellular organism, which is at least as resistant to point mutations as a Shakespeare play. On the other hand, without 'mutating' a single word, one could transform the script of *Hamlet* into a legal document, a love letter, a declaration of independence, or more likely gibberish by simply shifting and shuffling, copying and deleting numerous individual words, sentences and whole paragraphs.

In contrast with the somatic gene mutation hypothesis, we have previously proposed that the genetically balanced euploid state is intrinsically stable and not prone to aneuploidy and transformation *in vivo* [1,3]. Aneuploid cells are damaged cells that almost never survive in competition with euploid cells. Nevertheless, there are rare aneuploid cells that win the genetic lottery by evolving into autonomous, single cell organisms that are extremely capable of rewriting their own scripts as evidenced by the extensive genetic instability inherent in these cells.

In spite of the fact that aneuploidy is an efficient mechanism for altering the phenotypes of complex systems, the presence of point mutations in a handful of genes continues to be viewed as a significant, even causal factor in carcinogenesis. However, a more likely reason for the appearance of these point mutations is that they are innocuous, hence readily accommodated during the clonal expansion of barely viable aneuploid cells as they compete with their more viable euploid counterparts. The current emphasis in cancer research on seeking mutant genes in a perpetual background of aneuploidy is a classic example of not seeing the forest for the trees.

Aneuploidy, in contrast to somatic gene mutation, offers a simple and coherent mechanism for the transformation of a range of complex phenotypes [1–4]. The auto-catalysed progression of aneuploidy is sufficient to explain: (1) the time course of primary cells in culture [eqn. (4), Figure 1]; (2) the difference in the spontaneous transformation rates between primary human and mouse cells in culture [eqn. (5), Figure 2]; (3) why the DNA indices of cancer cells are between 1.5 and 2 [eqn. (5), Figure 3]; (4) the time course for the appearance of tumours in carcinogen-treated mice [eqn. (7), Figure 4]; and (5) the age distribution of cancers in man [eqn. (7), Figure 5].

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