How aneuploidy affects metabolic control and causes cancer

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The complexity and diversity of cancer-specific phenotypes, including de-differentiation, invasiveness, metastasis, abnormal morphology and metabolism, genetic instability and progression to malignancy, have so far eluded explanation by a simple, coherent hypothesis. However, an adaptation of Metabolic Control Analysis supports the 100-year-old hypothesis that aneuploidy, an abnormal number of chromosomes, is the cause of cancer. The results demonstrate the currently counter-intuitive principle that it is the fraction of the genome undergoing differential expression, not the magnitude of the differential expression, that controls phenotypic transformation. Transforming the robust normal phenotype into cancer requires a twofold increase in the expression of thousands of normal gene products. The massive change in gene dose produces highly non-linear (i.e. qualitative) changes in the physiology and metabolism of cells and tissues. Since aneuploidy disrupts the natural balance of mitosis proteins, it also explains the notorious genetic instability of cancer cells as a consequence of the perpetual regrouping of chromosomes. In view of this and the existence of non-cancerous aneuploidy, we propose that cancer is the phenotype of cells above a certain threshold of aneuploidy. This threshold is reached either by the gradual, stepwise increase in the level of aneuploidy as a consequence of the autocatalysed genetic instability of aneuploid cells or by tetraploidization followed by a gradual loss of chromosomes. Thus the initiation step of carcinogenesis produces aneuploidy below the threshold for cancer, and the promotion step increases the level of aneuploidy above this threshold. We conclude that aneuploidy offers a simple and coherent explanation for all the cancer-specific phenotypes. Accordingly, the gross biochemical abnormalities, abnormal cellular size and morphology, the appearance of tumour-associated antigens, the high levels of secreted proteins responsible for invasiveness and loss of contact inhibition, and even the daunting genetic instability that enables cancer cells to evade chemotherapy, are all the natural consequence of the massive over- and under-expression of proteins.

Key words: carcinogenesis, genetic instability, mutation, transformation.

INTRODUCTION

Cancer cells differ from normal counterparts in several fundamental phenotypes: (1) loss of some or all differentiated function; (2) expression of 'neoantigens' that are not typical of the differentiated state [1]; (3) ability to invade non-native tissue; (4) significantly, but not specifically, different metabolism from that of normal counterparts [2]; (5) orders of magnitude greater genetic instability compared with normal cells [3,4]; (6) abnormal morphology [5], and (7) progression to malignancy [5].

Over 100 years ago, aneuploidy, a numerical abnormality of chromosomes, was proposed as the cause of cancer [6,7]. Aneuploidy was originally discovered in cancer cells and has since been observed in virtually all of the over-20 000 solid human cancers that have been analysed [8–10]. Since each chromosome carries thousands of genes, aneuploidy is the most massive genetic alteration of cancer cells. In 1968, de Grouchy and de Nava proposed a chromosomal theory of carcinogenesis, where '... chromosome aberrations are the common pathway through which carcinogenic factors induce malignancy...' [11]. However, the aneuploidy hypothesis has since lost support because it was unable to provide a specific mechanism for how the characteristic phenotypes of cancer cells could be generated without gene mutation [9,12,13].

The overwhelming success of genetics in reducing phenotypes to specific genes has led to the alternative hypothesis that cancer is caused by mutation of specific genes, now termed oncogenes and tumour-suppressor genes [12,14–17]. However, despite unprecedented efforts [3,18,19], the gene-mutation hypothesis has not succeeded in demonstrating that one or a combination of mutated genes is sufficient to transform a normal human or animal cell into a cancer cell [20–23]. Furthermore, since spontaneous mutations are common [24,25], and cancer is inevitably fatal [5], the existence of one or more genes that can be converted into cancer genes by mutation would be incompatible with the survival of multicellular organisms.

In view of this, we have re-examined the aneuploidy hypothesis to investigate how cancer could be caused without gene mutations. According to this hypothesis, the multiplicity of structural and functional cancer-specific phenotypes are the direct consequence of aneuploidy. Transforming a cell via aneuploidy would be analogous to transforming the sound of an orchestra by randomly altering the composition of instruments, rather than by 'mutating' specific players. Supporting this view, Lindsley et al. [26] showed that aneuploidy readily produces numerous complex phenotypes of *Drosophila*, including reduced survival, small size and a variety of morphological abnormalities such as rough eyes, abnormal wings and bristle patterns, and a mishapen abdomen. The authors concluded that '... The primary point of theoretical interest to emerge from these studies is that the deleterious effects of aneuploidy are, in the main, caused by the additive effects of genes that slightly reduce viability and not by the individual effects of a few aneuploid-lethal genes among a large array of dosage insensitive loci...' [26,27]. Likewise, trisomy of the smallest human chromosome, no. 21, has the dramatic consequence of Down's syndrome, which '... cannot be

explained as a direct result of one or some very few loci on chromosome 21 …' [28]. Since Down's syndrome is phenotypically much less aberrant than cancer, the threshold of aneuploidy necessary for cancer is expected to be relatively high.

A theory for the analysis of phenotypes generated by complex assembly lines of genes was developed by Kacser and Burns [29,30] and independently by Heinrich and Rapoport [31,32]. According to this theory, the control of normal phenotypes is distributed to various extents among all the genetic components of complex systems [29–37]. We have adapted the approach of Kacser and Burns to assess the role of aneuploidy in determining the phenotypes of cancer cells. The results show that transformation to a cancer cell requires alterations of massive numbers of gene functions, which is exactly what aneuploidy does. The results also demonstrate that the level of aneuploidy that is detected in cancer cells exceeds that found in certain abnormal, non-cancerous cells, e.g. Down's syndrome, which suggests that there is a threshold of aneuploidy for cancer.

ANEUPLOIDY AFFECTS THE METABOLIC CONTROL OF CELLULAR PHENOTYPES

Over the past 25 years the formalisms of Metabolic Control Analysis have become sophisticated to the point of being inaccessible to the majority of those working in cancer research. For that reason, and in the spirit of Kacser and Burns, we have chosen to keep the mathematical formulations as simple and intuitive as possible.

