Regulation of G_2 by cell size contributes to maintaining cell size variability within certain limits in higher plants

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Summary

The variability of (1) surface area projection (size) at which cells terminate DNA replication, (2) the area at which they initiate mitosis, (3) the area at which they divide, (4) the duration of G_2, and (5) the duration of G_2 plus mitosis (in fact, prophase + metaphase + anaphase) has been estimated in steady-state cell populations of *Allium cepa* root meristems. The coefficient of variation of cell area at termination of DNA synthesis was found to be 14 % while the coefficient of variation of cell area at mitosis initiation was 13 %. As there is also a substantial variability of G_2 (the coefficient of variation was estimated to be 38 %), the combination of these data indicates that cell size regulation of G_2 contributes to maintaining cell size variability (and therefore DNA concentration) within certain limits. Mitosis also varies but less than G_2 (the coefficient of variation of G_2 + mitosis was found to be 31 %). As the coefficient of variation of cell area at division (14 %) is hardly larger than the coefficient of variation of cell area at initiation of mitosis, it can be suggested that coordination between cell size and mitosis duration helps to avoid a significant increase in the variability of cell size at the end of the division cycle.

Key words: regulation of G_2, cell size, plant cells, cell cycle, cell proliferation.

Introduction

Synthesis of DNA, nuclear division and cell division appear to be a dependent sequence of events. Each event does not occur unless the preceding one has been completed. Cell growth does not lie in this sequence because it is well known that it will continue when DNA replication is blocked. Mitchison (1971) has formalized this in terms of two sequences: the growth cycle and the division cycle, which are normally coupled but can be dissociated. There is a substantial body of evidence for the coordination of cell growth and cell division (for reviews see Mitchison, 1977; Fantes & Nurse, 1981; Tyson, 1985).

Interaction of the growth cycle and the division cycle at the level of G_1 results from data suggesting that cells must attain a critical size to initiate DNA synthesis. This hypothesis was first proposed for bacteria (Donachie, 1968; Pritchard, 1968; Helmstetter *et al.* 1968) and it explains the negative correlation between cell size at birth and the length of G_1 that has been observed in most materials (for a review, see Baserga, 1984) including *Allium cepa* root meristems (Navarrete *et al.* 1983; Cuadrado *et al.* 1985, 1986). Interaction of both cycles in the postreplicative portion of the cell cycle has been proposed for bacteria (Bremer & Chuang, 1983; Puyet & Cánovas, 1984; Bremer, 1986), yeasts (Nurse, 1975; Fantes & Nurse, 1981; Thuriaux *et al.* 1978), *Amoeba* (Hartman, 1928; Prescott, 1956*b*), and *A. cepa* root meristems (Navarrete *et al.* 1983; Cuadrado *et al.* 1985, 1986). Cell size-dependence of G_2 duration is also supported by experiments with mammalian cells (Stancel *et al.* 1981; Rao *et al.* 1984) though the authors prefer to consider it as non-significant.

Previous results supporting cell size regulation of G_2 in *A. cepa* root meristems (Navarrete *et al.* 1983; Cuadrado *et al.* 1985, 1986) were obtained by drug treatments. In our opinion, they were not entirely conclusive because secondary effects due to the presence of the drugs could not be discarded. In addition, they do not show whether such a regulatory system has

a physiological role or not. The possibility exists that cell size regulation of G_2 is only significant in special circumstances, and does not occur under physiological growth; for instance, in very big cells originated by drug treatments. The aim of this paper is to confirm that G_2 regulation by cell size operates during physiological growth, contributing to maintaining cell size variability within certain limits. The rationale of our experimental work is based on the argument that a significant variability of G_2 duration, if random, must produce an increase in cell size variability between the beginning and end of G_2. However, if there is a negative correlation between G_2 time and cell size, the variability of G_2 must be also significant but cell size variability at the end of G_2 should be smaller or equal to that at the beginning of this period. We have measured the variability of G_2 and G_2 plus the first three phases of mitosis, together with the variability of cell size at DNA synthesis termination, at initiation of mitosis, and at cell division. Techniques to estimate cell size from measurements of cell surface area projection have proved to be suitable in *A. cepa* root meristems (Cuadrado *et al.* 1986).

