MITOTIC ASYMMETRY:
DIFFERENTIAL BEHAVIOUR OF
SISTER NUCLEI

By

STUART WILLIAM ARMSTRONG, B.Sc.

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AUTHOR: Stuart William Armstrong, B.Sc. (McMaster University)

SUPERVISOR: Dr. D. Davidson

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ABSTRACT

Caffeine, a trimethylxanthine, is a potent inhibitor of cytokinesis in plant cells; it induces the formation of binucleate cells. The use of binucleate cells is particularly revealing since they make it possible to compare the behaviour of sister nuclei in a binucleate cell with the behaviour of nuclei in sister cells, i.e. to compare the behaviour of sister nuclei in one cytoplasmic environment or in two separate environments. We have used this technique to study the asymmetry of mitosis and the consequences of this asymmetry. With binucleate cells we have shown that a number of differences exhibited by the nuclei of sister cells, i.e. differences in nuclear size, protein content and RNA content, are also exhibited by the sister nuclei of binucleate cells. The fact that these differences occurred in binucleate cells indicates that differences between sister nuclei are inherent and arose as a result of the mitosis from which the nuclei were formed.

The subsequent behaviour of the sister nuclei is also affected by the asymmetrical mitoses. Sister nuclei of binucleate cells showed a differential growth pattern and when supplied with ^{3}H-uridine a differential ability to synthesize RNA. An analysis of nuclei of sister cells revealed an identical pattern of behaviour. This suggests that the asymmetry of division not only produces sister nuclei of different

size and macromolecular content but also results in functional differences between the two sister nuclei.

It is proposed that a large degree of heterogeneity in cell size, nuclear size, macromolecular contents of cells and nuclei, and in cell cycle duration is the result of asymmetrical mitoses. The asymmetrical mitoses are responsible for generating, at every mitosis, physical differences between sister cells and these physical differences are functionally related to the differential behaviour of sister nuclei and sister cells.

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INTRODUCTION

During a mitotic cell cycle a cell, on average, doubles its constituents and then divides, producing two daughter cells. Nuclear DNA follows this pattern; DNA content doubles over the cell cycle and during mitosis sister chromatids segregate to opposite poles of the cell to form the daughter nuclei. These are sister nuclei; they are genetically identical and each contains half the DNA of the parent nucleus. Thus, in proliferative populations of cells such as root meristems, a 2-fold range of DNA values exist; however, for many other cell and nuclear parameters there is a much greater than 2-fold range of values. In lateral root meristems of V. faba there is a 6-fold range in interphase nuclear volume values and a 10-fold range in cell area (Davidson, Golding and Armstrong, 1978). Similar results have been obtained from root meristems of other plant species (Davidson, Pertens and Eastman, 1978; Lyndon, 1967; Webster, 1979b; White and Davidson, 1976). RNA content, protein content and dry mass of nuclei also show this high degree of variability (Bansal and Davidson, 1978a; Lyndon, 1967; Bennett, 1970).

Since cells, on average, double their size and mass over a cell cycle this high degree of variability would at first seem to be paradoxical. However, this variability might arise by asymmetrical mitoses, which would result in pairs of sister cells differing in size. This appears to be the case; sister cells differ in size (Davidson, Pertens and Eastman, 1978; Davidson and Pertens, 1981a; Ivanov, 1971;

Lehtonen, 1980), and sister nuclei differ in volume (Davidson, Pertens and Eastman, 1978; Davidson and Pertens, 1981a; Webster, 1979b). These differences in cell and nuclear sizes can be related to differences in behaviour of sister cells. Ivanov (1971) has shown that the majority of divisions in the cortex of Zea mays are asymmetric, producing a larger apical cell and a smaller basal cell; it is the larger, apical cell which has the shorter cell cycle. Analogous to this is the situation found in the root cap initials of Zea mays. Both the cell and its nucleus are larger in Row I than in the sister cell in Row II (Davidson, Pertens and Eastman, 1978; Ivanov, 1979). The mean cell doubling time for Row I cells is 10 hr. while for Row II cells it is 25 hr. (Clowes, 1976). Differences in cell cycle duration for sister cells have also been observed in a number of other species (Kubitschek, 1962; Lehtonen, 1980; López-Sáez, Giménez-Martín and González-Fernández, 1966; Prescott, 1959; Webster, 1979a). Differences in cell cycle kinetics have been cited as the possible source of the variation in cell and nuclear size in proliferative populations (Bansal and Davidson, 1978a, 1978b; Davidson, Pertens and Eastman, 1978; Thomas, 1980).

Differences in size of sister cells can be achieved in plant cells by the positioning of the new cell plate or cross wall at late telophase. If the new cross wall is asymmetrically located in the dividing cell then the resulting sister cells will differ in length and most probably in volume as well. Inequality in the size of sister nuclei is more difficult to explain, however. Davidson et al. (1978) have pointed out that the variation in nuclear volume could be generated by variation in the volume of the chromatids that make up the post-telophase nucleus or variation in the nuclear growth rate. In Pisum

sativum mean telophase volume is 93 μm^3(Lyndon, 1967) while mean nuclear volume of G1 cells is 239 μm^3(Webster, 1979b). These results have been interpreted as showing that nuclei undergo a considerable degree of hydration as they enter G1 (Lyndon, 1967; Webster, 1979b). Thus any differences in chromatid volumes would have little affect on nuclear volume once the nuclei were fully hydrated. However, variation in nuclear growth rates could have significant affects on the size of sister nuclei.

The initial phase of nuclear growth, i.e. in early G1, involves the sequestering of macromolecules by the post-telophase nucleus from the cytoplasm (Goldstein, 1976; Phillips, 1972; Rao and Prescott, 1970). This stage is dependent on the ability of the nuclei to regulate this uptake of material and also on the amount of material available to the nuclei. If the sister nuclei have different abilities to regulate the uptake of molecules, or if the cytoplasmic environment in which the nuclei find themselves is different, then there will be a differential uptake of material. This in turn could affect the second phase of nuclear growth, which is the doubling of its constituents during the cell cycle.

For pairs of sister cells, known to be in G1, the ratio of larger to smaller nuclear volume was 1.15:1 in Allium cepa (Davidson and Pertens, 1981a) and 1.16:1 in Pisum sativum (Webster, 1979b). Clearly, volume differences exist between sister nuclei early in interphase. To study these differences in sister nuclei and how they arise we have examined sister nuclei in sister cells and in caffeine-induced binucleate cells. The use of binucleate cells is particularly reveal-

ing since they make it possible to compare the behaviour of sister nuclei in a binucleate cell with the behaviour of nuclei in sister cells: i.e. to compare the behaviour of sister nuclei in one cytoplasmic environment or in two separate environments. In cells induced to become binucleate, sister nuclei have different volumes and this difference in size is established early in G1 (Davidson and Pertens, 1978, 1981a, Stallwood and Davidson, 1977; Wellwood and Davidson, 1977; White and Davidson, 1978) as it is in nuclei of sister cells (Davidson and Pertens, 1981a; Webster, 1979b).

Furthermore, the difference in volume of sister nuclei of binucleate cells is maintained throughout interphase and even into prophase, with the sister nuclei exhibiting differential growth both in absolute and relative terms (Davidson and Pertens, 1981a; Wellwood and Davidson, 1977; White and Davidson, 1978). Thus the regulation of nuclear growth must, to a large extent, reside within nuclei since, if cytoplasmic factors were controlling nuclear size, one would expect sister nuclei to adopt identical volumes as they progress through interphase and increase in size.

Finally sister nuclei in a binucleate cell can show asynchrony in entry into S in $\sim$ 20% of the cases (Benbadis, Ribsztejn and Deysson, 1974) or if sister nuclei enter S synchronously, as they do in polynucleate cells, the duration of S varies from nucleus to nucleus (González-Fernández et al., 1971). Variation in the duration of G1, S and G2 have also been reported between sister cells (Demchenko and Ivanov, 1977, 1978). These results confirm that sister nuclei are capable of acting autonomously, even when occupying a common cytoplasm.

Thus with binucleate cells we have a marked population of cells whose age as they proceed through a cell cycle is known and whose behaviour parallels that of sister cells.

In this study, we have used two species of plants, Vicia faba, the English broad bean, and Pisum sativum, the common garden pea. Once roots had reached a steady state of growth they were treated with caffeine, an inhibitor of cytokinesis (Giménez-Martín et al., 1965; Paul and Goff, 1973). The caffeine treatment results in the formation of binucleate cells, however, one problem with caffeine is the low number of binucleate cells formed during a 1 hr. treatment. In order, in the present study, to increase the number of binucleate cells formed the roots were pretreated with 5-aminouracil (5-AU). This results in a partially synchronous population of cells entering mitoses (Davidson, Golding and Armstrong, 1978; Jakob and Trosko, 1965; McLeish 1969; Rudolph and Davidson, 1975). At the peak of mitotic activity of this population of cells the caffeine treatment is performed thereby enhancing the frequency of binucleate cells formed.

In roots of V. faba it was found that of the binucleate cells produced, only 15% of them completed a cell cycle and divided again. These cells took 14 hours to complete a cell cycle; 14 hours is the estimated cell cycle duration for fast cycling cells (Webster and Davidson, 1968). At this time the remaining 85% of the binucleate cells still had a DNA content equivalent to that of G1 or the pre - S level. It had been expected that approximately 75% of the binucleate cells would behave with the cycle kinetics of fast cycling cells. Thus about 60% of the binucleate cells exhibited altered cycle

kinetics, now being either slow or non-cycling cells. In binucleate cells nuclear volume was found to decrease over this 14 hour period. The decrease in nuclear volume of sister nuclei was a differential one; i.e. as mean nuclear volume decreased so did the mean volume difference between sister nuclei. This means that the absolute reduction in volume was greater in the larger nucleus than in the smaller nucleus of each cell: this is further evidence of differential behaviour of sister nuclei within a common cytoplasm.

This differential behaviour of the sister nuclei within a binucleate cell suggested that they might differ in their ability to take up macromolecules. This possibility was tested in the following way. RNA was labelled with ^{3}H-UR and the uptake of labelled, preformed RNA was studied in binucleate cells. It will be reported here that the uptake of ^{3}H-RNA into the late telophase - early G1 nuclei the sister nuclei was unequal. Also, analysis of RNA synthesis indicates that the rates of RNA synthesis are different in the two sister nuclei; this was indicated by the relative differences in grain counts over the sister nuclei with time. This difference in level of RNA synthesis may result from the differences established in the sister nuclei when they are formed. Such differences can come about by a differential uptake of preformed RNAs and/or proteins into the forming nuclei (Goldstein, 1976; Phillips, 1972; Rao and Prescott, 1970).

On the basis of these results it was decided to determine the macromolecular contents of the sister nuclei. Bennett and his colleagues have shown that nuclear RNA and protein content are positively correlated with the activity of the nucleus, specifically with cell

cycle time (Bennett, 1970; Bennett and Rees, 1969; Bennett, Smith and Smith, 1972). Thus the differences in nuclear volume and behaviour of sister nuclei could be due to differences in RNA content, protein content or possibly the degree of hydration of the sister nuclei. While different degrees of hydration would be interesting and suggestive of autonomous regulation, by a nucleus, of its water content, differences in macromolecular content would provide a basis for an analysis of the functional differences between the sister nuclei in binucleate cells or the nuclei of sister cells.

For this study P. sativum was used, for in contrast with V. faba, the majority of caffeine induced binucleate cells in this species complete a cell cycle and divide. However, as in V. faba, sister nuclei did not have identical volumes, nor did the volumes come to equilibrium with time. RNA and protein contents were determined microspectrophotometrically in the sister nuclei of binucleate cells and in the nuclei of sister cells. With this data we were able to compare the relative behaviour of pairs of sister nuclei in binucleate cells, in which they share a common cytoplasm, with that in sister mononucleate cells, in which each nucleus has its own cytoplasmic environment. In both cases, sister nuclei differed in macromolecular content; the mean ratio of RNA content between sister nuclei in binucleate cells 2 hr. after the caffeine treatment was 1.74:1 while for protein content it was 1.14:1.

The results from studies of V. faba and P. sativum lead to the conclusion that mitosis is asymmetrical for molecules that regulate rates of macromolecular synthesis, nuclear growth and progress through a cell cycle. Once this initial asymmetry has been established, it

is maintained throughout interphase, even in binucleate cells in which the two nuclei share a common cytoplasm.

MATERIALS AND METHODS

II.1 Germination and Culturing of Seedlings

Vicia faba seeds were germinated and grown as previously described (Rudolph and Davidson, 1975). The seeds were soaked in distilled water for 24 hr , the testae were then removed and the seeds planted in sterile sand containing 25 ml H_2O per 100 ml of sand. The seeds were left in sand for 72 hr at which time the seedlings were removed from the sand, washed and suspended over tanks of distilled water. The tanks were kept at 20°C and aerated continually; the water was changed every 24 hr. When the lateral roots had formed and reached a length of 1-1.5 cm. the treatments were begun.

Root cultures of *Pisum sativum* were set up as described previously (Scadeng and MacLeod, 1976; Webster, 1979a; White, 1943). Seeds were surface sterilized with 6% sodium hypochlorite for 5 min., rinsed in distilled water and placed in moist, sterile vermiculite for 3-5 days. When roots were 4-5 cm. long the seedlings were removed from the vermiculite and the apical 10 mm of the roots were excised and placed in White's medium supplemented with sucrose to a final concentration of 2%, 50 ml of sterile medium and 10 roots were placed in 125 ml Bellco culture flasks under aseptic conditions. The flasks were then placed in a shaking water bath set at 1 cycle/sec. and 20°C. The roots were allowed to grow for 3 days before treatments were started.

II.2 Induction of Binucleate Cells

Once roots had achieved a steady state condition the treatments

were begun. Seedlings of V. faba were transferred from tanks of distilled water to a 3.93×10^{-3}M solution of 5-aminouracil (5-AU) for 6 hr. They were then washed and returned to tanks of distilled water. After 15 hr recovery the peak of mitotic activity was reached and at this point the roots were treated with a 0.1% solution of caffeine for 1 hr. At the end of the treatment the roots were washed and transferred back to tanks of distilled water. Roots were fixed at various times during the recovery period.

Once roots of P. sativum had become acclimatized to the culture conditions they were placed in sucrose supplemented White's medium containing 5-AU (3.93×10^{-3}M) for 6 hr, then returned to fresh medium and allowed to recover. After 13 hr recovery the peak of mitotic activity was reached and roots were placed in sucrose supplemented White's medium containing 0.1% caffeine for 1 hr. The roots were then washed and returned to fresh medium. Fixations were made at various times of recovery following the caffeine treatment.

All fixations were made in 10 ml of acetic acid-ethanol (1/3, v/v) containing 0.1 ml of formaldehyde.