Since all gene products are enzymes, or substrates and products of enzymes, or modifiers of enzyme activity, variations in the levels of gene products of biological systems are kinetically equivalent to changes in effective enzyme concentrations [29–32,38]. For algebraic convenience, the simple straight chain of enzymes shown in Scheme 1 will be used in this analysis. However, the results apply equally to systems of interlocking pathways, cycles, feedback loops [29,30], regulatory cascades [39] and control of gene expression [40], except that the formulations become more tedious [33].

$$X_1 \xrightarrow{\overleftarrow{E_1}} S_1 \xrightarrow{\overleftarrow{E_2}} S_2 \ldots S_{n-1} \xrightarrow{\overleftarrow{E_n}} X_2$$
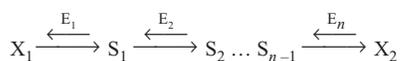
Scheme 1

Scheme 1 represents a steady state of n enzyme-catalysed steps with a 'source' X_1 and a 'sink' X_2. Using the fact that, at steady state, each intermediate flux is equal to the overall flux, F, for the production of X_2, Kacser and Burns (and independently Heinrich and Rapoport) were able to derive eqn. (1) for the overall steady-state flux, F, of Scheme 1 [29,31].

$$F = \frac{X_1 - \dfrac{X_2}{K_1 K_2 \ldots K_n}}{\dfrac{K_{m_1}}{V_1} + \dfrac{K_{m_2}}{V_2 K_1} + \ldots + \dfrac{K_{m_n}}{V_n K_1 K_2 \ldots K_{n-1}}} \tag{1}$$

Eqn. (1) can be simplified. Since all terms in the numerator of eqn. (1) are constants, they can be combined into a single constant term, C_n, which represents the environmental and constitutive parameters for the specific system or phenotype being considered. Furthermore, since $V_i = E_i k_{cat(i)}$, all the V_i terms are proportional to their respective enzyme concentrations. Each fraction in the denominator of eqn. (1), then, can be

replaced by the composite e_i terms, all of which are proportional to enzyme concentration. These modifications result in a remarkably simple equation, eqn. (2), which gives the overall metabolic output or flux of Scheme 1 [30–32]. The e_i terms represent the genetically determined parameters for each of the gene products contributing to the flux. The connectedness of all the gene products shown in eqn. (2) for the simple linear system of Scheme 1 is equally true for an enzyme system of any complexity [33,41]:

$$F = \frac{C_n}{\dfrac{1}{e_1} + \dfrac{1}{e_2} + \ldots + \dfrac{1}{e_n}} \tag{2}$$

Since each phenotype has its own characteristic F, the ensemble of fluxes of complex systems will be used to represent the specific phenotypes that they control. When n is for a eukaryotic genome, eqn. (2) represents the phenotype of a eukaryotic cell in a particular environment. Eqn. (2) could just as easily describe higher-order phenotypes: tissues, organs or organisms [42]. Eqn. (2) can be rearranged to eqn. (3), which shows that the reciprocal of the cellular phenotype F multiplied by a constant is the linear combination of the reciprocals of all the elemental phenotypes, e_i, that comprise a eukaryotic cell. Furthermore, the sums of reciprocals of phenotypes (fluxes) are themselves the reciprocal elements of higher-order phenotypes (fluxes) [42–44].

$$\frac{C_n}{F} = \frac{1}{e_1} + \frac{1}{e_2} + \ldots + \frac{1}{e_n} \tag{3}$$

For a system at least as complex as a cell, the number of gene products necessary to determine its phenotype is on the order of tens of thousands. For systems this complex, the $1/e_i$ terms make only small individual contributions [29–34,45] and can be approximated by replacing them with $1/\bar{e}$, the mean of all the $1/e_i$ terms [38]. Making this substitution in eqn.(3) gives eqn.(4), which represents the phenotype of a normal eukaryotic cell for a given environment:

$$\frac{C_n}{F} = \left(\frac{1}{\bar{e}} + \frac{1}{\bar{e}} + \ldots + \frac{1}{\bar{e}} \right)_n = \frac{n}{\bar{e}} \tag{4}$$

Since the production of gene products is, in a first approximation, proportional to gene dose [46], eqn. (4) should be sensitive to the effects of aneuploidy, which increases or decreases substantial fractions of the genetic material of a cell. If only a subset of the gene pool is involved, the fluxes in eqn. (4) can be partitioned into those that are affected by aneuploidy and those that are not to give eqn. (5):

$$\frac{C_n}{F_a} = \frac{n-m}{\bar{e}} + \frac{m}{\pi \bar{e}} \tag{5}$$

F_a is the phenotype of a eukaryotic cell resulting from aneuploidy. The number of genes experiencing a change in expression levels due to aneuploidy is m. The variable π is the segmental ploidy factor, reflecting the change in the number of gene copies or expression levels for m. For example, $\pi = 1.5$ for trisomy of m. The difference $n-m$ is the number of genes not experiencing aneuploidy or a change in gene expression. Eqn. (5) can be simplified by dividing it by eqn. (4) to give eqn. (6).

$$\frac{F}{F_a} = \frac{n-m+\dfrac{m}{\pi}}{n} = 1 - \frac{m}{n} + \frac{m}{n\pi} \tag{6}$$
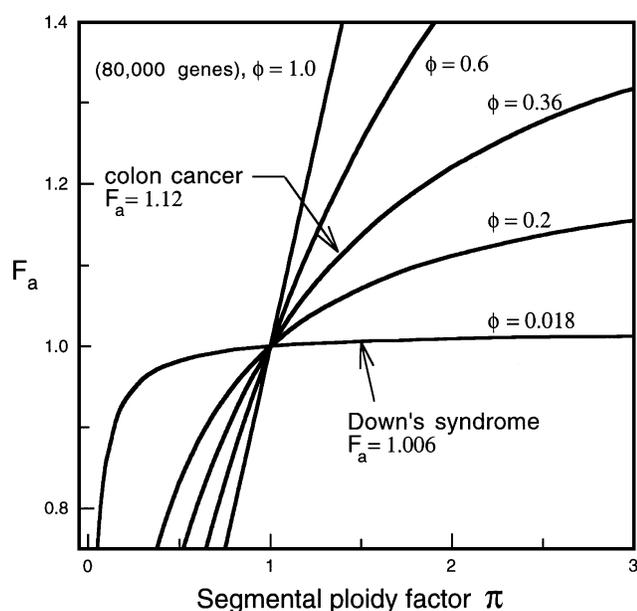
Figure 1   Change in phenotype due to aneuploidy

A plot of eqn. (7) shows how the normal phenotype, F, is perturbed by varying the ploidy factor, π, and the genome fraction, ϕ, to produce an ensemble of aneuploid phenotypes F_a, including Down's syndrome [27] and colon cancer [47]. $F_a > 1$ represents gain-of-flux and $F_a < 1$ represents loss-of-flux relative to the wild-type. In phenotypic analysis, ϕ is a measure of the extent to which a given segment of the genome controls phenotypic transformation. The values of $\pi = 1.5$ and $\phi = 0.018$ for Down's syndrome are readily determined from the fact that every cell has one extra copy of chromosome 21. About 36% ($\phi = 0.36$) of the genes of the primary-colon-cancer cells were differentially making transcripts compared with normal colon cells (see eqn. 11 and and Table 1). However, there is no simple derivation of an overall π value for colon cancer, since:

$$\frac{1}{F_a} = 1 - \Sigma\phi_i + \Sigma\frac{\phi_i}{\pi_i}$$

(see Appendix A), showing that π need not be constant over the region of differential expression. The equation for the flux of the colon-cancer cells can be approximated by:

$$\frac{1}{F_a} = 1 - \Sigma\phi_i + \frac{\Sigma\phi_i}{\pi_{effective}}$$

which assumes a constant or effective ploidy factor over the region of differential expression. This approximation gives $\pi_{effective} = 1.4$ for colon cancer.