Materials and methods

Allium cepa root meristems were used. Bulbs were grown at 15°C (as described by Navarrete *et al.* 1983), with a generation time of 30 h, allowing enough time to attain a steady state of growth; this occurs approximately when roots are 3 cm long.

Pulse labelling of DNA

Thirty-minute pulses with 40 μCi ml^{-1} of [^{3}H]thymidine (21 Ci mmol^{-1}; Radiochemical Centre, Amersham, Bucks, UK) were given just before fixation to roots cut from the bulbs. For autoradiography, slides were dipped in diluted Kodak nuclear track emulsion (1:1, v/v, with water).

Samples size

Several roots were taken from each bulb and cells from the second half of the meristem were scored in each root. The total number of anaphase/telophase cells counted was 212. To analyse cell size variability at mitosis initiation or DNA synthesis termination, cells were classified for their surface area projection in intervals of 1 arbitrary unit (a.u.), between 5 and 26 a.u.; in each interval, 60–120 cells were scored for their position in the cell cycle. To estimate the variability of cell cycle periods, the fraction of labelled prophases or labelled telophases was determined at 1-h intervals after a pulse of [^{3}H]thymidine; the number of cells analysed in each sample was 300–400.

Histochemical techniques and microdensitometry

Squashed meristems were stained by the method of ninhydrin–Schiff (Cuadrado *et al.* 1986). This method is compatible with autoradiographic identification of replicating cells. Individual isolated cells were scored for surface area projection with a M85 scanning microdensitometer (Vickers Ltd) as described by Cuadrado *et al.* (1986). Microdensitometry was also used to differentiate G_1 and G_2 cells among non-labelled cells.

Results

Variability of cell size at various points of the cell cycle

An approximate estimation of cell size at division can be obtained by measuring surface area projection of anaphase/telophase cells (A_d). The number of these cells in each A_d interval was counted. A plot of the cumulative frequency (F_d) of anaphase/telophase cells *versus* A_d is shown in Fig. 1. Experimental points fitted rather well to a sigmoid curve (Fig. 1) given by:

$$F_d = 1 - e^{-a(A_d - c)^b}, \qquad (1)$$

in which a, b and c are specific coefficients, and e is the base of natural logarithms. Statistical data (mean, $\bar{A}_d$; standard deviation, S.D.$_d$; and coefficient of variation, C.V.$_d$ = 100 S.D.$_d$/$\bar{A}_d$) were calculated graphically from the continuous curve and they are shown in Table 1.

To analyse cell size distribution at initiation of mitosis (A_m), cells were classified in different size intervals and the ratio of mitotic cells to total cells was determined in each interval. This ratio gives the cumulative frequency (F_m) of cells that have terminated G_2 at a given A_m if there is no overlapping between distributions of cell size at the start of mitosis and at division, or in a theoretical population in which cells do not divide. Neither of these situations occurs in our system. Therefore, F_m values were corrected for

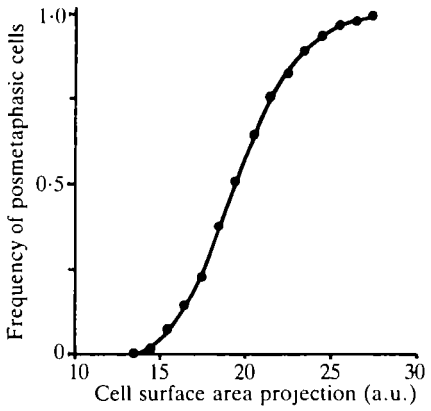

Fig. 1. Cumulative frequency of anaphase/telophase cells per surface area projection class. The continuous curve is the regression line corresponding to equation (1). The coefficients a and b of this sigmoid function (the primitive function of Weibull equation; Quinet, 1983) were calculated, after its conversion into a linear form, by the minimum square method. The coefficient c was calculated by testing different values in order to find the one that gives the best regression coefficient (r). Resulting values were: a = 0·0749, b = 2·4332, c = 13·0, r > 0·999.