II.3 Staining Procedures

Several staining procedures were employed for the various parameters measured. For nuclear volume measurements, excess fixative was washed from roots; they were hydrolysed in 1N HCl at 60°C for 8 min., stained with Feulgen's reagent 1 hr, dissected into columns and lightly tapped to produce a monolayer of cells. The cells were then counterstained with Fast Green and the slides made permanent. At no point in

preparation of the slides were the cells squashed; this was to ensure that nuclear size and shape were not distorted during preparation of the slides (Bansal and Davidson, 1978a).

For DNA determinations in V. faba, standard microspectrophotometric methods were followed (Chayen and Denby, 1968; Gottlieb-Rosenkrantz and O'Brien, 1971; McLeish and Sutherland, 1961; Patau, 1952). Fixed roots were washed free of fixative, hydrolysed for 1 hr in 5 N HCl at room temperature and stained in freshly made Feulgen's reagent for 1 hr. Slides were made as described above, however, no counterstain was used; the slides were passed through three 10 minute rinses in 0.25% $K_2S_2O_5$ in 0.05 N HCl to remove any unbound leuco-basic fuchsin and washed for 5 minutes in distilled H_2O before being prepared as permanent mounts in Permount.

For determinations of DNA and nuclear protein content in P. sativum, roots were again washed free of fixative, then stained using the combined dinitrofluorobenzene and Feulgen technique of Mitchell (1967). Washed roots were placed in the DNFB solution at 65°C overnight. After staining, the roots were washed in 70% ethyl alcohol and then rinsed in warm (~30°C) water. The roots were then placed in 5N HCl at room temperature for 1 hr, transferred to Feulgen's reagent for 1 hr, then slides were made as described for DNA determination in V. faba roots.

To determine the RNA content of nuclei the roots were stained using the Gallocyanin-chrome alum method (Mitchell, 1968; Pakkenberg, 1962). The stain was prepared by shaking 300 mg of Gallocyanin in 100 ml of H_2O for 1 min., to this solution was added 100 ml of a 10% chrome

alum aqueous solution (w/v). The resulting mixture was boiled for 30 min. in a water bath, allowed to cool and then filtered using a Buchner Funnel. The filtrate was then adjusted to pH 1.6 using 1N HCl. Roots, washed free of fixative, were placed in the Gallocyanin-chrome alum solution for 12 hr at 40°C. The stained roots were then washed in distilled water adjusted to pH 1.6 using 1N HCl. Permanent slides were then made as described above. Though Gallocyanin-chrome alum is specific for nucleic acids it stains both RNA and DNA, therefore a second set of roots were pretreated with RNase (1 mg/ml) for 1 hr at 60°C to remove RNA. These roots were then stained with Gallocyanin-chrome alum and permanent slides made of the preparations.

II.4 Nuclear Volume Determinations

Nuclear volumes were determined as described by White and Davidson (1976). Major and minor axes of nuclei were measured using an ocular micrometer at a magnification of 1250 X and the shape of the nucleus was classified as: 1) spherical; 2) semi-spherical; 3) oval; or 4) elongate. Nuclear volumes were then calculated based on the shape class using the major and minor size measurements:

1) Spherical Nuclei; $NV = 4/3\ \Pi\ r^3$ where r = ½ diameter

2) Semi-Spherical Nuclei; $NV = 4/3\ \Pi ab^2$ where a = ½ major axis
b = ½ minor axis

3) Oval Nuclei; $NV = 4/3\ \Pi\ ab\ \frac{(a+b)}{2}$ where a = ½ major axis
b = ½ minor axis

4) Elongate Nuclei; $NV = 4/3\ \Pi b^3 + 2\Pi b^2\ (a-b)$
where a = ½ major axis
b = ½ minor axis

II.5 Nucleolar Volume Estimates

Area measurements of individual nucleoli were made using a MOP-3 digitzier (Carl Zeiss, Inc.). By the use of a camera lucida projection, the area of each nucleolus was traced on the measuring tablet of the MOP system; the machine delivers the area values in square microns. Each nucleolus was assumed to be a sphere, thus the radius of each nucleolus could then be calculated from the area values and then nucleolar volume by the formula:

$$\text{Nucleolar Volume} = 4/3\ \Pi\ r^3$$

Since some nuclei contained up to 4 nucleoli, total nucleolar volume per nucleus was calculated by summing the individual nucleolar volumes found within each nucleus.

II.6 Cell Area Determinations

Cell areas were determined in the same manner as nucleolar areas using the MOP-3 digitizer.

II.7 Microspectrophotometric Determinations

All macromolecular determinations, i.e. of DNA, nuclear protein and nuclear RNA content, were carried out using microspectrophotometry. For DNA determinations in V. faba the two-wavelength method of Patau (1952) was used, the wavelengths were 565 and 500 nm, the peak and the 50% peak of absorption respectively. DNA measurements on 30 bi-nucleate cells were made at 1 + 3, 1 + 6, 1 + 12 and 1 + 14 hr after the caffeine treatment. 2C and 4C DNA contents were obtained by measuring 24 telophase and 24 prophase nuclei from control cells.

For DNA, nuclear protein and nuclear RNA content in cells of P. sativum the one-wavelength method was used (Bansal and Davidson,

1978a; Tobin, Yun and Naylor, 1974). DNA and nuclear protein were measured in the same cells using the combined DNFB-Feulgen technique (Mitchell, 1967); 30 binucleate cells were measured at 1 + 2, 1 + 6, 1 + 10, 1 + 12 and 1 + 14 hr after the caffeine treatment. DNA was measured at a wavelength of 550 nm and protein determinations at 405 nm.

For Gallocyanin stained material peak absorbency was found to be 600 nm. Since Gallocyanin-chrome alum is specific for nucleic acid but stains both DNA and RNA, RNA content was established by subtracting the values for RNase treated material from the values of total nucleic acid:

RNA Content = Total Nucleic Acid Content - RNase Treated Material

At 1 + 2 hr 50 binucleate cells were measured to determine total nucleic acid content and 25 binucleate cells from RNase treated roots at 1 + 2 hr were used to establish the DNA content; these values were then used to determine nuclear RNA content using the above formula.

II.8 Autoradiography

RNA synthesis was studied by supplying roots with a ^{3}H-uridine (^{3}H-UR) solution having a radioactivity of 2μ Ci/ml (S.A. 10 Ci/mmol). V. faba roots were incubated in ^{3}H-UR for 1 hr either during the caffeine treatment or immediately following the caffeine treatment.

Repopulation of nuclei with preformed RNA was examined by supplying the roots with ^{3}H-UR for 1 hr after 12 hr recovery from 5-AU. This was 3 hr before the peak of mitotic activity and before the time when the caffeine treatment was employed. Most of the cells

would be in G2.

Fixations were made at 1 hr, 1 + 1 hr and 1 + 3 hr i.e. immediately after exposure to caffeine and after 1 and 3 hr recovery. Autoradiographs of the fixed material were prepared in the standard manner for plant material (Davidson, 1964; Zuk and Swietlinska, 1973). Cell columns were teased apart and preparations were tapped to give a large number of isolated cells, separated from other cells. In this way, pairs of nuclei seen in squash preparations were known to be from binucleate cells. To ensure maximum contact of cellular material with the emulsion coat, the preparations were squashed. Squash preparations were coated with liquid emulsion (Kodak, NTB2) and exposed for 10 days. Slides were then placed in developing solution (D19), rinsed in distilled water, put in fixer (Rapid Fix) and then given a final rinse in running water. The slides were stained with toluidine blue, run through a dehydration series and made permanent. Correction for background grains was made by counting the number of grains in an area adjacent to and equal in size to the cell being scored.

II.9 Cytoplasmic Gradients

To determine if cytoplasmic gradients existed in dividing cells the cellular protein and RNA contents were measured at the spindle poles of metaphase cells stained with either DNFB or Gallocyanin-chrome alum respectively. A constant plug size was used in measuring, microspectrophotometrically, the amount of bound dye at the poles of the mitotic spindle of metaphase cells. The metaphase cells were from control, untreated material of P. sativum;

50 cells were measured for both protein and RNA content.

II.10 Identification of Sister Cells and Sister Cells in G1

When root cells are teased apart there is a tendency for older cell walls in a column of cells to separate (Thomas, 1980; Webster, 1979a). Thus cells derived from a common ancestor remain in distinct cohorts of varying sizes. In the preparation of slides for this study the roots were sufficiently teased apart so that doublets and triplets were found. The doublets represent sister cells, if both cells are in interphase the exact phase of the cell cycle that the cells occupy is not known. However, sister cells in G1 can be found in the triplets (Davidson and Pertens, 1981a;Webster, 1979b).

In P. sativum the average difference between cycle durations of sister cells is 0.14 times the average cell cycle time (Webster, 1979a, 1979b). Thus with triplets, when one cell of a sister pair has divided and produced two new sister cells, in G1, the other cell of the original sister pair will be in G2 or mitosis. It is the pair of newly formed sister cells in the triplets which we are interested in; if triplets are chosen so that one of the cells is in mitosis we know, from the difference in cycle time of sister cells, that the two cells in interphase are sisters and are in G1.

To determine total nuclear protein content of nuclei from sister cells only doublets, with both cells in interphase, were scored from control roots; they were stained by the DNFB-Feulgen technique. By microspectrophotometric determination of the Feulgen content of the nuclei we were able to classify nuclei as being in G1, S or G2. Their

nuclear protein contents were determined and so estimates were obtained of protein contents of G1, S and G2 nuclei.

Sister cells in G1 from triplets were used to determine nuclear RNA content in sister nuclei from control roots. They were stained with Gallocyanin-chrome alum and the total nucleic acid content was measured by microspectrophotometry. The nuclear RNA content was then estimated based on the G1 DNA content calculated from measurements of binucleate cells in G1.

II.11 Statistical Analysis

Cummulative percentage frequencies of nuclear volumes were calculated as described by Bansal (1975). These frequencies were then plotted against their corresponding linear or log-transformed values of nuclear volume to generate a probit plot. The probit plots were then analysed to determine goodness of fit to a normal or log-normal distribution using EDF statistics (Stephens, 1974).

Frequency distribution histograms were generated for DNA content and nuclear protein content; these histograms were plotted by computer (HP-85, Hewlett Packard). Each sample was divided into 8 classes; the class interval was determined by the number of classes and the minimum and maximum values across the samples that were compared. The height of the bar for each class interval represents the frequency of that class interval.

Groups of paired data were analysed in several ways. For the comparison of mean values the t-test for the differences between two

means was used (Sokal and Rohlf, 1969). The Mann-Whitney U-test was used to determine if the distributions between two samples were significantly different (Sokal and Rohlf, 1969). Paired data were also compared by calculating a regression coefficient, slope and correlation coefficient. These were calculated on an HP-85 computer using the Paired Sample Analysis program from the General Statistics Pac (Hewlett Packard Company, 1979). To determine if regression coefficients were significantly different an F-test for the differences between two regression coefficients was employed (Sokal and Rolhf, 1969). For all statistical tests the 5% level of significance ($p = 0.05$) was used.

RESULTS

III.1 Cell Cycle Kinetics of Caffeine Induced Binucleate Cells of V. faba

Roots were treated with 5-AU and then with caffeine. The 5-AU treatment produces a wave of cells that enter mitosis together (Fig. 1). The caffeine treatment was then given at the peak of mitotic activity. This double treatment was successful in increasing the frequency of binucleate cells from 0.9% to 3.7%.

With this population of binucleate cells, nuclear and cellular behaviour were followed over a period of one cell cycle. The following parameters were studied:

1) the duration of the cell cycle in the binucleate cells
2) the proportion of binucleate cells that divided
3) the DNA content of sister nuclei in binucleate cells
4) changes in nuclear volume as the cells grow older
5) RNA synthesis in binucleate cells

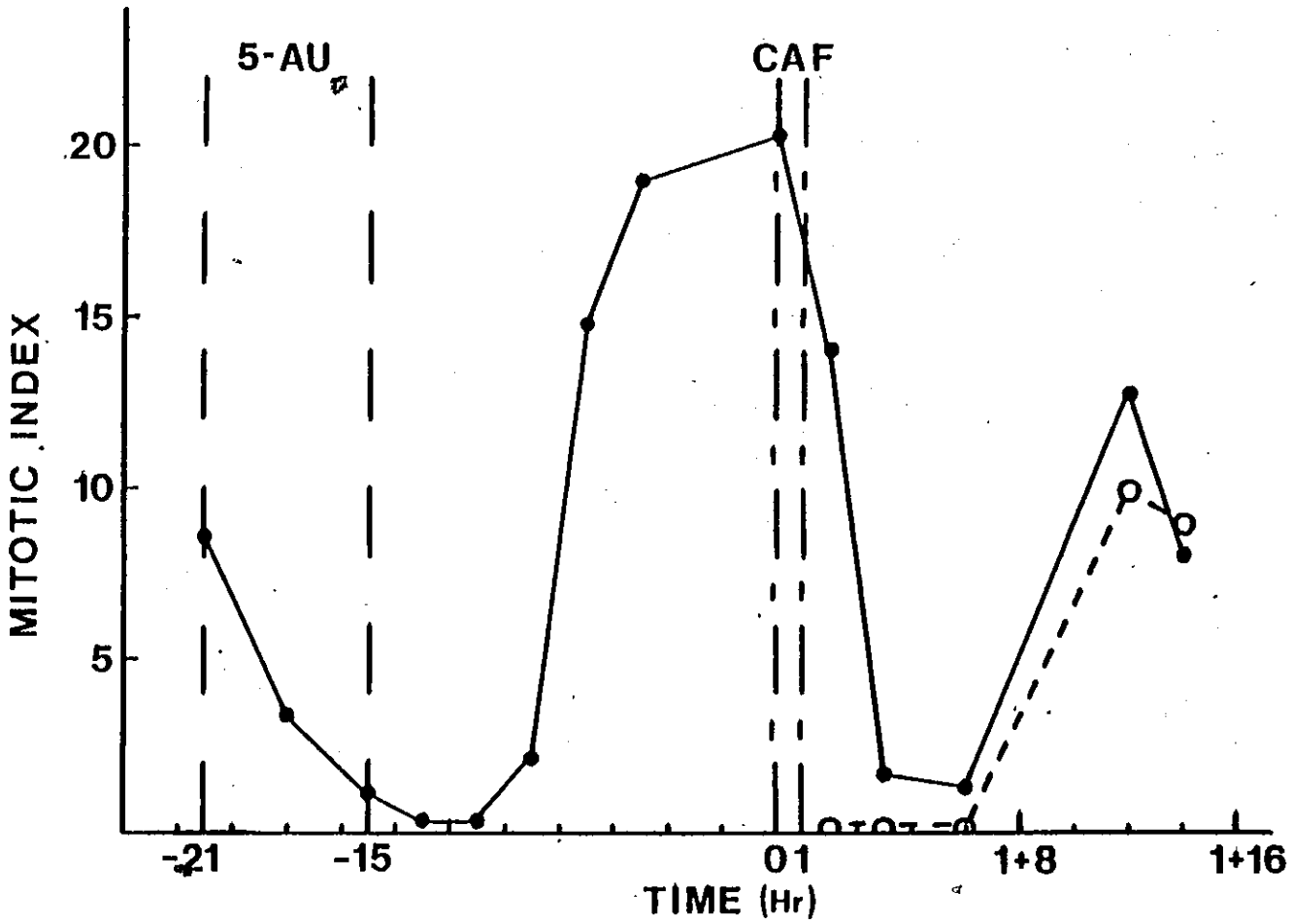
III.1.1 Duration of the Cell Cycle in Binucleate Cells

At 1 ± 1 hr there were 37 binucleate cells per 1000 cells scored but their frequency decreased between 1 + 1 and 1 + 3 hr and remained around 10 per 1000 from 1 + 3 to 1 + 14 hr. This decrease occurred because: a) no new binucleate cells were formed after the roots were removed from caffeine, and b) a number of mononucleate cells divided, adding new cells to the population in the period 1 ± 1 to 1 + 3 hr (Fig. 1). Between 1 + 3 and 1 + 14 hr the number of cells which entered and completed a division was low, thus few new mononucleate were added to the population; consequently the

Figure 1

Mitotic Index of Mononucleate and Binucleate Cells After 5-AU and Caffeine Treatments in V. faba

The mitotic index of mononucleate (●) and binucleate (O) cells at various times after treatment with 5-AU and caffeine (CAF). The vertical bars indicate the periods of 5-AU and caffeine treatments; all times are given in relation to the 1 hour caffeine treatment. Each M.I. is based on 3000 cells in the case of mononucleate cells and 100 cells in the case of binucleate cells. Data for the 5-AU treatment and recovery taken from Davidson, Golding and Armstrong (1978).