To further simplify eqn. (6), we can arbitrarily set the normal phenotype $F = 1$, and replace the quotient m/n with ϕ, which is just the fraction of the cell's gene products experiencing changes in expression relative to the normal cell. These modifications give the dimensionless eqn. (7), the fundamental equation of the analysis of phenotypes, where F_a is now the relative flux (see Appendix A for the general form of eqn. 7):

$$\frac{1}{F_a} = 1 - \phi + \frac{\phi}{\pi} \tag{7}$$

The $1 - \phi$ term represents the fraction of unaffected gene products. The composite term ϕ/π is the fraction, ϕ, of gene products undergoing a π-fold change in expression. The product $\phi\pi$ is a measure of the increase or decrease in the gene products themselves. Assuming that the production of gene products is proportional to gene dose, the DNA index is $1 - \phi + \phi\pi$. Eqn. (7) will be used to investigate Theodor Boveri's proposal that aneuploidy causes cancer [7]. Specifically, we investigate how the

ploidy factor, π, and the aneuploid fraction, ϕ, of the genome determine the phenotype of an aneuploid cell.

Figure 1 is a graphical representation of eqn. (7), where the normal phenotype, F, of a eukaryotic cell is perturbed by varying the ploidy factor, π, and the genome fraction, ϕ, to produce an ensemble of aneuploid phenotypes, F_a. The variable ϕ defines the shape of the curve as well as the limiting metabolic flux at the plateau for a genome fraction $\phi < 1$. The ploidy factor, π, determines the specific values of F_a within the limits set by ϕ. The positions of two specific examples of human aneuploidies, i.e. Down's syndrome [27] and cases of colon cancer [47], are identified in Figure 1.

Figure 1 also shows that, for $\pi < 1$, there is a decline in F_a, indicating a loss of function compared with the normal phenotype, and, for $\pi > 1$, there is a gain.

The genome fraction, ϕ, turns out to be identical with the Flux Control Coefficient of Metabolic Control Analysis (see Appendix A) [41,44]. A Flux Control Coefficient ranges from 0 to 1 and is a measure of the sensitivity of the flux of Scheme 1, for example, to changes in the activities of component gene products. In phenotypic analysis, ϕ is a measure of the extent to which a given segment of the genome controls phenotypic transformation.

A measure of phenotypic robustness (i.e. resistance to changes in gene dose) is the slope of the metabolic flux F_a for particular values of ϕ and π (Figure 1). The slopes for the most robust phenotypes (i.e., $\phi \cong 0$) are close to zero (Figure 1). The slopes are steeper for $\pi < 1$ than for $\pi > 1$, which is consistent with a loss of gene dose being more deleterious than a gain for both *Drosophila* and humans [26,27,48].

Figure 1 does not indicate an obvious relationship between genome fraction, ϕ, and the ploidy factor, π. For any given aneuploid phenotype there is an infinite number of combinations of ϕ and π. The question is, are there optimal combinations of these two variables? In other words, are there values of the genome fraction, ϕ, and the ploidy factor, π, that result in a minimal production of translation products, $\phi\pi$, necessary for a given phenotypic transformation?

To investigate the dependence of $\phi\pi$ on π and ϕ respectively at constant F_a, eqn. (7) can be rearranged to give eqns. (8) and (9). The partial derivatives of eqns. (8) and (9) are set equal to zero in search of minima (see Appendix B for the calculation of the values of π and ϕ at the minimum for $\phi\pi$).

$$\phi\pi = \frac{\pi\left(\frac{1}{F_a}-1\right)}{\frac{1}{\pi}-1}, \; F_a, \pi \neq 0, 1; \; \text{for} \; \frac{\partial\phi\pi}{\partial\pi} = 0, \; \pi = 2 \tag{8}$$

$$\phi\pi = \frac{\phi}{1-\frac{1}{\phi}\left(1-\frac{1}{F_a}\right)}, \; F_a, \phi \neq 0, 1; \; \text{for} \; \frac{\partial\phi\pi}{\partial\phi} = 0, \; \phi = 2\left(1-\frac{1}{F_a}\right) \tag{9}$$

Eqn. (8) shows that the minimum production of gene products to achieve a given phenotype is always at a constant value, $\pi = 2$, regardless of the genome fraction, ϕ, and the aneuploid phenotype, F_a. On the other hand, eqn. (9) shows that the value of ϕ at the minimum is not constant but is dependent on the aneuploid phenotype, F_a.

Figure 2 is a plot of eqn. (8) that shows how the various products of $\phi\pi$ for two specific phenotypes, i.e. $F_a = 1.07$ and $F_a = 0.97$, depend on the ploidy factor. The loss-of-flux ($\pi < 1$, $F_a = 0.97$) and the gain-of-flux ($\pi > 1$, $F_a = 1.07$) aneuploid phenotypes F_a of Figure 2 were calculated from the segmental
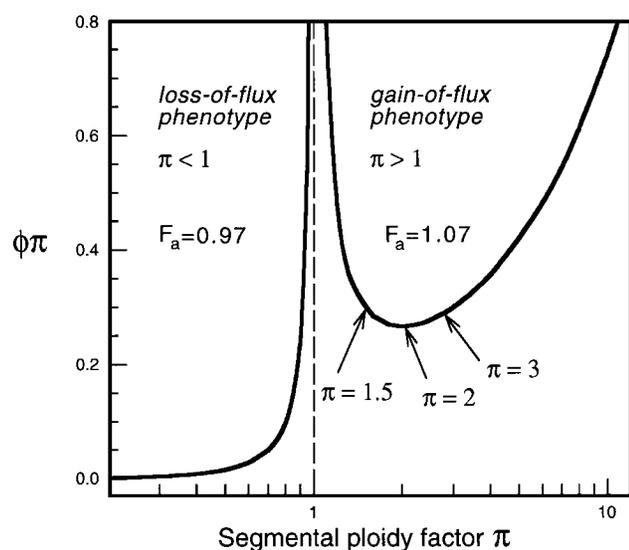
Figure 2   Translation products versus ploidy factor

A plot of eqn. (8) shows how the level of translation products, $\phi\pi$, depends on the ploidy factors for the loss-of-flux ($\pi < 1$) and for the gain-of-flux ($\pi > 1$) aneuploid phenotypes F_a calculated from the segmental aneuploidy data of Lindsley et al. for the whole organism *Drosophila* [26] (see the text for details). The most economical or likely values of π are about 2 for gain-of-flux phenotypes of any species. The value of the translation products, $\phi\pi$, tends towards 0 for loss-of-flux phenotypes for any genome fraction, ϕ.

aneuploidy data of Lindsley et al. for the whole organism *Drosophila* [26].