Table 1. *Statistical data of cell surface area projection at DNA synthesis termination, initiation of mitosis and initiation of anaphase*

Corrections	$\bar{A}_t$	S.D.$_t$	c.v.$_t$	$\bar{A}_m$	S.D.$_m$	c.v.$_m$	$\bar{A}_d$	S.D.$_d$	c.v.$_d$	Duration of periods (h)*		
										G_2	Pf+Mf	G_2+Pf+Mf
None	17·2	3·0	17	21·2	4·6	22	19·6	2·8	14	9	−3·4	5·6
Division†	16·4	2·3	14	18·2	2·4	13	—	—	—	4·5	3·2	7·7
Labelling pulse‡	16·2	2·3	14	—	—	—	—	—	—	5·0	—	8·2

* Periods were calculated as follows; $P = \ln(\bar{A}_x/\bar{A}_y)\ln 2/T$ (equation (2)), in which P is the period time, $\bar{A}_x$ and $\bar{A}_y$ are the average area at, respectively, the end and the beginning of the period, and T is the cell generation time. The actual duration of these periods is: $G_2 = 5 \pm 0{\cdot}5$ h; Pf+Mf = $3 \pm 0{\cdot}3$ h (Giménez-Martín *et al* 1977).

† Corrections for cell loss caused by division were made using equation (3).

‡ Corrections for labelling pulse (t) were made as follows: $A = A' - 1/2\Delta A$ and c.v. = $100\ [(A'\text{c.v.}'/100)^2 - \Delta A^2/12]^{\frac{1}{2}}$, in which A' and c.v.' are the uncorrected parameters and $\Delta A = \ln 2 A' t/T$ (Koppes & Nanninga, 1980).

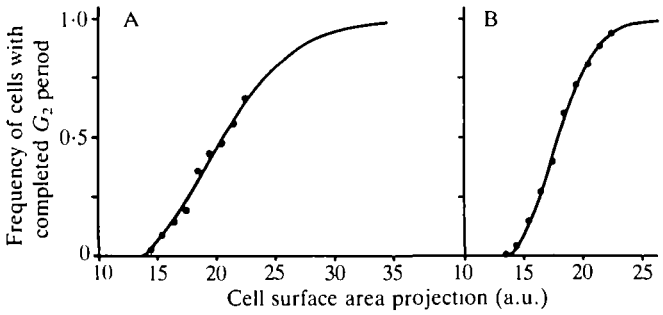

Fig. 2. Cumulative frequency of cells with completed G_2 period per surface area projection class. A. Experimental frequencies; B, frequencies corrected for cell loss caused by division (equation (3)). The continuous curves are the regression lines corresponding to an equation analogous to equation (1) (see legend to Fig. 1). The coefficients of the equation were: in A, $a = 0{\cdot}0237$, $b = 1{\cdot}717$, $c = 13{\cdot}4$, $r = 0{\cdot}996$; in B, $a = 0{\cdot}0294$, $b = 2{\cdot}0616$, $c = 13{\cdot}3$, $r > 0{\cdot}999$.

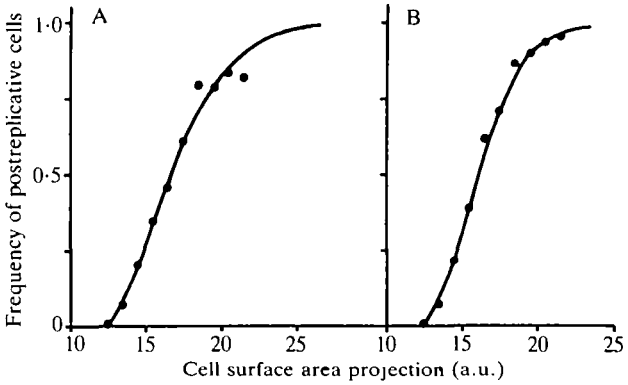

Fig. 3. Cumulative frequency of postreplicative cells per surface area projection class. A. Experimental frequencies; B, frequencies corrected for cell loss caused by division (equation analogous to equation (3)). The continuous curves are the regression lines corresponding to an equation analogous to equation (1) (see legend to Fig. 1). The coefficients of the equation were: in A, $a = 0{\cdot}0547$, $b = 1{\cdot}6894$, $c = 12{\cdot}2$, $r = 0{\cdot}995$; in B, $a = 0{\cdot}0432$, $b = 1{\cdot}9889$, $c = 12{\cdot}1$, $r = 0{\cdot}998$.