5-AU
CAF
20
15
10
5
MITOTIC INDEX
-21
-15
0 1
1+8
1+16
TIME (Hr)

frequency of binucleate cells remained fairly constant (Table 1). Large numbers of mononucleate cells were seen in mitosis at 1 + 12 and 1 + 14 hr and their division resulted in an increase in the relative proportion of mononucleate cells subsequent to 1 + 14 hr. This increase made it difficult to find appropriate numbers of binucleate cells for analysis. The mononucleate cells in mitosis at 1 + 12 and 1 + 14 hr were, for the most part, cells that were in division at the time of the caffeine treatment but that were not induced to become binucleate: at 1 + 12 and 1 + 14 hr they were undergoing a second wave of semi-synchronous division.

At 1 + 12 and 1 + 14 hr binucleate cells entered mitosis, but only in low numbers. It was found that only 9 or 10% of the binucleate cells divided at these times (Table 1). In V. faba 75% of the cells seen in mitosis are fast cyclers with a mean cell cycle duration of 14 hr (Webster and Davidson, 1968). Therefore, it was expected that if the binucleate daughter of a fast cycling cell was also fast cycling, it should have entered mitosis in approximately 14 hr; in addition, it was expected that approximately 75% of the binucleate cells would have completed a cell cycle by 14 hr. The observation that only 10% of the binucleate cells are in mitosis shows that many of the binucleate cells induced by caffeine in V. faba do not complete a cell cycle and enter mitosis, or at least not within 14 hr. Thus, in addition to its ability to produce binucleate cells, caffeine also alters the cell cycle kinetics of the cells induced to become binucleate.

The ability of caffeine to alter cell cycle kinetics, however,

Table 1

Changes in Nuclear Volume of Mononucleate and Binucleate Cells During Recovery from Caffeine

Mean volume (μm^3) of nuclei in mononucleate and binucleate cells in interphase (I) and prophase (P) are given. Lateral roots were treated with 0.1% caffeine for 1 hour and allowed to recover for 1 to 14 hours. Each mean ± S.D. is based on 300 nuclei. The range of nuclear volumes, the percentage of binucleate cells and the M.I. of mononucleate and binucleate cells are also given.

		Mononucleate Cells			Binucleate Cells			
		Nuclear Volume (μm^3)		M.I.	Percent	Nuclear Volume (μm^3)		M.I.
Time		Mean ± SD	Range			Mean ± SD	Range	
1+1	I	1392 ± 706	449-3338	14.1	3.7	1074 ± 402	384-2910	0
	P	2014 ± 896	742-4831	-	-	-	-	-
1+3	I	-	-	1.7	1.3	766 ± 276	173-1521	0
1+6	I	673 ± 321	116-2120	1.3	1.1	770 ± 305	231-1871	0
	P	1235 ± 475	390-2573	-	-	-	-	-
1+12	I	-	-	12.8	0.4	559 ± 221	173-1490	10.0
1+14	I	557 ± 189	225-1145	8.1	1.0	534 ± 203	173-1278	9.0
	P[a]	1063 ± 314	570-2235	-	-	994 ± 506	285-1676	-
Control								
	I	730 ± 261	269-1752	9.8	0	-	-	-
	P	1450 ± 397	629-2686		-	-	-	-

[a] Binucleate cell prophase value based on 5 cells, i.e. 10 nuclei

appears to vary from species to species. Caffeine induced binucleate cells in A. cepa come back into division in high numbers, approximately 75% of the binucleate cells were observed in division 13 hr after the caffeine treatment (Giménez-Martín et al., 1965) and from our own studies on caffeine induced binucleate cells in roots of P. sativum, grown in root culture, over 60% of the binucleate cells completed a cell cycle (see Section III.3.1.). Thus, though caffeine induced binucleate cells cannot be considered to be physiologically normal (Clowes, 1980; Davidson and Pertens, 1978, 1981a), nevertheless they present a unique opportunity to study the behaviour of pairs of sister nuclei. This opens up a number of avenues which have largely been unexplored and some of them will be examined in the course of this study (i.e. Sections III.2 and III.4).

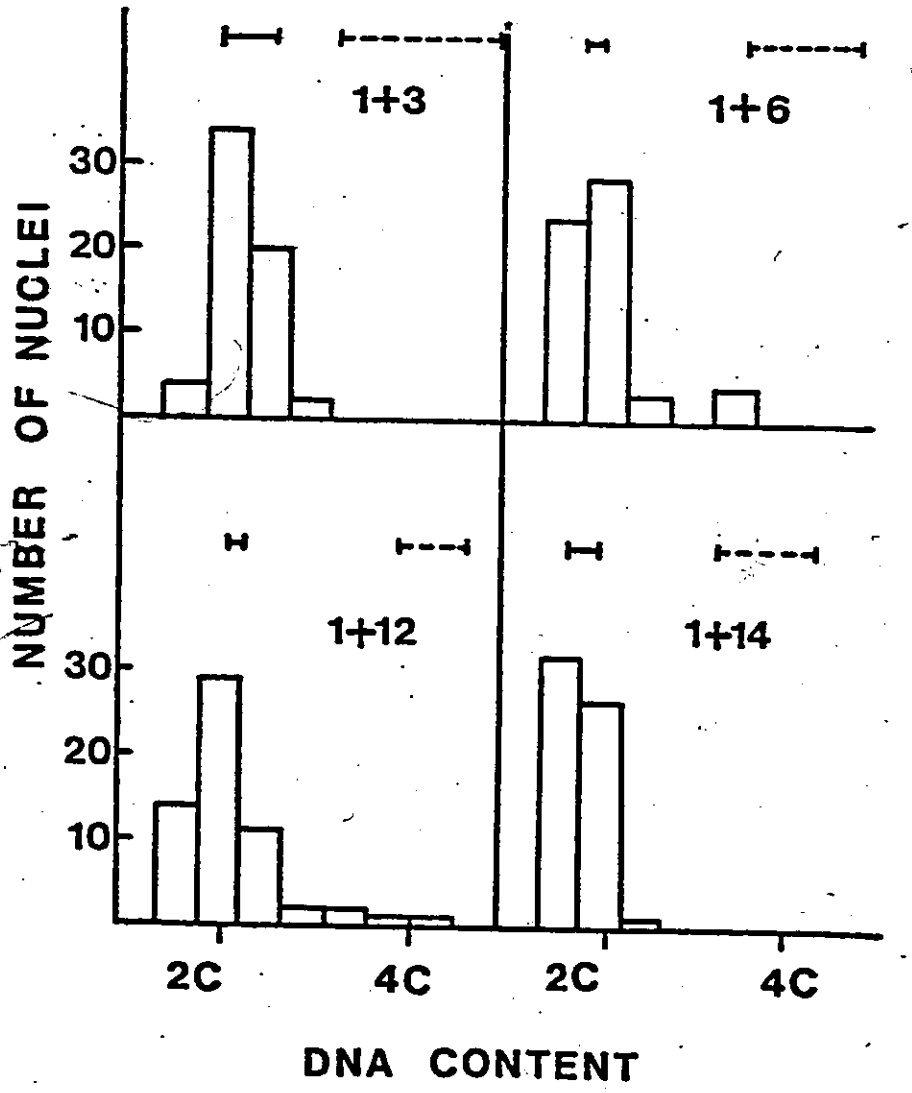
III.1.2 DNA Content of Binucleate Cells

About 10% of the binucleate cells complete a cell cycle by 1 + 14 hr. To determine where in the cell cycle, i.e. G1, S or G2, the remaining 90% of the binucleate cells were located and the DNA content of the sister nuclei in binucleate cells was determined. Figure 2 shows the DNA values for the nuclei of binucleate cells at 1 + 3, 1 + 6, 1 + 12 and 1 + 14 hr. At 1 + 3 hr most of the nuclei appeared to have a G1 DNA content with a few nuclei possibly in the early stages of S, i.e. the nuclei with DNA contents greater than 404 a.u. By 1 + 6 hr it can be seen that a few nuclei were definitely in S, while at 1 + 12 hr, 10% of the nuclei were either in G2 or completing S. It was at this time that the first binucleate cells were seen in mitosis. At 1 + 14 hr all nuclei of the binucleate cells had G1 DNA contents. This means

Figure 2

DNA Content of Nuclei in Caffeine Induced Binucleate Cells

Mean DNA content in arbitrary units of nuclei in binucleate cells induced by a 1 hour treatment with 0.1% caffeine. Measurements were taken in G1 (1 + 3 hr), S (1 + 6 hr) and G2 (1 + 12 and 1 + 14 hr). At each time 60 nuclei were measured, from 30 binucleate cells. The 2C and 4C DNA values are each the mean of 24 telophase or 24 prophase nuclei.


1+3
1+6
1+12
1+14
30
20
10
30
20
10
NUMBER OF NUCLEI
2C
4C
2C
4C
DNA CONTENT

that the 10% of the binucleate cells in S and G2 at 1 + 12 hr must have entered mitosis. We see, therefore, a close agreement between M.I. and the percentage of binucleate cells with a 4C DNA content. Since there is probably some overlap of cells, based on the MI scored at both 1 + 12 hr and 1 + 14 hr, we estimated that about 15% of the binucleate cells divided. These results can be taken as direct evidence of a significant inhibitory effect of caffeine on many of the cells that are binucleate: 85% of these cells have not entered S by 1 + 14 hr, they have been blocked in G1. It appears that they have been changed from fast cycling cells to either slow cycling or non-cycling cells. The remaining 15% enter S by 1 + 6 hr and by 1 + 14 hr all of these binucleate cells must have been in mitosis, or had divided, since no binucleate cells were seen whose nuclei had 4 C DNA contents (Fig. 2).

III.1.3 Nuclear Volume

Nuclear volumes were determined for both binucleate and mononucleate cells (Table 1). Mean nuclear volume in both binucleate and mononucleate cells at 1 + 1 hr was significantly ($p = 0.05$) larger than the mean nuclear volume from untreated, control roots; i.e. 1074 um^3 and 1392 um^3 as compared to 730 um^3. This increase in mean nuclear volume was due to the 5-AU pretreatment. In roots of *V. faba* and *Z. mays* a 5-AU treatment causes an increase in both mean nuclear volume and mean cell area (Davidson, Golding and Armstrong, 1978; Davidson and Pertens, 1981b). Thus the caffeine treatment was carried out on a population of cells whose nuclear volume was larger than normal.

The most striking feature of the study of nuclear volume in caffeine treated roots was a reduction in mean volume of nuclei in binucleate and mononucleate cells over a cell cycle (Table 1). In binucleate cells mean nuclear volume decreased from 1074 ± 403 μm^3 at 1 + 1 hr to 543 ± 203 μm^3 at 1 + 14 hr; in mononucleate cells the values decreased from 1392 ± 706 μm^3 to 557 ± 189 μm^3 over the same time period. Not only was there a decrease in mean nuclear volume but there was a decrease over the entire range of nuclear volumes. Even in nuclei that must have been progressing towards mitosis, there was a reduction, over the period 1 + 1 to 1 + 14 hr, in their volume (Fig. 3).

These results are unexpected since during a cell cycle nuclear volume normally doubles: e.g. in colchicine induced tetraploid cells there is a 2.7 fold increase in nuclear volume over one cell cycle (Bansal and Davidson, 1978b) and in caffeine induced binucleate cells in A. cepa there is a 1.7 fold increase in nuclear volume from mid-G1 to mid-G2 (Sacristan-Garate et al., 1974). It is possible that this decrease in nuclear volume in the binucleate cells was because 85% of them were arrested in G1 or, perhaps, were no longer cycling. However, the same can not be said about the mononucleate cells which continue to cycle. This reduction in nuclear volume could arise either by a loss of macromolecules from the nuclei or by a change in the degree of hydration. The possible loss of RNA from nuclei undergoing contraction was tested in the following way. Cells were allowed to incorporate ^{3}H-UR and ^{3}H-RNA moved from the nucleus into the cytoplasm. When cells with ^{3}H-RNA were induced to become binucleate, the ^{3}H-RNA was taken up by the nuclei as they entered G1. Immediately after the treatment the

Figure 3

Probit Plot of Log-transformed Nuclear Volumes of Mononucleate and Binucleate Cells