Lindsley et al. [26] showed that the viability of the segmental trisomies declined regularly with the amount of the triplicated material, becoming generally lethal when more than one-half an autosomal arm of either chromosome 2 or 3 was present in three doses. *Drosophila* is estimated to have 110 Mb of genomic DNA [49]. Chromosomes 2 and 3 contribute 39.5 Mb and 47.9 Mb [49,50] respectively to the total genomic DNA, for an average of 43.7 Mb per chromosome. Half of the average genomic DNA for these two chromosomes is 21.9 Mb, which represents 20 % of the total genomic DNA of *Drosophila*. For the segmental mono-somies the limit of viability was a loss of 'six-eighteiths' of a chromosome, or 3 % of the genomic DNA. Plugging these values of ϕ (0.20, 0.03) and π (1.5, 0.5) respectively into eqn. (7) gives $F_a = 1.07$ for the gain-of-flux, and $F_a = 0.97$ for the loss-of-flux, aneuploid phenotypes showing that a loss of gene dose is more deleterious than a gain (Figures 1 and 2). While the minimum is always at $\pi = 2$ for all gain-of-flux phenotypes of any species, the actual value of $\phi\pi$ in Figure 2 depends on the particular aneuploid phenotype, F_a.

Eqn. (9) can be used to estimate the smallest genome fraction, ϕ, necessary for the production of gain-of-flux phenotypes. In order to generate an aneuploid flux, $F_a = 1.05$, under optimal conditions (i.e. minimum aneuploidization), it is necessary to increase the metabolic activity of 9.5 % of the normal gene product pool (eqn. 10).

$$
\left.
\begin{aligned}
\phi = 2\left(1 - \frac{1}{1.05}\right), & \quad \phi = 0.095 \\[1em]
\phi = 2\left(1 - \frac{1}{1.01}\right), & \quad \phi = 0.02
\end{aligned}
\right\} \tag{10}
$$

The current estimate that the human genome encodes about 80 000 transcripts [51] means that a genome fraction, ϕ, of 0.095 corresponds to a maximum of 7600 genes. In other words, to produce a new phenotype with a flux only 5 % above the wild-type requires an approx. 2-fold increase in the expression of several thousand genes. Eqn. (10) shows that even as little as a 1 % increase in flux (i.e. $F_a = 1.01$) involves the overexpression of 2 % of the genome, corresponding to a maximum of 1600 genes (e.g. trisomy of chromosome 21 in Down's syndrome [27,28]).

It is clear from this analysis that transforming the robust normal phenotype into gain-of-flux phenotypes requires massive increases in the metabolic activity of a cell. Aneuploidy provides the necessary boost in genome dose responsible for the increased metabolic activity required for phenotypic transformation in-dependent of gene mutation.

THE COMPOSITION AND PHENOTYPES OF CANCER CELLS ARE A CONSEQUENCE OF ANEUPLOIDY

The current thinking among most investigators is that the many and complex cancer-specific phenotypes (see the Introduction) all result from a few specific mutations that either dominantly produce the numerous gain-of-flux cancer phenotypes or re-cessively disrupt genes that suppress cancer [16,17,24,52–55]. The consensus is that one or several mutations at specific genetic loci are necessary and sufficient for the production of cancer cells. For example, seven genetic events are currently thought necessary for colon cancer [56]. These mutations are also considered sufficient for the substantially increased or decreased levels of nuclear DNA [57,58], cytoplasmic RNA [47,59] and protein [57,59–61], as well as for the altered morphology, genetic instability, ability to metastasize and altered growth rates found in cancer cells (see the Introduction).

However, there is as yet no functional proof that any one or a combination, of these mutations can generate cancer-specific phenotypes. Indeed, Metabolic Control Analysis shows that it is unlikely that one or several mutated genes can have such pleiotropic powers over so many others (eqn. 2 and Figure 1). There is also no independent genetic evidence that mutations of these genes have such powers [25]. For example, mutated *ras* genes, which are currently taught to be dominant cancer genes [16,24,62], including colon cancer [63], have recently been found in cancer-free animals [64,65] and in benign human colon tissue, '…which has little potential to progress…' [66]. Even the view that mutated *ras* is necessary for cancer is hard to reconcile with (a) otherwise indistinguishable cancers with and without *ras* mutations [67,68], (b) metastases of the same tumours with and without *ras* mutations [69,70], (c) retroviral *ras* genes that are oncogenic without *ras* mutations [71] and (d) human tumour cells having spontaneously lost *ras* mutation but not tumouri-genicity [72].

Aneuploidy, in contrast with the mutation hypothesis, readily explains the tremendous increases or decreases in metabolic activity of cancer cells compared with their normal counterparts, as well as the other unique phenotypes of cancer cells.

For example, the high DNA indices, ranging from 1.5 to 2, that are found in most malignant cancers [73], are directly compatible with the high levels of cytoplasmic RNAs and proteins found in these tumours. Comparing the differences between normal and cancer cells in 1950s and 1960s, investigators found 10–100 % increases in the levels of cytoplasmic RNA and protein in solid cancers [57,59–61]. In view of this, Caspersson et al. concluded that a typical, active cancer cell '…is characterized by extreme stimulation and extreme activity of the system for protein formation … [The] different organelles of the cytoplasmic

Table 1 Phenotypic analysis of colon cancer: RNAs of colon cancer and normal cells by abundance class*

Calculated values: $F_a = 1.12$; $\text{DNA}_{\text{index}}$ ($\text{DNA}_{\text{index}} = 1 - \Phi + \Sigma\phi_i\pi_i$) = 1.71; $S = 0.65$ (the stability index, S, is defined as $S = F_a/\text{DNA}_{\text{index}}$; see Appendix C for the formal definition of S.

Abundance class (RNA copies/cell)	Number of genes		
	Normal	Cancer	(ϕ_i, π_i)
A (> 500)	62	54	(0.00054, 0.1)†
B (51–500)	645	470	(0.012, 0.1)
C (6–50)	4569	5011	(0.03, 9)
D ($\leqslant 5$)	9445	14155	(0.32, 2.5)
Total ...	14721	19690	$\Phi = 0.36$‡

* Data taken from Zhang et al. [47].