cell loss caused by division by adding to both mitotic cells (n) and total cells (N) in each size interval, the corresponding number of divided cells (d). Corrected values (F_m^*) were calculated with the following equation:

$$F_m^* = \frac{n+d}{N+d} = F_m\,(1 - F_d) + F_d\,. \qquad (3)$$

F_m and F_m^* data were fitted to sigmoid curves given by an equation analogous to equation (1). Both experimental points and continuous curves are illustrated in Fig. 2. Statistical data ($\bar{A}_m$, S.D.$_m$, c.v.$_m$) for both curves, F_m and F_m^*, are shown in Table 1. It must be pointed out that the duration of prophase (Pf) plus metaphase (Mf), as calculated from $\bar{A}_d$ and corrected values of $\bar{A}_m$, appears to be correct while a negative time was found for these phases when non-corrected $\bar{A}_m$ and $\bar{A}_d$ values were used in calculations (Table 1).

Cell size distribution at DNA synthesis termination (A_t) was analysed with the same method as that used to study A_m distribution. A population that had been labelled by a pulse of [^{3}H]thymidine was used to distinguish G_2 cells from replicating cells. The ratio of cells in G_2 + mitosis to total cells was determined in each size interval to obtain the cumulative frequency (F_t) of cells that have terminated DNA synthesis at a given A_t. As there is also overlapping between distributions of cell size at the end of the S period and at division, corrected values for this disturbance (F_t^*) were calculated with an equation analogous to equation (3). Experimental points for F_t and F_t^*, as well as the sigmoid curves to which they were fitted (equation similar to (1)), are represented in Fig. 3. Statistical data ($\bar{A}_t$, S.D.$_t$, c.v.$_t$) for both curves, F_t and F_t^*, together with data corrected for errors introduced by the labelling pulse time are shown in Table 1. Here also, correct values for G_2 and for G_2 + Pf + Mf were only obtained when corrected data were used in calculations.

Comparison of coefficients of variation reported in Table 1 shows that cell size variability seems to be slightly reduced from DNA synthesis termination to initiation of mitosis ($c.v._m < c.v._t$): a small increase in cell size variability appears to occur during mitosis, so that $c.v._d$ approximately equals $c.v._t$.

Variability of the duration of last cell cycle periods

The method devised by Koppes & Nanninga (1980) to analyse the variability of certain cell cycle periods in bacteria has been used. The variability of G_2 was studied in a population that had been pulse-labelled with [^{3}H]thymidine. The fraction of labelled prophases was determined at different times after isotope removal. Data were fitted to a sigmoid curve given by an equation analogous to equation (1). Experimental data and the regression curve are represented in Fig. 4. This procedure gives the G_2 distribution (cumulative frequency) if we assume that in each portion of S, the actual fraction (f_i) of cells with a given G_2 should not be different and that cells must stay in prophase for a time similar to the average duration of this phase (1·9 h: Giménez-Martín *et al.* 1977). However, a correction has to be made for the difference between the duration of intervals at which samples were taken and the time for passage through prophase. Experimental cumulative frequency of labelled prophases (F_i), if measured at intervals of 1 h, is related with f_i by the function:

$$F_i = f_i/\mathrm{Pf} + \sum_{i=1}^{i-1} f_i ,$$

in which Pf is the time of prophase. From this equation, the corrected value for the cumulative frequency of cells at a given G_2:

$$F_i^* = \sum_{i=1}^{i} f_i$$

can be obtained as follows:

$$F_i^* = \mathrm{Pf}(F_i - F_{i-1}^*) + F_{i-1}^* . \qquad (4)$$

The corrected curve is shown also in Fig. 4. Statistical data for both uncorrected and corrected curves, and data also corrected for the error introduced by the pulse-labelling time, are presented in Table 2. The variability of G_2 appears to be very high (c.v. = 38 %). Comparison of average G_2 (as measured by this procedure) with actual G_2 value under similar conditions (5 ± 0·5 h; Giménez-Martín *et al.* 1977) does not show significant differences.