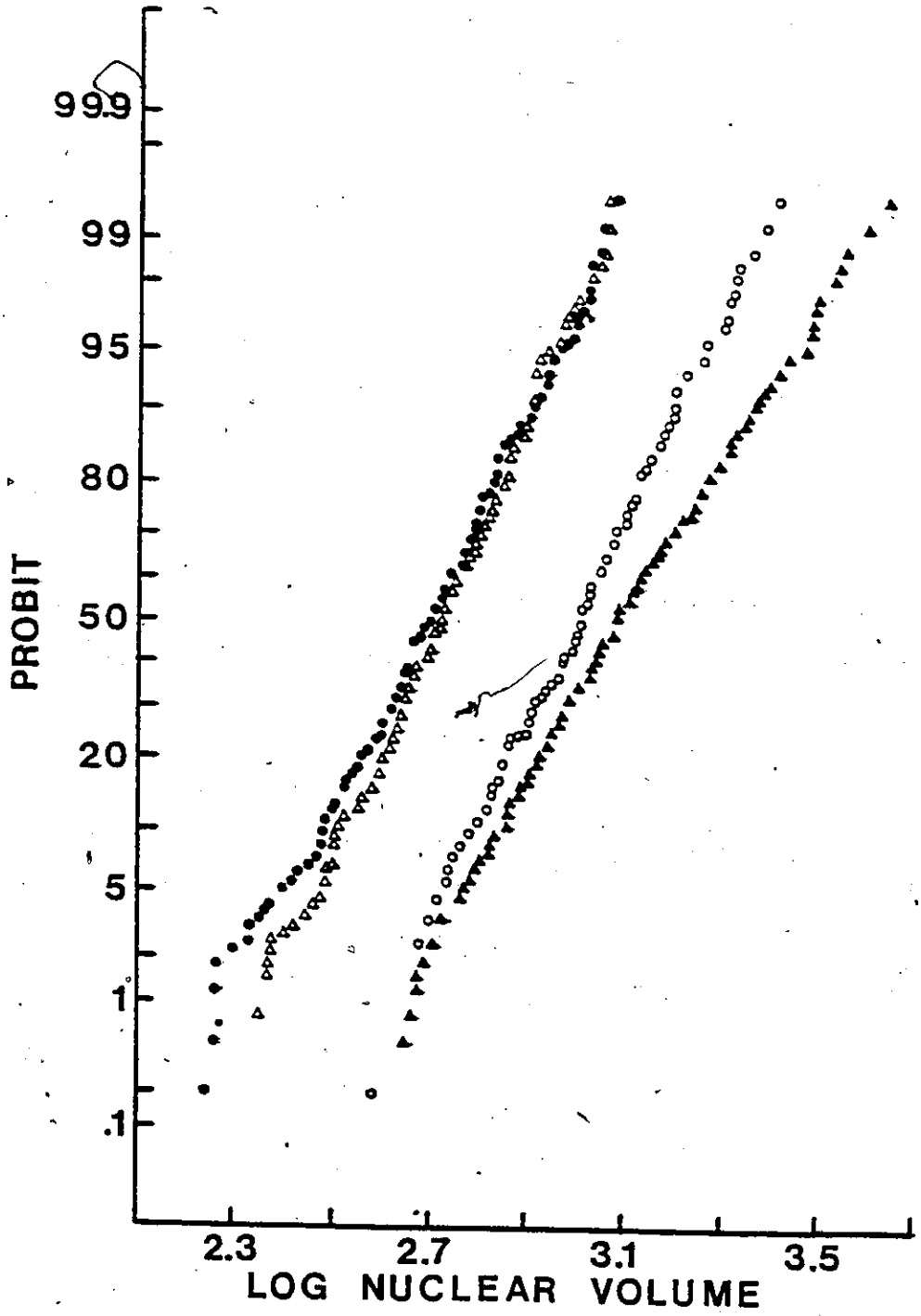
A cumulative frequency of log-transformed nuclear volumes of mononucleate and binucleate cells scored at 1 + 1 and 1 + 14 hr. Each sample was of 300 nuclei.

Binucleate Cells 1 + 1 hr ○ ;

Binucleate Cells 1 + 14 hr ● ;

Mononucleate Cells 1 + 1 hr ▲ ;

Mononucleate Cells 1 + 14 hr △ .


99.9
99
95
80
50
20
5
1
.1
PROBIT
2.3
2.7
3.1
3.5
LOG NUCLEAR VOLUME

the average grain count per nucleus was 14.7 ± 7.6 while at 1 + 1 hr the value had fallen to 11.1 ± 6.9 (Table 6). Thus, even though RNA synthesis is going on in these nuclei (see section III.1.4) and the number of grains per nucleus should be increasing, mean grain count per nucleus went down. This means that RNA molecules brought into the nucleus immediately after mitosis are being exported from the nucleus. The implication is that the macromolecular content of the nuclei must be decreasing and this contributes to the decrease in nuclear volume.

III 1.4 RNA Synthesis

In the presence of caffeine, macromolecular synthesis is depressed (Putrament et al., 1972; Zuk and Swietlinska, 1973); this may reduce the ability of the binucleate cells to complete a cell cycle. Therefore it was decided to examine RNA synthesis in the caffeine treated cells. Labelled precursor, ^{3}H-UR, was supplied to the roots during the caffeine treatment. Grain counts over nuclei exposed to caffeine were considerably lower than over nuclei from control roots (Table 2). This reduction in incorporation of labelled uridine into RNA is, in part, due to caffeine's ability to inhibit the uptake of precursors of macromolecules (Kilbert et al., 1973; Putrament et al., 1972; Odmark, 1972; Zuk and Swietlinska, 1973). However, RNA synthesis was also affected. During the 3 hr period studied the increase in grain count over nuclei from caffeine treated material was less than in controls, i.e. a 3.1 - fold increase in the total grain count for both nuclei of binucleate cells and a 3.3-fold increase for the mononucleate cells as compared with a 4.2-fold increase per nucleus from control cells (Table 2). These values are a measure of net synthesis and they

Table 2

Grain Counts from Autoradiographs of ^{3}H-UR Labelled Mononucleate and Binucleate Cells

Roots were treated for 1 hr with both 0.1% caffeine and ^{3}H-UR. Grain counts were determined over the cytoplasm (Cyto) and nuclei (Nuc) of both mononucleate and binucleate cells, 100 mononucleate and 30 binucleate cells were scored at each time. Control material was treated for 1 hr with ^{3}H-UR only. Nucleo-cytoplasmic ratios (N/C) are also given.

	Binucleate Grain Count			Mononucleate Grain Count			Control Grain Count		
Time	Cyto ± SD	Nuc ± SD[a]	N/C	Cyto ± SD	Nuc ± SD	N/C	Cyto ± SD	Nuc ± SD	N/C
1	12.23±7.09	8.76±6.36	0.71:1	7.82±5.50	5.38±3.57	0.69:1	12.22±8.78	15.72±9.47	1.29:1
1 + 1	21.10±16.88	15.00±6.23	0.71:1	13.63±7.71	7.32±3.98	0.54:1	27.41±13.25	22.51±9.95	0.82:1
1 + 3	42.93±17.24	27.17±12.56	0.63:1	36.26±18.01	17.61±8.11	0.49:1	148.66±47.44	65.75±27.48	0.44:1

[a] For the binucleate cells the nuclear grain count is the sum of the grain count over the two sister nuclei

do not include RNA exported from the nucleus to cytoplasm. This, however, can be measured by the accumulation of labelled RNA in the cytoplasm. In normal roots there was a 12.1-fold increase in cytoplasmic grain counts in 3 hr roots while in caffeine treated material it was much lower, i.e. in binucleate cells it was only 3.5-fold and only 4.6-fold in mononucleate cells (Table 2). These data clearly show that net RNA synthesis is higher in control cells than in caffeine treated cells.

The difference between the rate of RNA synthesis in the binucleate cells and the untreated control cells is even greater than the increase in grain counts suggests. It must be remembered that the binucleate cells were more than twice the size of the control cells (Table 3) and they have the equivalent of a 4C DNA content, i.e. 2 G1 nuclei. Thus, the surface area for the uptake of ^{3}H-UR was greater in binucleate cells than in normal cells; and the amount of DNA which can be transcribed to produce RNA is 4C in all binucleate cells but only 2C or 2C → 4C in many control cells. Thus the relative efficiency of the binucleate cells in synthesizing RNA was far less than that of the control cells.

From 1 to 1 + 3 hr mean nuclear volume in caffeine treated roots decreases by over 40% (Table 1). This led to an analysis of the rate of export of RNA from the nuclei of binucleate cells and a comparison with the rate of movement of ^{3}H-RNA from nucleus to cytoplasm in mononucleate cells (Table 2). In control cells the nucleus:cytoplasmic (N/C) ratio was 1.29:1 at the end of the 1 hr exposure to ^{3}H-UR; i.e. only 44% of the labelled RNA synthesized in that period moved into the cytoplasm.

Table 3

Comparison of Cell Area in Caffeine Treated Material and in Controls

Mean cell areas (μm^2) are given for binucleate and mononucleate cells 1 hour after a 1 hour treatment with 0.1% caffeine, i.e. 1 + 1 hr, and for untreated control cells in interphase and mitosis; 75 binucleate cells and 150 cells of all other types were scored. The ranges of cell areas are also given.

	Cell Area (μm^2)	
Cell Type	Mean $\pm$ S.D.	Range
Control Interphase	427 $\pm$ 196	143 - 1073
Control Mitotic	578 $\pm$ 200	264 - 1069
Binucleate	981 $\pm$ 336	362 - 1874
Mononucleate	613 $\pm$ 239	256 - 1518

In mononucleate cells of caffeine treated roots, however, the N/C ratio was 0.69:1. Though less ^{3}H-RNA was made in these cells than in controls, a greater proportion of it had migrated to the cytoplasm; the value was 59% of total ^{3}H-RNA. By 1 + 1 hr, the N/C ratio was 0.82:1 in control cells and only 0.54:1 in caffeine treated mononucleate cells (Table 2). The data show that 65% of ^{3}H-RNA was in the cytoplasm of caffeine treated mononucleate cells but the corresponding value was only 55% in control cells. By 1 + 3 hr the N/C ratio was 0.44:1 in control cells and 0.49:1 in mononucleate cells of caffeine treated material (Table 2). Therefore at 1 + 3 hr the relative amount of RNA which had moved out of the nucleus into the cytoplasm was the same in both control cells and mononucleate cells, i.e. 69% of the ^{3}H-RNA was found in the cytoplasm of controls and 67% in mononucleate cells from caffeine treated material. Even though the amount of labelled material in caffeine treated cells was less than in controls, initially the mononucleate cells are releasing more ^{3}H-RNA to the cytoplasm than are the control cells. However, by 1 + 3 hr the relative amount of ^{3}H-RNA released by both cell types was the same.

A similar pattern of ^{3}H-RNA release to the cytoplasm was found in the binucleate cells (Table 2). As in the mononucleate cells a relatively large proportion of the total ^{3}H-RNA was found in the cytoplasm at 1 hr; the N/C ratio at this time was 0.71:1 as compared with a ratio of 0.69:1 in mononucleate cells of caffeine treated material and 1.29:1 in controls (Table 2). By 1 + 3 hr the relative amounts of

^{3}H-RNA in the cytoplasm of binucleate and control cells were similar; the N/C ratios were 0.63:1 and 0.44:1 yet in terms of the relative amounts of ^{3}H-RNA in the cytoplasm of the two cell types the values were 61.2% and 69.3% respectively. These values, a reflection of the relative movement of material out of the nucleus into the cytoplasm, show that the rate of movement of ^{3}H-RNA is initially higher in the caffeine treated material. However, by 1 + 3 hr the relative amounts of ^{3}H-RNA found in the cytoplasm of control and caffeine treated cells are similar.

Since the values from the control cells are an indication of the normal rate of transport of RNA out of the nucleus into the cytoplasm it is evident that RNA was moving out of the nucleus at an enhanced rate in caffeine treated material. In part, the release of RNA into the cytoplasm in caffeine treated material must be due to normal export of RNA from the nucleus; in part, however, the enhanced rate of loss may be attributable to the decrease in nuclear volume. Since these results parallel those for the loss of preformed RNA (see section III.1.3, Nuclear Volume), it appears that the reduction in nuclear volume coincides with a loss of macromolecules, at least with the loss of RNA, from the nucleus.

III.2 Differential Behaviour of Sister Nuclei in Binucleate Cells of *V. faba*

Previously it has been shown that sister nuclei in binucleate cells showed differential growth (Wellwood and Davidson, 1977; White and Davidson, 1978). Thus, sister nuclei with the same genotype exhibit

phenotypic differences in size and behaviour while existing in a common cytoplasm. To analyse this phenomenon more fully, the decrease in nuclear volume of the sister nuclei was studied, as well as the rate of RNA synthesis in the sister nuclei and the amount of preformed RNA incorporated into the post-mitotic sister nuclei, i.e. into the sister nuclei in the binucleate cells formed during the caffeine treatment.

III.2.1 Nuclear Volume of Sister Nuclei

In the binucleate cells, volume differences between sister nuclei were clearly seen (Table 4). At 1 + 1 hr the mean difference in nuclear volume between sisters was 253 $\pm$ 201 μm^3; this difference dropped to 97 $\pm$ 89 μm^3 at 1 + 14 hr. Therefore, mean difference in nuclear volume decreased at the same time as mean nuclear volume decreased, c.f. Tables 1 and 3. Since mean difference in nuclear volume as a fraction of mean nuclear volume decreased the contraction shown by sister nuclei was a differential one. Thus over the 14 hr period studied the larger nucleus of the sister pair decreased in volume to a greater extent than the smaller nucleus, both in absolute and relative terms. This can be seen in the ratio of the mean nuclear volumes, large/small (L/S), which fell from 1.27:1 at 1 + 1 hr to 1.20:1 at 1 + 14 hr (Table 4). However, the sister nuclei never become equal in size, since pairs of prophase nuclei at 1 + 14 hr had an L/S ratio of 1.20:1 (Table 4).

III.2.2 RNA Synthesis in Sister Nuclei

The differential decrease in nuclear volume of pairs of sister nuclei indicates a certain degree of nuclear autonomy in behaviour. A

Table 4

Differences in Volume of Sister Nuclei of Binucleate Cells

Mean volume (μm^3) of the larger and smaller nucleus in 150 binucleate cells of V. faba roots treated with 0.1% caffeine for 1 hour and fixed 1 to 14 hours later. The mean differences, ranges of differences and the ratio of volumes (larger/smaller) between sister nuclei are also given.

Time	Nuclear Volume (μm^3) Mean Larger ± S.D.	Mean Smaller ± S.D.	Differences in Nuclear Volume (μm^3) Mean ± S.D.	Range	Ratio (L/S)
1 + 1	1201 ± 423	947 ± 338	253 ± 201	0-1121	1.27 : 1
1 + 3	847 ± 285	685 ± 242	162 ± 146	0- 820	1.24 : 1
1 + 6	850 ± 325	691 ± 262	159 ± 145	0- 808	1.23 : 1
1 + 12	612 ± 222	507 ± 209	105 ± 80	0- 343	1.21 : 1
1 + 14	582 ± 210	485 ± 183	97 ± 90	0- 521	1.20 : 1
1 + 14 Prophase[a]	1083 ± 544	904 ± 510	179 ± 119	17- 279	1.20 : 1

[a] Prophase value is based on 5 cells only, i.e. 10 nuclei

more direct approach to determine if the behaviour of the sister nuclei was differential was to study the synthetic capabilities of the sister nuclei of binucleate cells. Labelled precursor, ^{3}H-UR was supplied concurrently with the caffeine treatment, this ensured that labelled precursor would be available within the binucleate cells once the sister nuclei reformed at the end of mitosis and began RNA synthesis.