† The ϕ values are the absolute values of the difference in transcripts between normal and cancer cells divided by 14721 normal transcripts; see the text for derivation of π values.

‡ $\Phi = \Sigma\phi_i$

protein-forming system are very well developed, and the cell shows all indications of excessive protein formation ...' [59].

Elevations in cellular protein profoundly alter the physiology and biochemistry of cells. A 10% increase in cellular protein produces a 2-fold increase in membrane-bound proteins, and a 40% increase causes a 32-fold elevation in membrane-bound proteins [74]. In addition, a 10% increase in cellular protein causes a 5-fold boost in flux across membranes, and a 19% increase results in a 30-fold elevation in transmembrane fluxes [74]. Therefore, the membrane-bound tumour-associated antigens [75] and the high levels of secreted proteins [76] that are responsible for the invasiveness and loss of contact inhibition of cancer cells are the consequence of the over-expression of protein [57,59–61] due to aneuploidy.

While the altering of cellular phenotypes requires increases or decreases in the production of cellular protein, the size of $\phi\pi$ is limited by the space and solvating capacities of the cell. A typical normal cell contains 25% (17–40%) protein by weight [77]. Cancer cells, on the other hand, contain as much as 100% more protein than normal cells. To compensate for the osmotic stress caused by the extra protein, cancer cells increase their volume [59].

A spectrum of colon-cancer-specific RNAs has recently been quantified by Zhang et al. [47]. Using the new method called serial analysis of gene expression ('SAGE'), Zhang et al. identified 19690 RNAs in colon-cancer cells and 14721 in normal colon cells. The RNAs were divided into four convenient abundance classes, which made it possible to convert the colon-cancer-cell data of Zhang et al. into the variables ϕ and π of the analysis of phenotypes. The ϕ value for each abundance class of the colon-cancer cells was based on the 14721 measurable transcripts that determine the normal phenotype of healthy colon cells (Table 1).

Zhang et al. report that there were 181 genes in the cancer cells expressing on average 10-fold fewer copies of RNA than normal colon cells. These 181 'significant' under-expressing genes were among a total of 183 under-expressors in the primary colon tumours of abundance classes A and B (Table 1). Thus the reported 10-fold average decrease for the 181 genes can be used to approximate the π value for 183 under-expressors of abundance classes A and B ($\pi = 0.1$).

Estimating the π value for abundance class C is more difficult, because Zhang et al. reported the level of expression of only 108

genes out of a total of 442 over-expressors. The 108 genes were expressing RNA levels on average 13-fold higher than normal colon cells. The average π value for this class, then, must be below 13 and most likely above 5, the lower limit of abundance class C. The average ($\pi = 9$) will be used for abundance class C (Table 1).

Compared with normal cells, the colon-cancer cells had 4710 additional genes expressing five or fewer copies of RNA in abundance class D. By inspection, then, the average level of expression was 2.5-fold, which represents the π value for this class (Table 1). Inserting these values of ϕ and π (Table 1) into the general form of eqn. (7) (see Appendix A) yields eqn. (11) for the primary-colon-cancer cells.

$$\left.\begin{array}{l} \dfrac{1}{F_a} = 1 - \Phi + \dfrac{\phi_A}{\pi_A} + \dfrac{\phi_B}{\pi_B} + \dfrac{\phi_C}{\pi_C} + \dfrac{\phi_D}{\pi_D}, \ \Phi = \Sigma\phi_i \\[2mm] \dfrac{1}{F_a} = 1 - 0.36 + \dfrac{0.00054}{0.1} + \dfrac{0.012}{0.1} + \dfrac{0.03}{9} \\[4mm] \qquad + \dfrac{0.32}{2.5} = 0.89, \ F_a = 1.12 \end{array}\right\} \quad (11)$$

The calculated relative flux for the primary-colon-cancer cells is $F = 1.12$. The 12% increase in flux is the result of a 71% increase in protein compared to normal cells (Table 1). This increase in translation products is equivalent to an average of 79 chromosomes per primary colon cancer cell (1.71×46 average chromosomes = 79), which is within the range of 60–90 chromosomes frequently seen in human solid cancers [58].

By contrast, Zhang et al. consider, '... the genes exhibiting the greatest differences in expression are likely to be the most biologically important ...' in turning normal cells into cancer cells. On the basis of this thinking they limited their analysis to 289 (181 significant'' under-expressors + 108 'significant' over-expressors) of the most differentially expressed genes (an average of 10-fold and higher) out of a total of almost 20000 analysable transcripts in the colon-cancer cells. Since most of the transcripts (including K-*ras*, one of the seven genes thought to cause colon cancer; L. Zhang, personal communication) contributed five or fewer copies per cell, they were not included in the analysis of Zhang et al. [47].

Using only the 289 ($\phi = 0.02$) differentially expressed genes (considered by Zhang et al. [47] to be the most biologically important) in eqn. (11) gives $F_a = 0.91$. This represents a 9% loss of flux instead of a 12% gain when all transcribing genes are included. In other words, excluding the 4710 ($\phi = 0.32$) genes of abundance class D reduces the flux of a hypertriploid cancer cell to that of a hypodiploid phenotype. Thus the 4710 genes producing an average of 2.5 transcripts contribute the most to the colon-cancer phenotype, rather than the 289 genes with the greatest differential expression. This confirms the result of eqn. (8), that, for gain-of-flux phenotypes the most likely change in expression of a sizeable fraction of the normal genome is a 2-fold increase (eqn. 8 and Figure 2). This result demonstrates the counter-intuitive principle (implicit in Metabolic Control Analysis) that it is the fraction of the genome, ϕ, undergoing differential expression, and not the magnitude, π, of the differential expression, that controls phenotypic transformation (see Appendix A) [41,44].