The variability of G_2 plus the first three phases of mitosis (M') was studied using the same procedure by determining the fraction of labelled telophases at different times. The results are illustrated in Fig. 4 and statistical data are shown in Table 2. Variability of $G_2 + M'$ is also high (c.v. = 31 %) but lower than

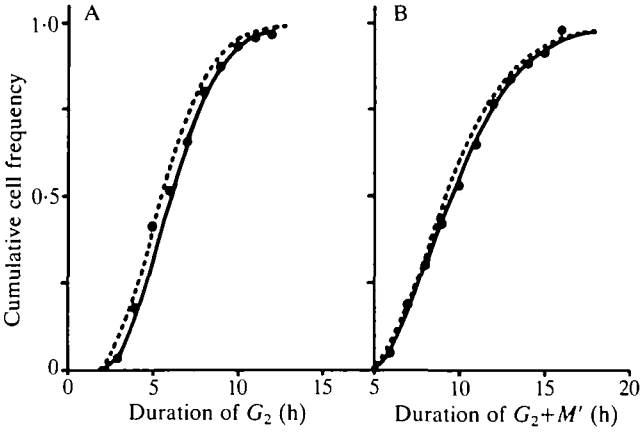

Fig. 4. Cumulative frequency of labelled cells as a function of time after a pulse labelling with [^{3}H]thymidine. A. Prophase cells; B, telophase cells. The continuous curves are the regression lines corresponding to an equation analogous to equation (1) (see legend to Fig. 1). The coefficients of the equation were: in A, $a = 0{\cdot}043$, $b = 1{\cdot}9944$, $c = 2{\cdot}0$, $r = 0{\cdot}992$; in B, $a = 0{\cdot}0543$, $b = 1{\cdot}6806$, $c = 5{\cdot}0$, $r = 0{\cdot}994$. The broken curves are the result of corrections for the duration of prophase (A) or telophase (B) with equation (4).

Table 2. *Statistical data of* G_2 *and* G_2 *plus the first three phases of mitosis* (M′)

	G_2			G_2+M'		
Corrections	$\bar{x}$	S.D.	c.v.	$\bar{x}$	S.D.	c.v.
None	6·2	2·2	35	10·0	3·0	30
Duration of prophase or telophase*	5·8	2·2	38	9·6	3·0	31
Labelling pulse†	5·7	2·2	38	9·5	2·9	31

*The difference between the duration of prophase or telophase (1·9 h and 1·5 h, respectively; Giménez-Martín *et al* 1977) and the intervals at which samples were taken (1 h) was corrected using equation (4).

† Corrections for labelling pulse were made as indicated in Table 1.

variability of G_2. Measured $G_2 + M'$ average time does not significantly differ from its actual value: 9 ± 0·9 h (Giménez-Martín *et al.* 1977).

Discussion

Coordination between cell growth and division probably serves to avoid cell production without the correct DNA concentration. Such coordination is supported by the fact that together with a high variability in the *c.v.* for cell division time (Tyson, 1985), there is reduction or maintenance of the c.v. for cell size between birth and division; the c.v. for cell size is smaller at division than at birth in mammalian cells

(Killander & Zetterberg, 1965) and *Schizosaccharomyces pombe* (c.v at birth = 8 % and $c.v._d = 5\,\%$: J. M. Mitchison, unpublished results), and practically equal in bacteria (Koppes *et al.* 1978; Koppes & Nanninga, 1980). It is less clear which cycle event or events are controlled in a way that cell size variability can be reduced. In fibroblasts, the minimum c.v. for cell size is attained at initiation of DNA synthesis (Killander & Zetterberg, 1965), which suggests that cell size variability is mainly controlled during G_1. In contrast, bacterial cells show the maximum c.v. for cell size at initiation of DNA replication while the minimum c.v. is found in constricted cells, that is at a stage close to division (Koppes *et al.* 1978; Koppes & Nanninga, 1980). From these results it appears that cell size variability is regulated in bacteria during DNA synthesis and/or the time between completion of chromosome replication and subsequent cell division. The results described in the present paper suggest that cell size regulation of G_2 (Navarrete *et al.* 1983; Cuadrado *et al.* 1985, 1986) contributes to maintaining cell size variability within certain limits in proliferating cells of *A. cepa* growing under physiological conditions. The reason is that together with a high variability of G_2, there is a reduction of cell size variability during this period. Other interpretations of the findings described in this work are those that take into account that cell size could alter the growth rate. Large cells may have smaller relative growth increments than small cells (Prescott, 1956*a*; Rønning & Seglen, 1982; Brooks & Shields, 1985); growth rate could decrease (and even stop) when cells approach mitosis or normal division size (Prescott, 1956*a*; Rønning & Seglen, 1982). These possibilities are compatible with a variability in G_2 duration without any relation to cell size. However, these interpretations are discarded by the theory of simplastic or coordinated growth of root cells, necessary to avoid distortions of root shape. During simplastic growth, neighbouring cells have identical growth rates per size unit (López-Sáez *et al.* 1975, 1983; Calvo *et al.* 1982; Carmona & Cuadrado, 1986). On the other hand, continuous and exponential growth through the cell cycle, including G_2, has been observed in *A. cepa* meristematic cells (Cuadrado *et al.* 1986).