The grain count per nucleus between sister nuclei was significantly different ($p = 0.05$, Table 5) at each time examined. The actual rates of increase of grain counts over the two nuclei of the binucleate cells were also different, for the nuclei with the higher grain count the rate was 3.6 ± 0.29 grains/hr while in the nuclei with the lower grain count it was 2.5 ± 0.86 grains/hr. Thus the pair of sister nuclei within a common cytoplasm showed a differential ability to synthesize RNA; this parallels the differential changes observed in the volume of the sister nuclei. One striking difference between sister nuclei lies in their relative abilities to synthesize RNA and in the degree of difference between their volumes. While the ratio of nuclear volume, L/S, for sister nuclei changed with time, on average it was 1.2:1; however, the average ratio of grain counts for sister nuclei, higher to lower, was 1.84:1 (Table 5). Thus, the metabolic capability of the sister nuclei showed a far greater difference than the difference in their nuclear volume would lead us to expect.

Analysis of sister cells from untreated, control roots supplied with ^{3}H-UR showed differences in nuclear grain counts similar to those between the sister nuclei of binucleate cells (Table 5). Analysis of

Table 5

Differential Synthesis of ^{3}H-RNA in Sister Nuclei of Binucleate Cells

Mean grain counts and the ratio of labelling intensity (High/Low) for the sister nuclei of the binucleate cells in Table 2 are presented. The rates of ^{3}H-RNA accumulation, as a measure of RNA synthesis, within the sister nuclei are also given; these rates, which are the increase in grain count per hour, were calculated by regression analysis of the grain counts between 1 and 1 + 3 hr. Control data came from pairs of sister cells from untreated material exposed to ^{3}H-UR for 1 hour, 30 pairs of sister cells were scored.

	Nuclear Grain Counts			
Time	Mean ± S.D.	High ± S.D.	Low ± S.D.	Ratio (H/L)
Control	9.65 ± 5.65	11.63 ± 6.00	7.67 ± 4.57	1.52 : 1
1	4.33 ± 3.55	5.57 ± 3.73	3.10 ± 2.94	1.80 : 1
1 + 1	7.50 ± 4.60	10.23 ± 4.30	4.77 ± 3.04	2.14 : 1
1 + 3	13.58 ± 7.29	16.60 ± 3.04	10.57 ± 5.37	1.57 : 1
Rate	-	3.6 ± 0.29	2.5 ± 0.86	1.44 : 1

the individual ratios indicated that the distribution of differences was greater in the binucleate cells than between sister cells, even though regression analysis showed the two cell populations to have almost identical slopes after 1 hr of labelling; i.e. 0.651 c.f. 0.645 for control and binucleate cells, respectively. There are several reasons for these apparently conflicting results: 1) these results deal with relative and not absolute differences, 2) in absolute terms grain counts per nucleus are significantly higher in controls than in binucleate cells. These two facts combine to indicate that the relative difference is not as great in controls, as it is in binucleate cells, but it also indicates that the differences are maintained over a cell cycle since the control sample was composed of G1, S and G2 cells. Further it shows that, early in G1, a difference is established between the two nuclei of a sister pair whether they are in a binucleate cell, and sharing a common cytoplasm, or are in separate cells with their own environment; also, that this difference is maintained over a cell cycle.

III.2.3 Repopulation of Post-Telophase Nuclei by Preformed RNA

Analysis of the uptake of preformed RNA showed that sister nuclei in binucleate cells did not take up equal amounts of the labelled RNA. At 1 hr, immediately at the end of the caffeine treatment, the binucleate cells showed mean grain counts of 18.57 $\pm$ 7.80 grains/nucleus over the more highly labelled nucleus of a pair and 10.87 $\pm$ 5.19 grains/nucleus over the more lightly labelled nucleus (Table 6). Since RNA synthesis does not occur during mitosis in *V. faba* (Davidson, 1964) most of the labelled RNA observed at 1 hr must have been synthesized in the previous

Table 6

Differential Uptake of Preformed ^{3}H-RNA Into Sister Nuclei After Mitosis

Roots were labelled for 1 hr with ^{3}H-UR 3 hours prior to the 5-AU induced wave of mitosis and during the peak of mitosis the roots were treated with 0.1% caffeine for 1 hour; the binucleate cells were then examined by autoradiography to determine grain counts over the sister nuclei. The mean grain count, the grain counts for the sister nuclei, the ratio of the labelling intensity (High/Low) as well as the total cell grain counts are given; 30 binucleate cells were scored at each time.

Time	Total Grains Per Cell	Nuclear Grain Count			
		Mean ± S.D.	High ± S.D.	Low ± S.D.	Ratio (H/L)
1	78.1 ± 27.0	14.72 ± 7.63	18.57 ± 7.80	10.87 ± 5.19	1.71:1
1 + 1	80.2 ± 28.2	11.07 ± 6.92	13.47 ± 7.50	8.67 ± 5.40	1.55:1
1 + 3	299.9 ± 93.8	38.75 ± 15.94	44.03 ± 16.75	33.47 ± 13.36	1:31

interphase. However, since RNA synthesis resumes in interphase nuclei it was difficult to determine if the changes in relative grain counts was due to 1) continued uptake of preformed RNA, 2) newly synthesized RNA or 3) both. Between 1 hr and 1 + 1 hr the total number of grains per binucleate cell was not significantly different ($p = 0.05$), i.e. 78.1 $\pm$ 27.0 c.f. 80.2 $\pm$ 28.2 grains/cell for 1 hr and 1 + 1 hr, respectively (Table 6). Thus, overall grain counts remained the same at these two times. The distribution of grains did change, however; grain counts per nucleus fell while the cytoplasmic grain counts increased (Table 6). These results suggest that RNA incorporated into the nucleus at the end of mitosis is being released from the nucleus during a period when nuclear volume in the binucleate cells is decreasing (cf. Tables 6 and 1).

The release of preformed ^{3}H-RNA from sister nuclei is also differential. Between 1 and 1 + 1 hr the mean grain count per nucleus fell from 14.7 $\pm$ 7.6 to 11.1 $\pm$ 6.9 grains (Table 6), at the same time the ratio of grain counts, high to low (H/L), between the pair of sister nuclei decreased from 1.71:1 to 1.55:1 (Table 6). After 1 + 1 hr grain counts per cell increased dramatically (Table 6), suggesting that RNA synthesis had started and that labelled precursor present in cytoplasmic pools was being used, resulting in much higher grain counts. Nevertheless, the results indicate that loss of preformed ^{3}H-RNA, between 1 and 1 + 1 hr, was differential; i.e. in a binucleate cell, the nucleus containing the greater amount of ^{3}H-RNA, as determined by

grain counts showed a greater loss in labelled material than its sister nucleus. These results parallel the observed differential decrease in nuclear volume exhibited by sister nuclei of binucleate cells.

III.3 Caffeine Induced Binucleate Cells in Roots of P. sativum

It had been our intention to further characterize the differences between sister nuclei of binucleate cells in V. faba by examining their nuclear protein and RNA contents. However, attempts to grow seedlings, using seeds from several new lots, proved to be impossible due to a fungal infection which appeared at the time of lateral root emergence. Because of the cytological effects produced by pesticides (Cortes et al., 1982; Upadhyaya and Noodén, 1977) rather than treating the seedlings with a fungicide, it was decided that another system should be found.

Root cultures looked appealing because of the ease of working with large numbers of roots and a constant environment could be maintained for root growth (Webster, personal communication). The choice of P. sativum as the experimental organism was made because of the extensive work which has been done with roots of this plant in culture (Scadeng and MacLeod, 1976; Webster and Van't Hof, 1970; Webster, 1979b).

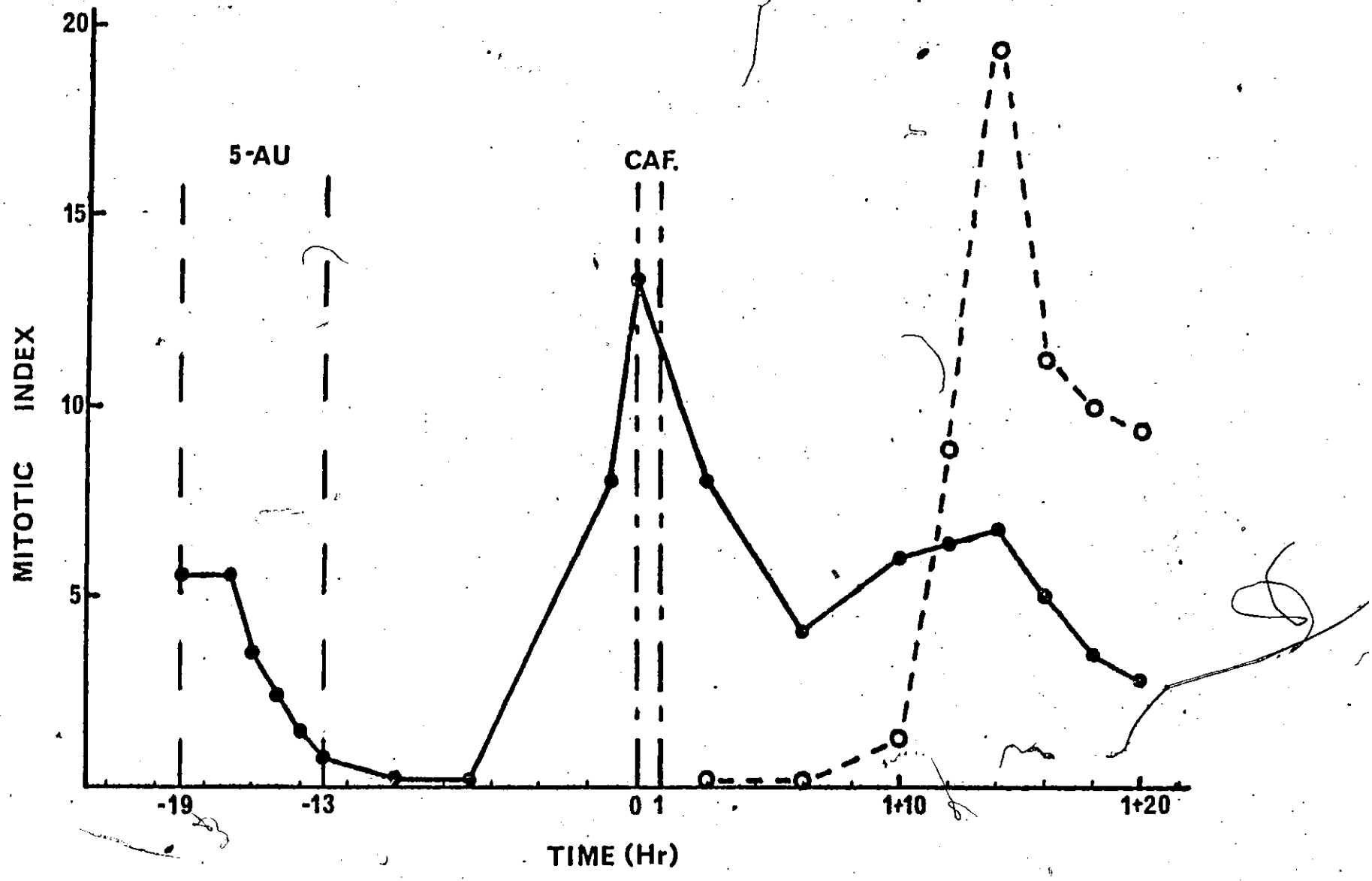
III.3.1 Cell Cycle Kinetics of Binucleate Cells of Pea Roots

Excised roots were cultured (see Methods and Materials) and treated with 5-AU, to increase the degree of mitotic synchrony. When the semi-synchronous wave of cells entered mitosis the roots were treated with 0.1% caffeine for 1 hr (Fig. 4). The roots were then allowed to recover and samples were taken at various times between 2 and 20 hours after the caffeine treatment.

Figure 4

Mitotic Index of Mononucleate and Binucleate Cells After 5-AU and Caffeine Treatments in P. sativum

The mitotic index of mononucleate (●) and binucleate (O) cells at various times after treatment with 5-AU and caffeine (CAF). Each M.I. is based on 3000 cells in the case of mononucleate cells and 100 cells in the case of the binucleate cells. The vertical bars indicate the periods of 5-AU and caffeine treatments; as in Figure 1 all times are given in hours and relate to the 1 hour caffeine treatment.


20
15
10
5
MITOTIC INDEX
5-AU
CAF.
-19
-13
0 1
1+10
1+20
TIME (Hr)

Binucleate cells were first seen in mitosis at 1 + 10 hr and reached their peak M.I., 19.3 at 1 + 14 hr (Table 7, Fig. 4). Mean cycle duration for binucleate cells, therefore, is approximately 14 hr. Microspectrophotometric determinations of DNA content showed that all binucleate cells were still in G1 at 1 + 6 hr (Fig. 5A and B). However, some had begun S at 1 + 10 hr (Fig. 5C) and some binucleate cells were even in mitosis at 1 + 10 hr (Table 7), indicating that a small proportion of the binucleate cells completed S and G2 in about 4 hr; i.e. they were in G1 at 1 + 6 hr but were in mitosis at 1 + 10 hr. The majority of binucleate cells were somewhat slower, i.e. at least 50% of them were still in S at 1 + 10 and 1 + 12 hours (Fig. 5C and 5D) and were in G2 at 1 + 14 hr (Fig. 5E). Between 1 + 14 and 1 + 20 hr about 50% of the binucleate cells underwent mitosis (Table 7, Fig. 4). This evidence indicates that there is variation in the cycle duration of binucleate cells.

The cycle kinetics of the binucleate cells that complete their cell cycle by 12-14 hr, i.e. the most rapidly cycling binucleate cells, are particularly interesting. Their G1 lasts at least 6 hr (Fig. 5B) and S + G2 lasts only 6-8 hr (Fig. 6 and 5B). In mononucleate cells of pea roots, grown under culture conditions similar to those used here, mean duration of G1 was 4.8 hr, mean duration of S + G2 was 11.8 hr and mean cycle duration was 18.7 hr (Scadeng and MacLeod, 1976). Thus, relative to the values for untreated cells (Scadeng and MacLeod, 1976), the most rapidly cycling binucleate cells show: I) a reduction in the duration of S + G2 and of a cell cycle and II) an

Figure 5

Changes in DNA Content Over a Binucleate Cell Cycle

Frequency histograms of the DNA content, in arbitrary units (a.u.), of the nuclei from binucleate cells induced by a 1 hour treatment with 0.1% caffeine; (A) 1 + 2 hr, (B) 1 + 6 hr, (C) 1 + 10 hr, (D) 1 + 12 hr and (E) 1 + 14 hr. The cells were stained by the combined DNFB-Feulgen technique; DNA content was measured by microspectrophotometry.