ORIGIN OF CANCER-SPECIFIC GENETIC INSTABILITY

Occasionally, mitosis can lead to cells containing 2, 4, 8, ... 2^n times the diploid number of chromosomes, all of which maintain the genetic balance of normal cells. Apparently, mitosis cannot
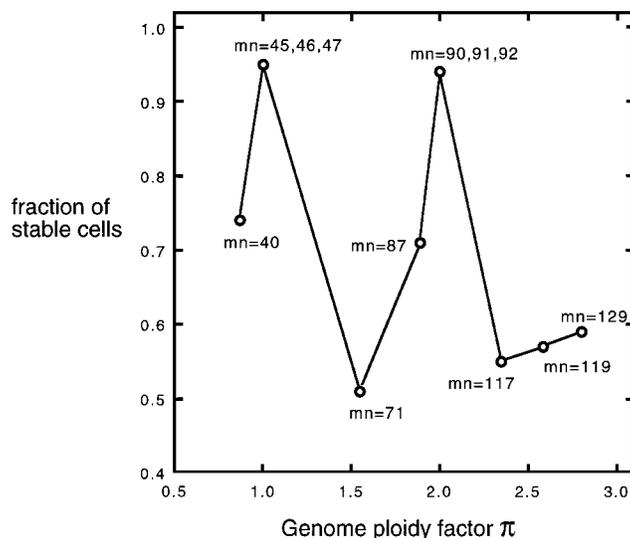
**Figure 3   Karyotypic stability of colon cancer cell lines**

The karyotypes of colon-cancer cell lines are unstable in proportion to their degree of aneuploidy, where mn = modal number of chromosomes. The karyotypes with a balanced number of chromosomes, i.e. $\pi = 1$ (diploid) or $\pi = 2$ (tetraploid), are stable. The cell lines represented are: $mn = 40$ (SW837), $mn = 45$ (HCT 116), $mn = 46$ (DLD1, pseudodiploid), $mn = 47$ (SW48), $mn = 71$ (HT29), $mn = 87$ (LoVo), $mn = 90$ ($2 \times$ HCT 116), $mn = 91$ (DLD1 $\times$ HCT 116), $mn = 92$ ($2 \times$ DLD1), $mn = 117$ (DLD1 $\times$ HT29), $mn = 119$ (SW480), $mn = 129$ ($2 \times$ HT29), $mn = 142$, but this hybrid lost chromosomes [79].

produce the odd multiples $3, 6, \ldots 3 \cdot 2^{(n-1)}$ of the diploid set because the number of chromosomes is farthest from the balanced values. Such cells are expected to be genetically unstable. Recently we have confirmed this prediction experimentally. The genetic instability of chemically transformed Chinese-hamster embryo cells is proportional to their degree of aneuploidy [78]. Analysis of the genetic instability of colorectal cancer also illustrates this principle.

The results of Lengauer et al. demonstrate that the genetic instability of human colon-cancer cell lines is a function of the level of aneuploidy, although the authors failed to emphasize this point [79]. Their data clearly show that diploid and tetraploid cells (2^n ploidy) are the most stable, and triploid and hexaploid cells [$3 \cdot 2^{(n-1)}$ ploidy] are the least stable (Figure 3) [78]. The HT29 colon-cancer-cell line, for example, has a modal number of 71 chromosomes, for a DNA index = 1.5. The observed 50 % genetic instability of the triploid HT29 cells [79] is due to the conflict between the most economical production of translation products on the one hand and the maintenance of chromosomal balance on the other.

The effect of aneuploidy on genetic stability can be quantified. The phenotypic output or flux of a normal cell is in balance with its genetic content. However, as Figure 1 shows, there is an imbalance between the phenotype and genotype of aneuploid cells. A measure of this imbalance is the relative aneuploid flux, F_a, divided by the DNA index. Since cancer-specific genetic instability has been shown to be proportional to the degree of genetic imbalance of aneuploid cells [78,79], the ratio of the aneuploid flux, F_a, to the DNA index defines the stability index, S. (See Appendix C for the formal definition of the stability index, S.)

The stability index, S, ranges from 0 to 1 (Figure 4). A stability index of 1 signifies a balanced genome. The stability index $S = 0.65$ for the primary-colon-cancer cells (Table 1) indicates substantial genetic instability. Figure 4 shows that the flux of an aneuploid cell is in balance with its genetic content only when the
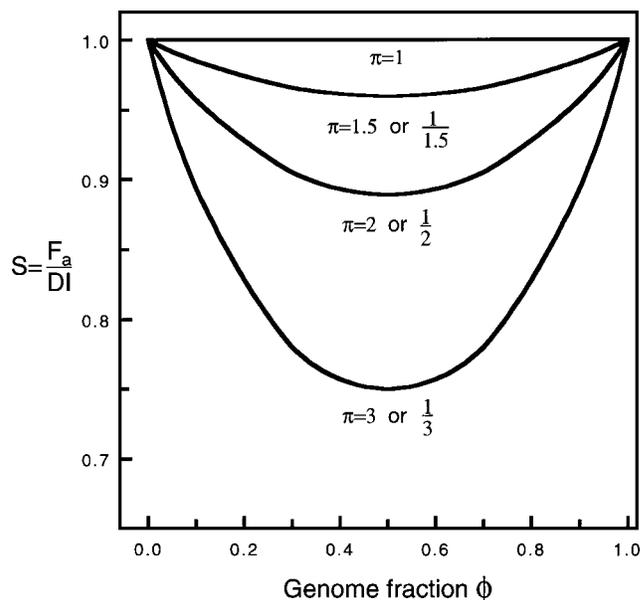
Figure 4   Stability index S

Plot of the stability index, S, versus the genome fraction, ϕ, shows that the flux of an aneuploid cell is in balance with its genetic content only when the genome fraction, ϕ, is 1. The stability index S ranges from 0 to 1. The greatest genetic imbalance is at $\phi = 0.5$, i.e. when half the genome is aneuploid. See Appendix C for the formal definition of S.

genome fraction, ϕ, is 1. Therefore, the most genetically balanced triploid HT29 cell requires $\phi = 1$ and $\pi = 1.5$. However, as we have seen, the most economical π value is 2. The aneuploid cells, then, are trapped in a Sisyphean effort of rearranging the genome in order to optimize the values of π, ϕ, and F_a of eqns. (8) and (9) to produce the most economical aneuploid states at the expense

of chromosomal balance. Aneuploidy, therefore, is the self-perpetuating source of the genetic instability of cancer cells [80].

ORIGIN OF ANEUPLOIDY

As Ohno so confidently asserted, '…malignant cells are either overtly or covertly aneuploid (genetically unbalanced)…' [81]. But what is the source of the aneuploidy seen in cancer? Aneuploidy may arise either spontaneously or by chemical induction of chromosome loss or gain during cell division in germ and somatic cells. All trisomies and most monosomies are thought to be generated by non-disjunction, that is, the failure of sister chromatids in mitosis or of paired chromosomes in meiosis to migrate to opposite poles at cell division [82].

As we have seen, a gain in genetic material is better tolerated than a loss. Therefore, hyperploidy is favoured over hypoploidy. The 60–90 chromosomes that are typical of late-stage cancer cells [58] may arise by a gradual, stepwise increase in the level of aneuploidy as a consequence of the autocatalysed genetic instability of aneuploid cells. This stepwise model of carcinogenesis could explain the decades-long progression of many cancers. Aneuploid cells are injured cells that are less viable than euploid cells. The stepwise progression of aneuploidy would, in general, produce fewer and fewer viable cells, which should inhibit the progression to cancer. But decades of genetic instability may eventually produce, through the stepwise accumulation of chromosomes, a viable cell with the level of aneuploidy required for malignancy. However, there is an alternative to the stepwise model of carcinogenesis that is based on chromosome doubling and subsequent loss of chromosomes [58,83].