To date we have had no method for studying the variability of mitosis but have analysed the variability of G_2 plus the first three phases of mitosis (M'). The c.v. for $G_2 + M'$ (31 %) is lower than the variability of G_2 alone (38 %), which indicates that variability of M' is smaller than variability of G_2. On the other hand, M' is not constant because, in this case, its length would be given by $M' = \text{S.D.}_{G_2}\,(1/\text{c.v.}_{G_2+M'} - 1/\text{c.v.}_{G_2})100 =$ 1·3 h, which is far away from its actual value (3·5 ± 0·3 h; Giménez-Martín *et al.* 1977). From these considerations and from the fact that $c.v._d$ is rather similar to $c.v._m$, it can be suggested that some control of cell size variability is exerted during mitosis but to a lesser extent than during G_2.

Theoretical cell size distributions at initiation of mitosis can be constructed from experimental cell size distribution at DNA synthesis termination if a given G_2 distribution is assumed. Three such theoretical curves were compared with the experimental cell size distribution at the beginning of mitosis. In one case G_2 was assumed to be constant (average G_2); in another case experimental variations in G_2 were supposed to occur randomly, without any relation to cell size; in the third case, an inverse proportionality between cell size and G_2 duration was assumed. The theoretical curve in which G_2 was supposed to be constant and the experimental curve were indistinguishable. None of the two other theoretical curves fit the experimental one (Fig. 5). Therefore, none of the simple G_2 distributions that we have tested is correct because the model that gives the best fit assumes a constant G_2, which is not what was found. In our opinion, G_2 regulation by cell size counteracts the increase in size variability that would produce random G_2 variations. However, the fact that there is not a strict inverse proportionality between G_2 duration and cell size suggests the existence of other factors, in addition to cell size, that regulate the length of G_2.

From previous work (Cuadrado *et al.* 1985, 1986) we know that mechanisms for cell size regulation of G_2 are different from those governing G_1, at least in *A. cepa*. A number of models have been proposed for the control of G_1 (for a review see Tyson, 1985) and some of them have also been suggested to govern cell division (Bremer, 1986; Tyson & Diekmann, 1986). An interesting observation can be made in this respect if tails of cell size distribution in *A. cepa* (expressed in relative frequencies) at DNA synthesis termination and at mitosis initiation are compared (Fig. 6). Cell size distribution at mitosis initiation ends with cells whose surface area projection is about 26 a.u., while the maximum area at DNA synthesis termination is about 24 a.u. These data indicate that the biggest cells have a G_2 period $\leq$3·5 h (equation (2) reported in Table 1 was used for this calculation). As G_2 in the whole population varies between 2·5 and 13·5 h (Fig. 4), it might be concluded that in such cells G_2 has a minimal duration and that it varies very little (from 2·5 to 3·5 h). If similar considerations are applied to the left part of the indicated curves, it gives a G_2 period $\geq$3·5 h for the smallest cells. This is rather striking because it means that some small cells can have almost the minimum G_2, like the big cells. This situation confirms our previous suggestion that in addition to cell size there are other factors controlling G_2 duration. Such factors are apparent mainly in small