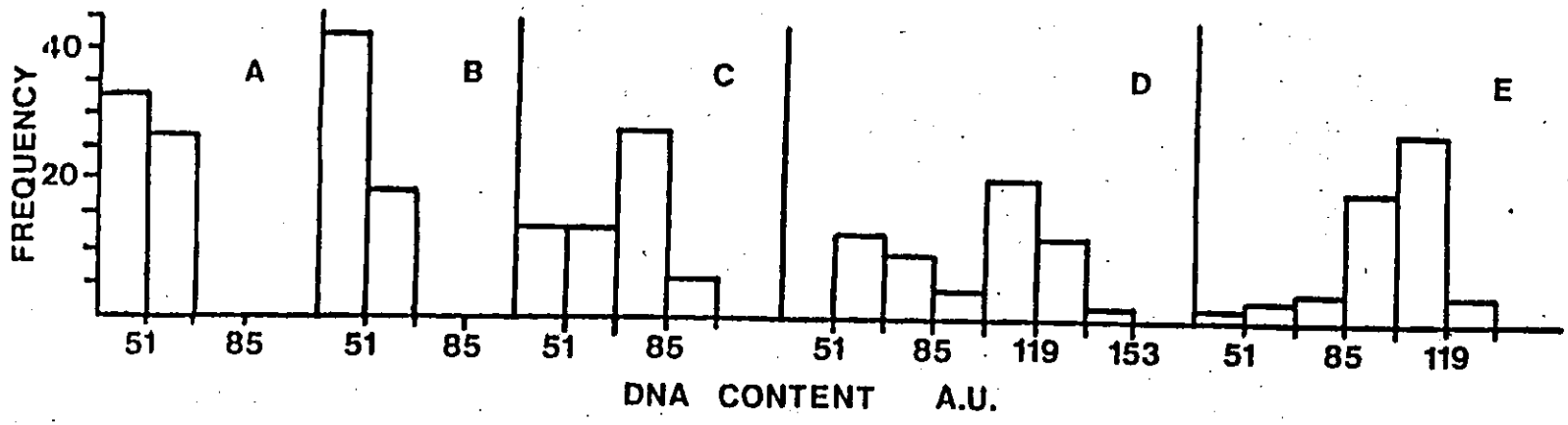
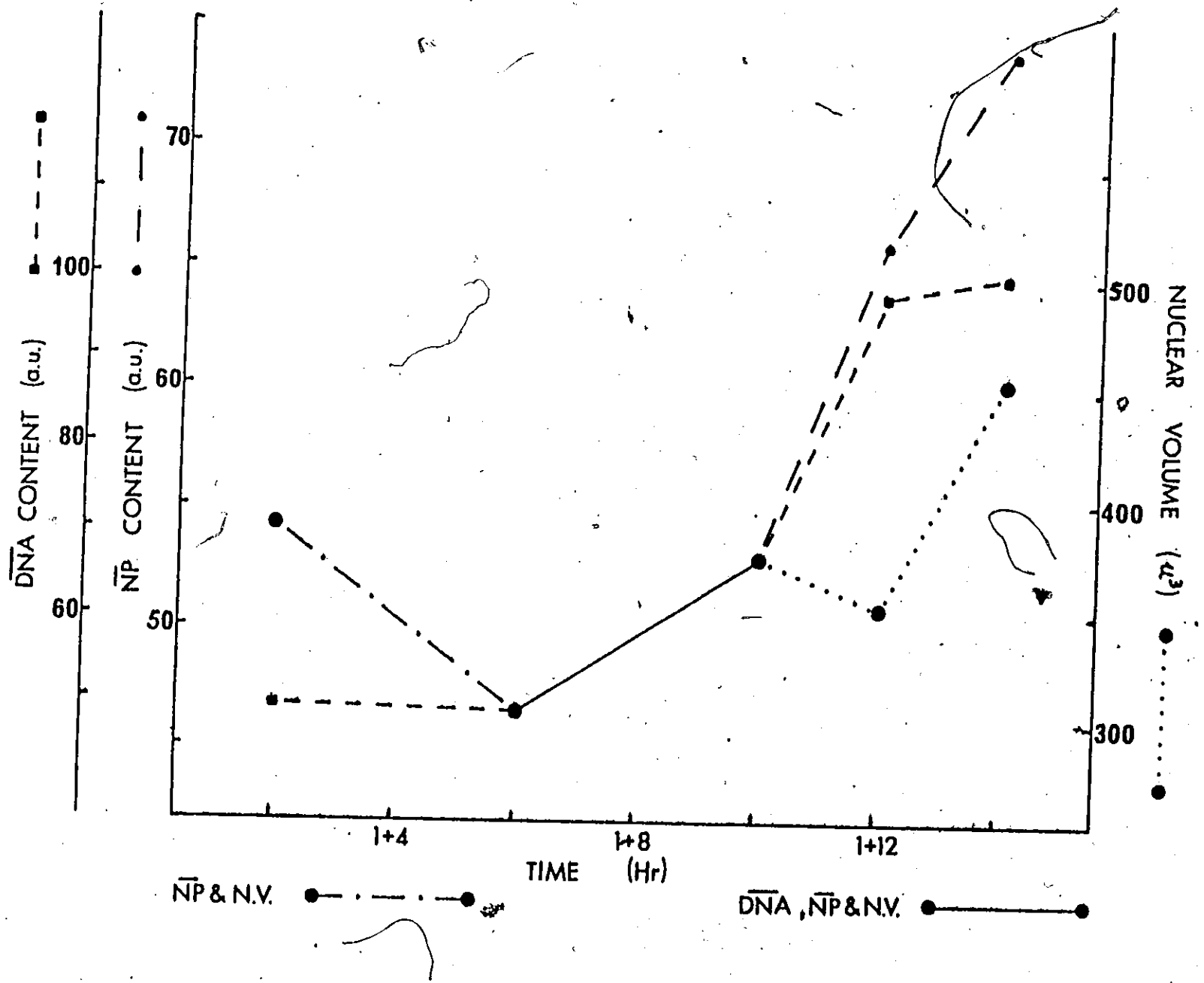

FREQUENCY
40
20
A
B
C
D
E
51
85
51
85
51
85
51
85
119
153
51
85
119
DNA CONTENT A.U.

Figure 6

Comparisons of Various Cell Parameters over a Binucleate Cell Cycle

The mean values of DNA content ($\overline{DNA}$), nuclear protein content ($\overline{NP}$) and nuclear volume ($\overline{NV}$) at various times of recovery are given for binucleate cells induced during a 1 hour, 0.1% caffeine treatment. The values have been plotted on the same relative scales thus facilitating comparisons between the various parameters.


DNA CONTENT (a.u.)
NP CONTENT (a.u.)
NUCLEAR VOLUME (μ³)
100
80
60
70
60
50
500
400
300
1+4
1+8
1+12
TIME (Hr)
NP & N.V.
DNA , NP & N.V.

increase in duration of G1. Further evidence that the duration of G2 in binucleate cells has been reduced is this: from the decrease in M.I. in the first 6 hr of treatment with 5-AU the mean duration of $G_2 + \frac{M}{2}$ can be calculated (Socher and Davidson, 1971) and was estimated to be 3.2 hr. Thus, G2 is shorter, in binucleate cells, than in the mother cells from which they were derived.

III.3.2 Nuclear Volume of Binucleate Cells

It has been shown that mean nuclear volume decreases during interphase in caffeine induced binucleate cells of V. faba (Table 1). A similar decrease in nuclear growth was seen with P. sativum roots grown in culture. Mean nuclear volume in binucleate cells fell from 387 ± 142 μm^3 at 1 ± 2 hr to 304 ± 108 μm^3 at 1 + 6 hr (Table 7). This period in which the decrease in nuclear volume occurred, coincides with the extended G1 period (cf. Table 7 and Figure 6). However, unlike the situation in V. faba, this reduction in nuclear volume was temporary. By 1 + 10 hr the trend of nuclear contraction appeared to be reversed (Table 7), and significantly, this coincided with the onset of DNA synthesis (Fig. 5C). From 1 + 10 to 1 + 20 hr nuclei of binucleate cells continued to grow and increases in their volume were seen particularly in the nuclei that formed the upper 50% of the distribution (Table 7). To determine if the reduction in nuclear volume was due to a loss of macromolecules, the protein and RNA contents of the nuclei of binucleate cells were measured.

Table 7

Changes in Nuclear Volume of Binucleate Cells in *P. sativum* During Recovery from Caffeine

Mean ± S.D., minimum, maximum and values from percentage cumulative frequency distributions of nuclear volume (μm^3). Each value is based on 150 binucleate cells. The frequency of binucleate cells, based on 3,000 cells, and their mitotic index (M.I.) are also given. The times given are hours after a 1 hour treatment with 0.1% caffeine.

	Nuclear Volume (μm^3)								Binucleate Cells	
Time	Mean ± S.D.	Min.	10	25	50	75	90	Max.	Frequ.	M.I.
1 + 2	387 ± 142	133	225	274	364	463	582	837	0.47	0
1 + 6	304 ± 108	83	186	226	297	361	431	780	0.63	0
1 + 10	374 ± 124	130	225	288	364	431	542	837	0.37	1.3
1 + 12	352 ± 114	95	225	274	336	427	510	723	0.23	8.8
1 + 14	453 ± 180	130	263	322	428	532	679	1189	0.20	19.3
1 + 16	562 ± 335	106	231	304	470	732	1039	2433	0.10	11.1
1 + 18	442 ± 200	101	226	304	413	511	713	1672	0.47	9.7
1 + 20	544 ± 268	134	274	369	497	659	850	2074	0.50	9.3
Control	274 ± 122	89	133	189	245	339	431	842	-	-

III.3.3 Nuclear Protein Content

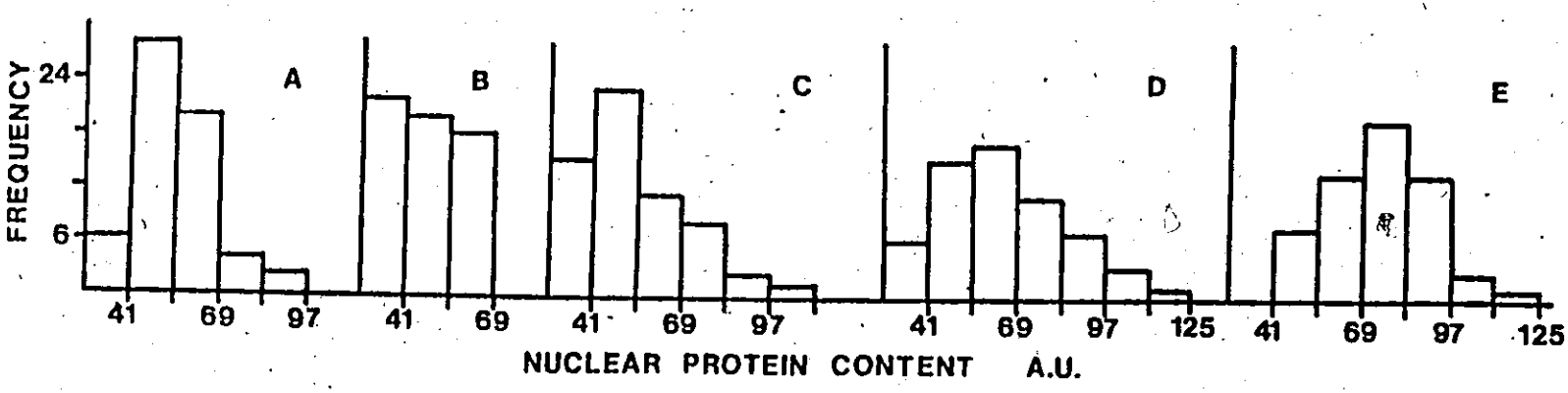
Nuclear volume is determined by the amounts of various low molecular weight molecules, by macromolecules, e.g. DNA, RNA and protein, as well as by the degree of hydration. Changes in nuclear volume must result from changes in one or more of these components. First we consider changes in protein content of nuclei; is there any correlation between the protein content and the volume of a nucleus? Histograms for nuclear protein content have been made for various points within the binucleate cell cycle (Fig. 7). The analysis of DNA contents showed the duration of G1 was extended while S + G2 was shortened and the combined effect of these changes was a reduction in total cell cycle time by some 4 hr.

During the extended G1 period there is a significant drop in nuclear protein content and this drop parallels the change in nuclear volume exactly (Figs. 6 and 7, c.f. 1 + 2 and 1 + 6 hr). Between 1 + 6 and 1 + 10 hr, when DNA contents are first seen to increase, mean nuclear protein and mean nuclear volume show a parallel increase. Mean nuclear protein content continues to increase with time while mean nuclear volume shows some fluctuation between 1 + 10 and 1 + 14 hr (for explanation see III 3.2. Nuclear Volume of Binucleate Cells). However, there was a strong correlation between the protein content and the volume of a nucleus; i.e. changes in mean protein content of nuclei paralled changes in their volume (Fig. 7). The correlation coefficient between these two parameters was $r = 0.79$ for the 5 determinations made between 1 + 2 and 1 + 14 hr. The correlation between

Figure 7

Changes in Nuclear Protein Content Over a Binucleate Cell Cycle

Frequency histograms of the nuclear protein content, in arbitrary units (a.u.), of the same cells whose DNA contents are given in Fig. 5. The times for A-E are also the same as those in Fig. 5. The cells were stained by the combined DNFB-Feulgen technique; protein content was measured by microspectrophotometry.


FREQUENCY
24
6
A
B
C
D
E
41
69
97
41
69
41
69
97
41
69
97
125
41
69
97
125
NUCLEAR PROTEIN CONTENT A.U.

nuclear volume and protein content is even stronger, r = 0.99, in the period 1 + 2 to 1 + 10 hr. The decrease in the r value towards the end of interphase reflects the increase in the range of protein contents as more and more nuclei enter S and G2 (Fig. 5D and E, c.f. Fig. 7D and E). These results are similar to those found in P. sativum where nuclear dry mass and nuclear volume had a correlation coefficient of 0.73 (Lyndon, 1967) and in V. faba where the correlation coefficient between nuclear protein content and nuclear volume was 0.74 (Bansal and Davidson, 1978a). Changes in protein content, therefore, do contribute to changes in nuclear volume.

III.3.4 Nuclear RNA Content

Total nucleic acid content of nuclei was measured in: 1) caffeine induced binucleate cells 2 hours after the end of the caffeine treatment, 2) mononucleate cells of untreated roots and 3) pairs of sister cells, known to be in G1, from untreated roots. Since at 1 + 2 hr the binucleate cells are still in G1 (see III.3.1 above) the pairs of mononucleate cells selected for analysis were also in G1. Thus a comparison could be made between pairs of sister nuclei that either shared a common cytoplasm or were in separate cells.

The method of selecting G1 cells has been described (Method and Materials). The preparations were stained with Gallocyanin-chrome alum; this is specific for nucleic acids, but it stains both DNA and RNA. Therefore other roots were pretreated with RNase to remove RNA and the RNA content of the nuclei was estimated by subtracting the

Table 8

Nuclear RNA Content

Mean values for total nucleic acid content (± S.D.) and DNA content (± S.D.), taken from RNase treated material for caffeine induced binucleate cells[a], sister cells and controls are given. Estimates of nuclear RNA content are derived from these values. Total nucleic acid content and DNA content were measured by micro-spectrophotometry on Gallocyanin-chrome alum stained material; 50 binucleate cells, 25 pairs of sister cells and 100 control cells were scored.