Oksala and Therman have described numerous routes to the production of polyploid cells [84]. While tetraploid cells are comparatively stable, both *in vitro* and *in vivo*, they are, nevertheless, associated with a degree of chromosomal instability leading to random loss of chromosomes [58,81,83,85–90]. The process of chromosome doubling followed by chromosome loss has been shown in numerous sequential studies of normal rodent fibroblasts undergoing spontaneous neoplastic transformation *in vitro*, in simian-virus-40 malignant transformation of normal human fibroblasts in culture, in established human epithelial cancer cell lines grown in tissue culture and in sequential studies of early human bladder cancer [58].

Interestingly, near-tetraploid cancer cell lines may continuously produce an appreciable number of near-diploid daughter cells, which in future generations revert back to the near-tetraploid state [81]. The production of pseudodiploid cancer cells from aneuploid precursors may contribute to the occasional reports that some apparently malignant tumours maintain the normal diploid complement of the species.

The hypertriploidy–hypotetraploidy characteristic of later-stage cancer cells [73] can either result directly from the tetraploidization of hypodiploid cells with DNA indices greater than 0.75, but less than 1, or, alternatively, by the tetraploidization of diploid and hyperdiploid cells followed by chromosome loss [83,91]. Tetraploidization preserves the nuclear balance of the near-diploid aneuploid cells, which should promote the viability of the cells with double the previous number of chromosomes. A DNA index of 0.75 appears to be the lower limit of viability for hypodiploid cells [58,83,92,93]. The tetraploidization of these barely viable cells produces the DNA index of 1.5 that is characteristic of the lower-limit aneuploidy peak of most malignant cancer cells.

On the basis of this analysis we propose that the two-step model of carcinogenesis [5] corresponds to two levels of aneuploidization. The initiation step in carcinogenesis is the production of a non-cancerous, aneuploid cell. In such a cell, the level of aneuploidy is below the threshold for cancer. In the promotion step the threshold of aneuploidy for cancer is reached or exceeded either by gradual, stepwise increase in the level of aneuploidy as a consequence of the auto-catalysed genetic instability of aneuploid cells, or by tetraploidization followed by a gradual loss of chromosomes. Thus the initiation step of carcinogenesis generates a level of aneuploidy below the threshold for cancer, and the promotion step increases aneuploidy above this threshold. It is too early to say which of the two mechanisms (gradual, stepwise or chromosome doubling) better characterizes carcinogenesis. Both mechanisms may contribute to carcinogenesis.

Regardless of the model that eventually turns out to best describe carcinogenesis, carcinogens are almost certainly aneuploidogens (aneugens). The power of a carcinogen is predicted to be proportional to its ability to cause aneuploidy.

DISCUSSION

Here we compare the aneuploidy and the mutation hypotheses as genetic theories of cancer. In addition, we recount the evidence that aneuploidy explains the seven cancer-specific phenotypes listed in the Introduction.

Aneuploidy versus mutation as the cause of cancer

The commonly held view that cancer is caused by the mutation of a few specific genes [12,14–17] derives from the widespread assumption that complex pathways must have rate-determining, or rate-controlling, or rate-limiting steps: a view that has dominated biochemical kinetics for nearly a century [94,95]. The search for oncogenes and tumour suppressor genes, then, is simply the search for the rate-determining molecular steps in carcinogenesis. However, the results of biochemical experiments of the last 25 years (e.g. glycolysis, the tricarboxylic acid cycle, photosynthesis, and the syntheses of fatty acids, urea, nucleotides and amino acids) have shown that complex systems are not controlled by slow or rate-determining steps [29–37,96].

In order to understand how complex biological pathways behave, it has become necessary to decrease the emphasis on mechanism [97]. While mechanisms such as co-operative feedback inhibition are still addressed by Metabolic Control Analysis, they are given less emphasis than in classical studies of metabolic regulation. One of the fundamental discoveries of Metabolic Control Analysis is that, even with a complete knowledge of the detailed properties of specific genes and gene products, it is not possible to either predict or describe the phenotypes of complex biological systems in terms of a few individual genetic components [29–37].

It is clear from eqns. (2) and (4) that the overall flux of a cell depends on the contributions of all its gene products. As the number of gene products required to produce a particular phenotype increases, the effect of varying any one becomes quite small. This result applies with equal force to genetic mutations. Therefore, alterations in a handful of 'gatekeeper' or 'caretaker' genes [98,99] are likely insufficient, if not irrelevant, for the generation of cancer-specific phenotypes, since their numbers are too few to alter the normal phenotype, and since there is as yet no independent genetic evidence that they are exceptionally pleiotropic. Indeed, not one mutated cellular gene, nor even a group of mutants, has ever been shown to transform a normal human or animal cell into a cancer cell [20–23].

While it is highly unlikely that individual genes directly lead to cellular transformation, there may be some mutant genes (yet to

be identified) that contribute indirectly to carcinogenesis: for example, growth control genes, since hyperplasia is a known risk factor for carcinogenesis [5].

Even if the flux of an essential gene product, e_i, goes to 0 as a result of mutation, carcinogenesis is an unlikely result. Instead, the corresponding phenotype (F) would go to 0, and either kill the cell or render it defective. An exception would be the inactivation of a tumour suppressor gene, if the loss of such a gene were a direct cause of cancer. However, this seems unlikely now, since non-cancerous mouse strains have been raised with null mutations of hypothetical tumour suppressor genes, as for example p53 [100]. According to Cairns '… one of the problems is that most mutations lead to loss of functions, rather than creation of new function…' [101], and thus are unlikely to explain the numerous cancer-specific phenotypes listed in the Introduction.

The attraction of the oncogene mutation theory was its promise of simplicity: that cancer resulted from a manageable number of specific mutations. A manageable number was the hoped-for key to unlocking the mysteries of cancer that should lead to the taming of the ever-growing modern scourge. Instead, we find that the seven mutations proposed to cause colon cancer [56] are drowned in a sea of nearly 5000 additional transcripts (see the section 'The composition and phenotypes of cancer cells are a consequence of aneuploidy' above) [47]. The feebleness of the seven mutations [56] is evident in Figure 1, where even the 1400 extra genes of Down's syndrome are nowhere near sufficient to produce the flux of the colon-cancer phenotype.