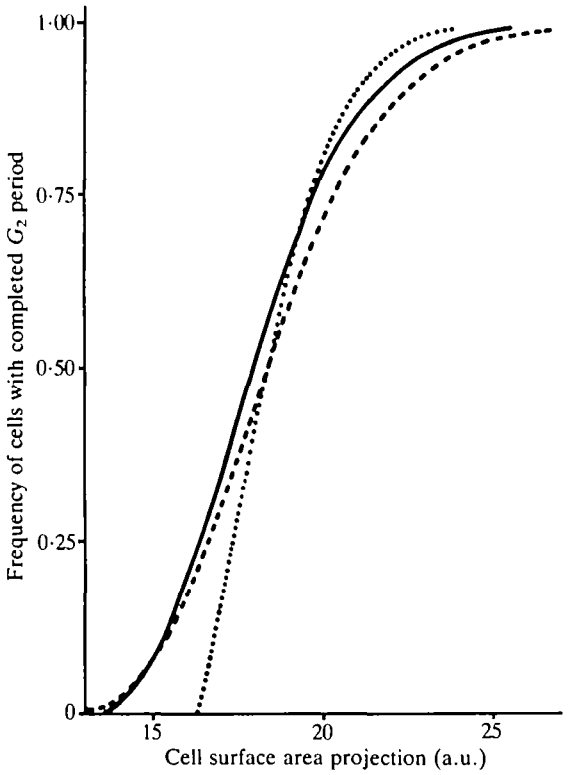

Fig. 5. Comparison of experimental distribution of A_m (——) and theoretical A_m distributions as obtained by assuming a random variation of G_2 (– – –) or a strict negative correlation between cell size at DNA synthesis termination and the duration of G_2 (· · · ·). The random G_2 curve was calculated by assuming that each cell group with a given A_t, independently of their size, had a G_2 variability comparable to that shown by the whole population. To construct the other curve, experimental distribution of A_t was correlated with experimental distribution of G_2 in the reverse way; last fraction in the right part of A_t curve was supposed to correspond to last fraction of equal value in the left part of G_2 curve, and so on. For both curves, the limits of an A_m interval (s_i, s_j), which is derived from a known A_t interval (s_i', s_j') after a given G_2 time (g) were calculated as follows: $s_i = s_i' 2^{g/T}$. The cell frequency in each A_m interval (f) was calculated from the cell frequency in the A_t interval from which it is derived (f') by assuming an exponential cell age distribution (Powell, 1956): $f = f' 2^{-g/T}$. Theoretical A_m intervals with their corresponding cell frequencies (f) were combined with computer help in order to group cells in regular intervals. Final frequencies were directly proportional to the previously calculated frequencies but corrected to give a total sum equal to unity.

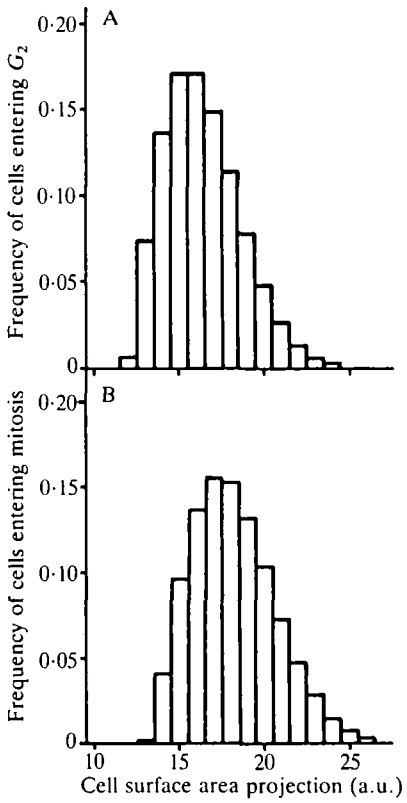

Fig. 6. Comparison of cell size distribution at DNA synthesis termination (A) and at mitosis initiation (B). Relative frequencies represented in A were obtained from cumulative frequencies illustrated in Fig. 3B. Identically, values represented in B were derived from data shown in Fig. 2B.

cells. In contrast, the key factor controlling the length of G_2 in big cells should be their size, the result of this control being a minimum G_2 for such cells and, therefore, the absence of cells with very low DNA concentration.

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