	Control Cells	Binucleate Cells	Sister Cells
Total Nucleic Acid Content	78.7 ± 24.2	74.8 ± 18.9	66.0 ± 11.3
DNA Content (RNase treated)	70.1 ± 19.1	57.7 ± 10.0	(57.7)[b]
RNA Content	8.6	17.1	8.3
RNA/DNA Ratio	0.12	0.30	0.14

a Binucleate cells were scored 2 hours after the caffeine treatment, i.e. at 1 + 2 hr, at this time binucleate cells are known to be in G1

b Since binucleate cells at 1 + 2 hr are in G1 the DNA value found for these cells was used in calculating the RNA value for sister cells, which were a selected sample consisting of G1 cells (see Materials and Methods)

value for the RNase treated material from the value of total nucleic acid.

The mean nucleic acid content per nucleus for the binucleate cells was 74.8 ± 18.9 a.u. while the means for control cells and the pairs of sister cells were 78.7 ± 24.2 a.u. and 66.0 ± 11.3 a.u., respectively (Table 8). When the binucleate cells were compared to the pairs of sister cells, mean nucleic acid content per nucleus was found to be significantly greater in binucleate cells than in the sister cells ($p = 0.05$, Table 8). Since both populations of cells were in G1, their DNA content per nucleus would be identical, these results indicate that the nuclei of the binucleate cells contained more RNA than nuclei from untreated cells.

The above observations were confirmed by the values obtained from the RNase treated material. Once the RNA had been removed the mean nucleic acid content per nucleus for binucleate cells was only 57.7 ± 10.0 a.u. while for the control mononucleate cells it was 70.1 ± 19.1 (Table 8). Since RNase removes RNA, the values obtained are an estimate of the DNA content per nucleus, and since the binucleate cells were in G1 it is not surprising that their DNA content was lower than that from control mononucleate cells which were made up of cells in G1, S and G2. This means that binucleate cell nuclei contain, on average, 17.1 a.u. of RNA per nucleus while control mononucleate cells and pairs of sister cells contained only 8.6 a.u. and 8.3 a.u. of RNA per nucleus, respectively (Table 8). This increase in the mean nuclear

RNA content in binucleate cells may be related to the increase in mean nuclear volume of the binucleate cells (c.f. Tables 7 and 8); this possibility is examined in the next section.

III.3.5 Relationship of Nuclear Volume to Nuclear Protein and Nuclear RNA Content

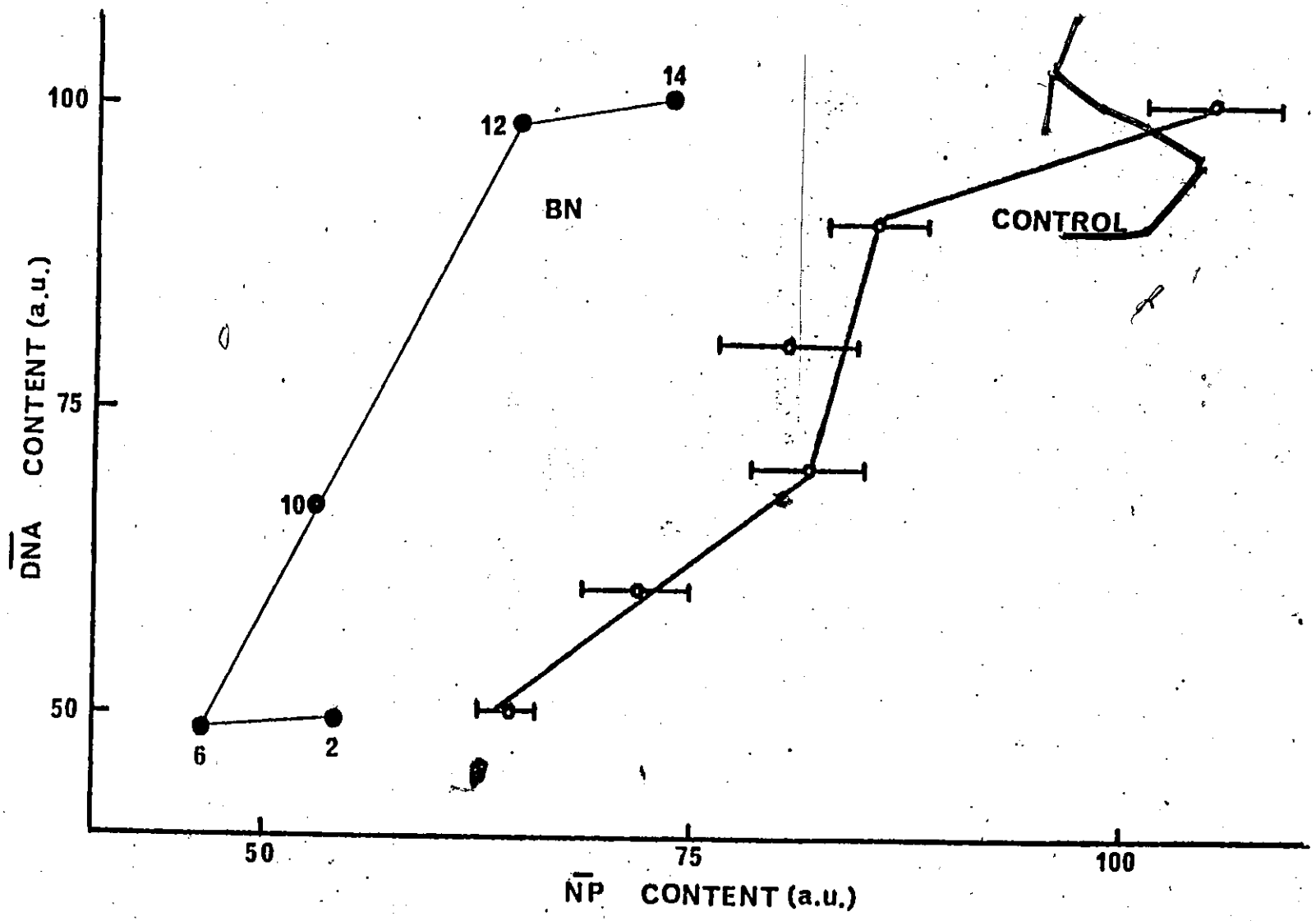
We have shown that for the most rapidly cycling binucleate cells nuclear protein and nuclear volume are highly correlated ($r = 0.788$, Fig. 6). However, it was also found that mean nuclear volume of binucleate cells at 1 + 2 hr was significantly greater ($p = 0.05$) than it was for control cells in G1 (c.f. 387 μm^3 and 189 μm^3, Table 9) yet mean nuclear protein content for these two populations of cells was not significantly different ($p = 0.05$, c.f. 54.2 a.u. and 56.6 a.u., Table 9). This situation was true for all times examined, in fact, nuclear protein content of binucleate cells tended to be lower than controls while nuclear volume was always greater in binucleate cells (c.f. Table 7 and Fig. 8). These results show that volume and protein content of nuclei can vary independently of one another and they suggest that protein content is not the most critical component governing nuclear volume.

RNA content and volume of nuclei; on the other hand, showed a strong correlation. There was a 2.06-fold increase in mean nuclear RNA content in binucleate cells over control cells in G1, mean nuclear volume increased by 2.05-fold in the binucleate cells (Table 9). Further, the correlation coefficient between the larger or small

Figure 8

Changes in Nuclear Protein Content in Nuclei With Increasing DNA Content in Binucleate and Control Cells

The protein and DNA content of a nucleus were determined, by microspectrophotometry, after staining by the combined DNFB-Feulgen technique. Binucleate cells, induced by a 1 hour 0.1% caffeine treatment, were scored 2, 6, 10, 12 and 14 hours after treatment; 30 binucleate cells were scored at each time. Control nuclei were divided into 6 classes on the basis of their DNA content and the mean nuclear protein content was calculated for each class; 100 control cells were scored.


100
75
50
$\overline{DNA}$ CONTENT (a.u.)
14
12
BN
10
6
2
CONTROL
50
75
100
$\overline{NP}$ CONTENT (a.u.)

nucleus and their corresponding nuclear RNA content, for binucleate and control cells, was 0.888. These results indicate that nuclear RNA content is a more important factor in determining the nuclear volume of a cell than is the nuclear protein content.

Of the fraction of RNA which is important in determining nuclear volume rRNA appears to be a likely candidate. While no direct measurement of the rRNA content of these nuclei was made, total nucleolar volume per nucleus was measured. Total nucleolar volume compared to nuclear volume in the same manner as nuclear RNA content, gave an even higher correlation coefficient, r = 0.998 (Table 9). The implication of these results is that the metabolic activity of the nucleolus, which must be controlled by the needs of the cell, sets the limits on nuclear volume. That is, a more active nucleolus would be producing a larger quantity of rRNA thus giving a larger nucleolus, which in turn results in a larger nucleus. However, how this process is initiated or controlled is still not known.

III.4 Differential Behaviour of Sister Nuclei in P. sativum

The two nuclei of a binucleate cell are the products of one mitosis and occupy a single cytoplasmic environment. In spite of this the sister nuclei of a binucleate cell differ in size in all but a small proportion of cells (Davidson and Pertens, 1978; Davidson and Pertens, 1981a; Wellwood and Davidson, 1977). We have already shown that this was the case for caffeine induced binucleate cells in V. faba

Table 9

Comparison Between Sister Nuclei of Binucleate Cells and Nuclei of Sister Cells

A comparison between sister nuclei of caffeine induced binucleate cells in G1 (i.e. at 1 + 2 hr) and nuclei of sister cells from untreated material. Mean values for nuclear protein content (NP), nuclear RNA content (NRNA), nuclear volume (NV) & nucleolar volume (nuc V) are given. The values for nuclear volume and nucleolar volume for G1 cells is based on 50 pairs of sister cells, the nucleolar volume for the binucleate cells is based on 50 cells.

Parameter	Binucleate Cells (G1)			Sister Cells (G1)		
	Mean ± S.D.	Large ± S.D.	Small ± S.D.	Mean ± S.D.	Large ± S.D.	Small ± S.D.
NV (μm^3)[a]	387 ± 142	420 ± 146	354 ± 131	189 ± 67	206 ± 67	172 ± 63
nucV (μm^3)	38.9 ± 15.8	43.3 ± 17.2	34.6 ± 13.2	17.3 ± 8.0	19.9 ± 8.6	14.7 ± 6.5
NP (a.u.)[b]	54.2 ± 11.6	57.6 ± 12.0	50.8 ± 10.2	56.6 ± 10.1	58.8 ± 10.1	54.4 ± 10.2
NRNA (a.u.)[c]	17.1	21.7	12.5	8.3	11.5	5.0

[a] Nuclear volume data for binucleate cells are from Tables 7 and 10

[b] Nuclear protein data are from Tables 11 and 12

[c] Nuclear RNA data are from Tables 8 and 13

(Table 4). In this section it will be shown that sister nuclei of caffeine induced binucleate cells have different volumes (Table 10) and that the differences between sister nuclei are maintained as nuclei change in volume (Table 10). These differences in volume between sister nuclei led to an examination of the extent of differences in the macromolecular content of sister nuclei; microspectrophotometric determinations revealed that the sister nuclei of binucleate cells differed both in protein content (Table 11) and RNA content (Table 13).

III.4.1 Nuclear Volume and Nuclear Protein Content of Sister Nuclei in Binucleate Cells

Sister nuclei in binucleate cells induced by caffeine in *P. sativum*, as in *V. faba*, had different volumes. Mean volumes of larger and smaller nuclei in the pairs of sister nuclei in binucleate cells have been compared at each of the 8 times studied (Table 10): each pair of means is significantly different ($p = 0.05$). This establishes that the differences are real and that they are maintained as binucleate cells increase in age from 1 + 2 to 1 + 20 hr (Table 10). Regression analysis of mean nuclear volume of sister nuclei, smaller versus larger, for the 8 observations between 1 + 2 and 1 + 20 hr showed a linear relationship (Fig. 9). The correlation coefficient between pairs of mean values is high, $r = 0.97$; this shows that pairs of sister nuclei show the same relative change in volume over an 18 hr period. Confirmation of this relationship was obtained by plotting mean difference in nuclear volume against mean volume; this yielded a

Table 10

Differences in Volume of Sister Nuclei of Binucleate Cells

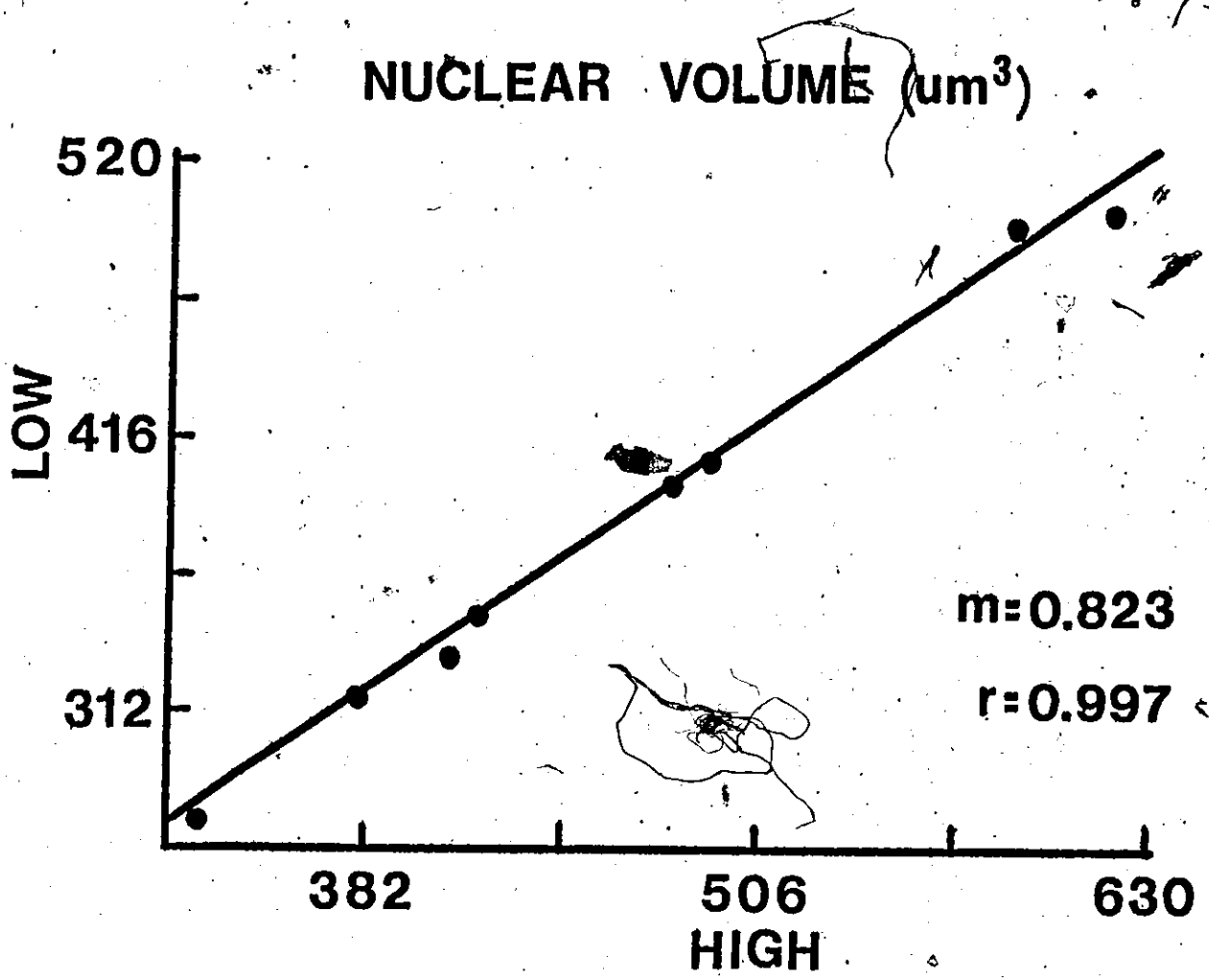
Mean volume (μm^3) of the larger and smaller nucleus for the binucleate cells given in Table 7. The mean difference and the ratio of volumes (Larger/Smaller) between sister nuclei are also given.