Far from providing insights into the nature of cancer, and hence insights into prevention and more effective treatments, the oncogene theory is now so burdened with the complexity of its details that it has become an empirical exercise devoid of theoretical and explanatory power. Analysing close to 49 000 genes of normal and cancer cells (colon and pancreas), Zhang et al. acknowledged that '… most of the transcripts could not have been predicated to be differentially expressed in cancers…' [47]. The authors were also surprised to discover that '… two widely studied oncogenes, c-*fos* and c-*erbb3*, were expressed at much higher levels in normal colon epithelium than in [colorectal] cancers, in contrast to their up-regulation in transformed cells …' The expression levels of mutated K-*ras*, which was said to be a 'dominant' oncogene of colon cancer in 1987 [63], was not even listed. According to one of the authors (L. Zhang, personal communication), K-*ras* was not included in the analysis because it contributed fewer than five copies per cell.

The experimental results of Zhang et al., however, provide unintended confirmation that it is the genome fraction, ϕ, and not the magnitude of differential expression, that controls phenotypic transformation. While Zhang et al. [47] chose to focus on the 289 genes ($\phi = 0.02$) undergoing the largest differential expression between normal and colon cancer cells, it is clear from eqn. (11) and Table 1 that the 4710 ($\phi = 0.32$) additional transcripts of abundance class D make the largest and decisive contribution to the colon-cancer phenotype. The value of $\pi = 2.5$ for the decisive abundance class D of the primary colon tumour cells is close to the value of 2, which offers a maximum of abnormality for a minimum of ploidy alteration (see eqn. 8 and Figure 2).

The aneuploidy hypothesis offers a simple and coherent explanation for all cancer-specific phenotypes

The aneuploidy–cancer hypothesis provides a simple and coherent explanation for all seven cancer-specific phenotypes listed in the Introduction, independent of gene mutation.

(1) The loss of differentiated function would be a direct consequence of the abnormal gene and chromosome balance of aneuploid cancer cells. The consequences of aneuploidy are analogous to the 'de-differentiation' of an orchestra by randomly altering the number and type of instruments.

(2 and 3) The increased protein synthesis due to aneuploidy produces a bizarre array of secreted and membrane-bound tumour-associated antigens that overcome the contact inhibition of normal differentiated cells and lead to invasiveness. In other words, aneuploidy scrambles balance-sensitive genetic programs leading to the de-repression of neoantigens and suppression of normal, tissue-specific antigens.

(4) The abnormal composition and metabolism of cancer cells is a direct consequence of the abnormal gene dosages conferred upon the cell by aneuploidy. The effects of abnormal dosages would be multiplied by abnormal regulatory effects.

(5) Aneuploidy is also sufficient to explain the 'genetic instability' of cancer cells as a consequence of aneuploidy-induced karyotypic instability. By unbalancing balance-sensitive components of the spindle apparatus, aneuploidy destabilizes chromosome disjunction. Aneuploidy leads to a conflict between the economical production of gene products on the one hand and chromosomal balance on the other, which causes a perpetual regrouping of the genome. The final result is the karyotypic heterogeneity that is the hallmark of cancer cells.

(6) The sum of the effects of abnormal chromosome mass due to aneuploidy produces the abnormal RNA and protein concentrations that lead to the abnormal morphologies of cancer cells. The aneuploidy-induced abnormal surface proteins also add to the bizarre and varied features of cancer cells.

(7) The progression of cancer cells to malignancy is the product of (a) the inherent karyotypic instability of aneuploid cells and (b) the selection of ever more aggressive variants. In other words, 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. It is this intrinsic genetic instability of aneuploid cells that fuels the progression to malignancy of cancer cells.

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APPENDIX A

The general form of eqn. (7) is:

$$\frac{1}{F_a} = 1 - \Sigma\phi_i + \Sigma\frac{\phi_i}{\pi_i}$$

Small and Kacser derived the equation

$$\frac{1}{F} = 1 - \frac{r-1}{r}C^J$$

to predict the relative flux, F, of Scheme 1 for large r-fold changes of enzyme concentrations [44]. The parameter C^J is the flux control coefficient of Metabolic Control Analysis. This equation can be rearranged to

$$\frac{1}{F} = 1 - C^J + \frac{C^J}{r}$$

which is formally identical with eqn. (7). Therefore, $r = \pi$ and the genome fraction, ϕ, is identical with the flux control coefficient, C^J.

Small and Kacser derived the general equation:

$$\frac{1}{F} = 1 - \Sigma C_i^J + \Sigma\frac{C_i^J}{r_i}$$

By strict analogy, then, the general form of eqn. (7) is:

$$\frac{1}{F_a} = 1 - \Sigma\phi_i + \Sigma\frac{\phi_i}{\pi_i}$$

APPENDIX B

Calculate the values of the genome fraction, ϕ, and the segmental ploidy factor, π, for the minimum production of gene products $\phi\pi$ subject to the condition

$$\frac{1}{F_a} = 1 - \phi + \frac{\phi}{\pi}$$

Using the method of Lagrange multipliers leads to the variational equations:

$$\frac{\partial}{\partial\pi}\left[\phi\pi + \lambda\left(1 - \phi + \frac{\phi}{\pi} - \frac{1}{F_a}\right)\right] = \phi - \lambda\frac{\phi}{\pi^2} = 0, \ \lambda = \pi^2 \tag{i}$$

$$\frac{\partial}{\partial\phi}\left[\phi\pi + \lambda\left(1 - \phi + \frac{\phi}{\pi} - \frac{1}{F_a}\right)\right] = \pi + \lambda\left(-1 + \frac{1}{\pi}\right) = 0 \tag{ii}$$

where λ denotes the Lagrange multiplier. Dividing eqn. (i) by ϕ gives $\lambda = \pi^2$.

Since eqn. (i) gives $\lambda = \pi^2$, λ in eqn. (ii) can be replaced by π^2 to give eqn. (iii) for the minimum production of gene products, $\phi\pi$.

$$\pi + \pi^2\left(-1 + \frac{1}{\pi}\right) = 0, \ \pi = 2 \tag{iii}$$

Inserting $\pi = 2$ into eqn. (7) gives

$$\frac{1}{F_a} = 1 - \phi + \frac{\phi}{2}$$

This rearranges to give

$$\phi = 2\left(1 - \frac{1}{F_a}\right)$$

for the minimum production of gene products, $\phi\pi$.

APPENDIX C

The stability index, S, is defined as:

$$S = \frac{F_a}{DNA_{index}}$$

Since

$$\frac{1}{F_a} = 1 - \phi + \frac{\phi}{\pi}$$

and

$$DNA_{index} = 1 - \phi + \phi\pi$$

the ratio of the aneuploid flux, F_a, to the DNA_{index} gives:

$$S = \frac{F_a}{DNA_{index}} = \frac{1}{(1 - \phi + \phi\pi)\left(1 - \phi + \frac{\phi}{\pi}\right)}$$

By inspection it is clear that:

$$S_\pi = S_{\left(\frac{1}{\pi}\right)}$$