Time	Nuclear Volume (μm^3)		Differences	
	Large ± S.D.	Small ± S.D.	Mean ± S.D. (μm^3)	Ratio (L/S)
1 + 2	420 ± 146	354 ± 131	65 ± 64	1.19:1
1 + 6	332 ± 115	277 ± 94	55 ± 50	1.20:1
1 + 10	411 ± 131	338 ± 105	73 ± 69	1.22:1
1 + 12	382 ± 120	323 ± 99	59 ± 56	1.18:1
1 + 14	492 ± 186	413 ± 163	79 ± 80	1.19:1
1 + 16	619 ± 373	505 ± 283	114 ± 148	1.23:1
1 + 18	481 ± 215	403 ± 176	78 ± 81	1.19:1
1 + 20	588 ± 291	500 ± 235	88 ± 97	1.18:1

Figure 9

Comparison of Nuclear Volume of Sister Nuclei of Binucleate Cells

A plot of nuclear volume, low versus high, is given for sister nuclei of binucleate cells. The values plotted here are the values given in Table 10. A linear regression of these values was generated by computer analysis; the correlation coefficient (r) and slope (m) are also given.


NUCLEAR VOLUME (um3)
520
416
312
LOW
m=0.823
r=0.997
382
506
630
HIGH

correlation coefficient of r = 0.930. Protein content of sister nuclei were then compared and a slightly different relationship emerged.

The protein contents of sister nuclei were compared using the t-test and the Mann-Whitney test. Both showed that protein contents were significantly different (p = 0.05) at 1 + 2 and 1 + 6 hr. At all other times the difference between sister nuclei was not statistically significant. Nevertheless, sister nuclei differed in mean protein content in all fixations (Table 11). Also, the absolute increase in protein content was almost identical in both the higher and lower content nuclei, i.e. 19.4 a.u. and 19.7 a.u. (Table 11). Thus, an absolute difference of 6.8 a.u. between sister nuclei is established by 1 + 2 hr, but between 1 $\pm$ 2 and 1 + 14 hr, the increase in protein content is almost identical in both nuclei. This was confirmed by regression analysis of mean nuclear protein content, lower versus higher; the slope was 0.955 and the correlation coefficient was 0.998. Thus the absolute changes in nuclear protein content were virtually identical in both nuclei over the period from 1 $\pm$ 2 to 1 + 14 hr.

This means that the nucleus containing the lower amount of protein at 1 $\pm$ 2 hr takes up more protein, on a relative basis, than the nucleus with the higher protein content at 1 + 2 hr. The result of this was, that for the 5 determinations between 1 + 2 and 1 + 14 hr, the correlation between mean nuclear protein content and mean differences in nuclear protein content was not very strong; i.e. r = 0.544. Change in protein content of sister nuclei, therefore, does not clearly parallel change in nuclear volume. For the 5 determinations of nuclear

Table 11

Differences in Protein Content of Sister Nuclei of Binucleate Cells

The mean protein content of the sister nuclei of binucleate cells are given; each value, based on 30 pairs of nuclei, is given in arbitrary units. The mean difference and the ratio (High/Low) of protein content between sister nuclei are also given. The protein content was measured by microspectrophotometry. The increase in mean protein content between 1 + 2 and 1 + 14 hr is given as the increase over a 12 hr period.

	Nuclear Protein (a.u.)		Differences	
Time	High ± S.D.	Low ± S.D.	Mean ± S.D. (a.u.)	Ratio (H/L)
1 + 2	57.6 ± 12.0	50.8 ± 10.2	6.8 ± 7.5	1.14:1
1 + 6	49.2 ± 11.2	43.9 ± 10.5	5.4 ± 4.1	1.13:1
1 + 10	55.3 ± 17.4	50.5 ± 14.5	4.8 ± 7.5	1.09:1
1 + 12	68.0 ± 20.4	61.8 ± 18.1	6.2 ± 5.0	1.10:1
1 + 14	77.0 ± 16.6	70.5 ± 16.8	6.5 ± 5.4	1.10:1
12 hr increase	19.4	19.7	-	-

volume for which there are corresponding protein values, i.e. between 1 ± 2 hr and 1 + 14 hr, the relative changes in the large and small nucleus were equal and mean nuclear volume and mean differences in nuclear volume showed a high correlation, r = 0.900. Furthermore the correlation between mean differences in nuclear volume and the mean differences in nuclear protein content was only 0.067, i.e. cf Tables 10 and 11. This r value is lower than the r value, 0.788, between mean nuclear volume and mean protein content. The difference between these two r values suggests that the difference in volume between sister nuclei is not due entirely to differences in their protein contents: other factors must also contribute.

III.4.2 Nuclear Protein Content of Nuclei in Sister Cells

The behaviour of sister nuclei can also be studied by determining nuclear parameters from pairs of sister cells. Sister mononucleate cells were selected and the DNA and protein content of each nucleus were measured microspectrophotometrically. On the basis of their DNA content, pairs of nuclei in G1, S and G2 were selected and their protein contents were determined. In 9 pairs of cells, out of a total sample of 50 pairs, the two nuclei were out of synchrony, e.g. one was in G2 and one in S (Table 12). Protein contents of the sister nuclei of two mononucleate cells were not identical in most cases and the mean difference ranged from 4.4 a.u. in G1/G1 pairs to 9.8 a.u. in G2/G2 pairs (Table 12). A linear regression plot of the lower against the higher protein content of sister cell nuclei (Fig. 10A) yielded a

Table 12

Differences in Protein Content of Nuclei in Sister Cells

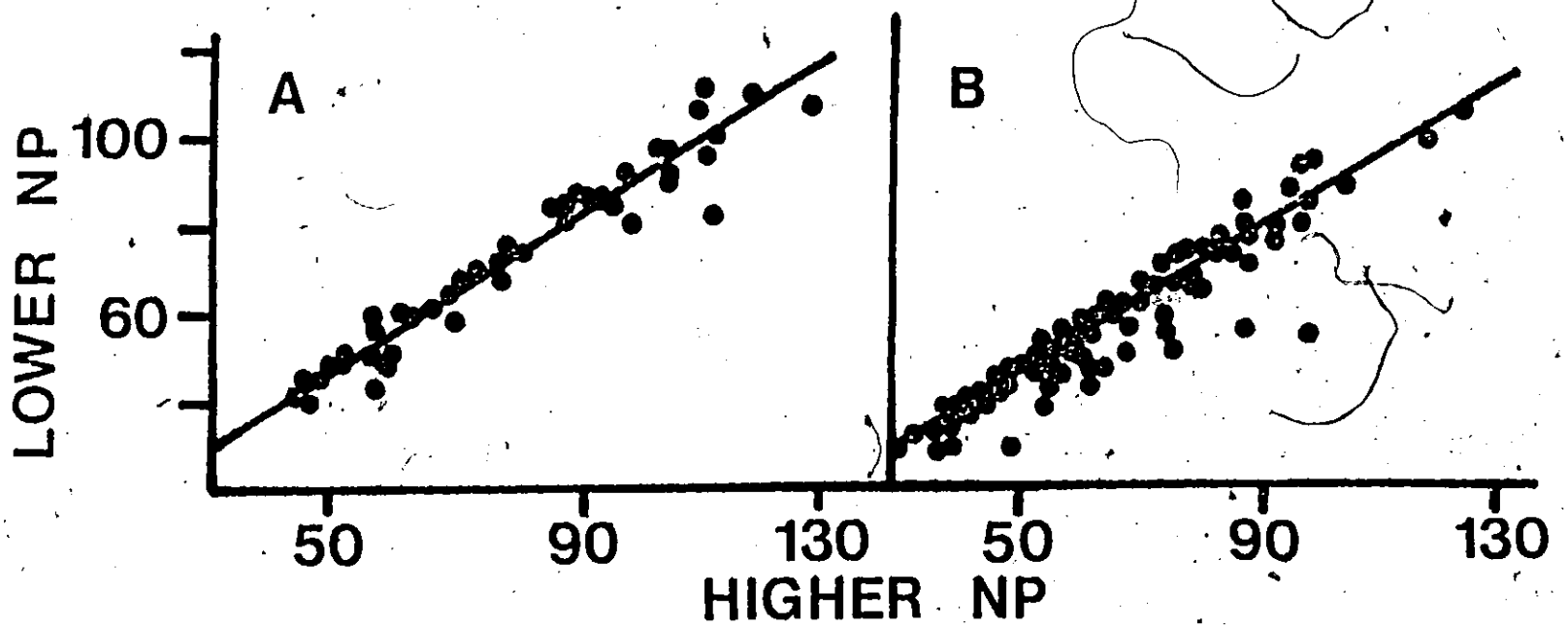
The mean protein content of the sister nuclei and the ratio of protein content (High/Low) between nuclei of sister cells. N is the number of sister pairs scored. The sample of 50 pairs of sister cells is grouped according to DNA content of the sister nuclei. The DNA and protein contents were measured by microspectrophotometry; values are given in arbitrary units (a.u.). *In ODD pairs sister nuclei were in different phases of interphase.

		Nuclear Protein (a.u.)		
Pairs	N	High ± S.D.	Low ± S.D.	Ratio (H/L)
G1 - G1	22	58.8 ± 10.1	54.4 ± 10.2	1.09:1
S - S	14	90.7 ± 12.4	84.2 ± 10.2	1.08:1
G2 - G2	5	108.4 ± 6.2	98.6 ± 11.8	1.11:1
ODD	9	92.4 ± 20.4	85.0 ± 16.2	1.08:1
Mean of all pairs	50	78.7 ± 22.3	72.6 ± 20.3	1.08:1

Figure 10

Nuclear Protein Content of Pairs of Sister Nuclei

These plots are linear regressions for nuclear protein contents (NP) of pairs of sister cells. The value from the nucleus with the lower protein content is plotted against the value from the sister with the higher protein content. (A) Pairs of sister nuclei from sister mononucleate cells of untreated roots. (B) Pairs of sister nuclei from binucleate cells induced by a 1 hr treatment with 0.1% caffeine. The correlation coefficient (r) and slope (m) are also given.


A
B
LOWER NP
100
60
50
90
130
50
90
130
HIGHER NP

slope value of 0.876. The correlation coefficient for protein contents of these pairs of sister nuclei was r =-0.965. A plot of protein contents from sister nuclei of binucleate cells yielded similar values. The slope of the linear regression (Fig. 10B) gave a value of 0.870 and r = 0.946. The regression coefficients of these two plots were not significantly different (p = 0.05). The two nuclei produced by one mitosis behave, overall, in a similar fashion whether they are in one cell or in two. This suggests some degree of autonomous regulation, by nuclei, of their growth and behaviour.

III.4.3 Nuclear RNA Content of Sister Nuclei in Binucleate Cells

At 1 + 2 hr microspectrophotometric determination of Feulgen stained material has shown that the DNA content of sister nuclei in binucleate cells is identical and at the 2C level (see Section III.3, Fig. 5A). Since the DNA contents of the sister nuclei are identical, any differences in their total nucleic acid content, as measured microspectrophotometrically on Gallocyanin-chrome alum stained material, must reflect a difference in their RNA content.

Sister nuclei in binucleate cells did show a difference in total nucleic acid content (Table 13). While the mean nucleic acid content per nucleus was 74.8 $\pm$ 18.9 a.u. for binucleate cells, the sister nuclei with the higher nucleic acid content had a mean of 79.4 $\pm$ 19.3 a.u. and the sister nuclei with the lower nucleic acid content had a mean of 70.2 $\pm$ 17.5 a.u.; these values were significantly different (p = 0.05). The mean ratio, high/low nucleic acid contents was 1.14:1, since the

Table 13

Differences in the Nuclear RNA Content of Sister Nuclei

Mean values for total nucleic acid and the estimated nuclear RNA content are given for the sister nuclei of the binucleate and the sister cells given in Table 8. The ratios of macromolecular content (High/Low) are also given. All values are in arbitrary units (a.u.)

	Nuclei of Binucleate Cells			Nuclei of Sister Cells		
	High ± S.D.	Low ± S.D.	Ratio (H/L)	High ± S.D.	Low ± S.D.	Ratio (H/L)
Total Nucleic Acid Content	79.4 ± 19.3	70.2 ± 17.5	1.14:1	69.2 ± 11.6	62.7 ± 10.1	1.11:1
DNA Content[a]	57.7	57.7	-	57.7	57.7	-
RNA Content	21.7	12.5	1.74:1	11.5	5.0	2.30:1

[a] DNA content was estimated from binucleate cells treated with RNase; these cells were known to be in G1.

DNA content of the two nuclei is identical, this difference between the two must be due to different RNA contents. This was confirmed by examination of the RNase treated material (Table 13), which gave an estimated DNA content for the two sister nuclei of 59.0 ± 10.2 and 56.4 ± 9.9 a.u.; these values were not significantly different ($p = 0.05$), therefore, the mean DNA content for G1 cells was estimated to be 57.7 ± 10.0 a.u.

From the values of total nucleic acid content and of DNA, from RNase treated material, the estimated RNA values were 21.7 a.u. for the nucleus containing the greater amount of RNA and 12.5 a.u. for the nucleus containing the lesser amount; this gave a ratio of 1.74:1 (Table 13). While this ratio between the sister nuclei appears quite large and is greater than their nuclear volume ratio, 1.19:1 (see Section III.4.1, Table 10) it must be remembered that the RNA content of a nucleus makes up only a small fraction of the total macromolecular content of a nucleus, i.e. the RNA/DNA ratio is 0.12 in controls and 0.30 in nuclei of binucleate cells. Thus the direct contribution to the actual physical size of the nucleus by the RNA is small; however, the differences in RNA content are, in addition to the protein content differences, further evidence that molecular contents of sister nuclei are not identical and that they contribute to the volume differences. The RNA and protein content differences may also reflect functional differences between sister nuclei.

III.4.4 Nuclear RNA Content of Nuclei in Sister Cells

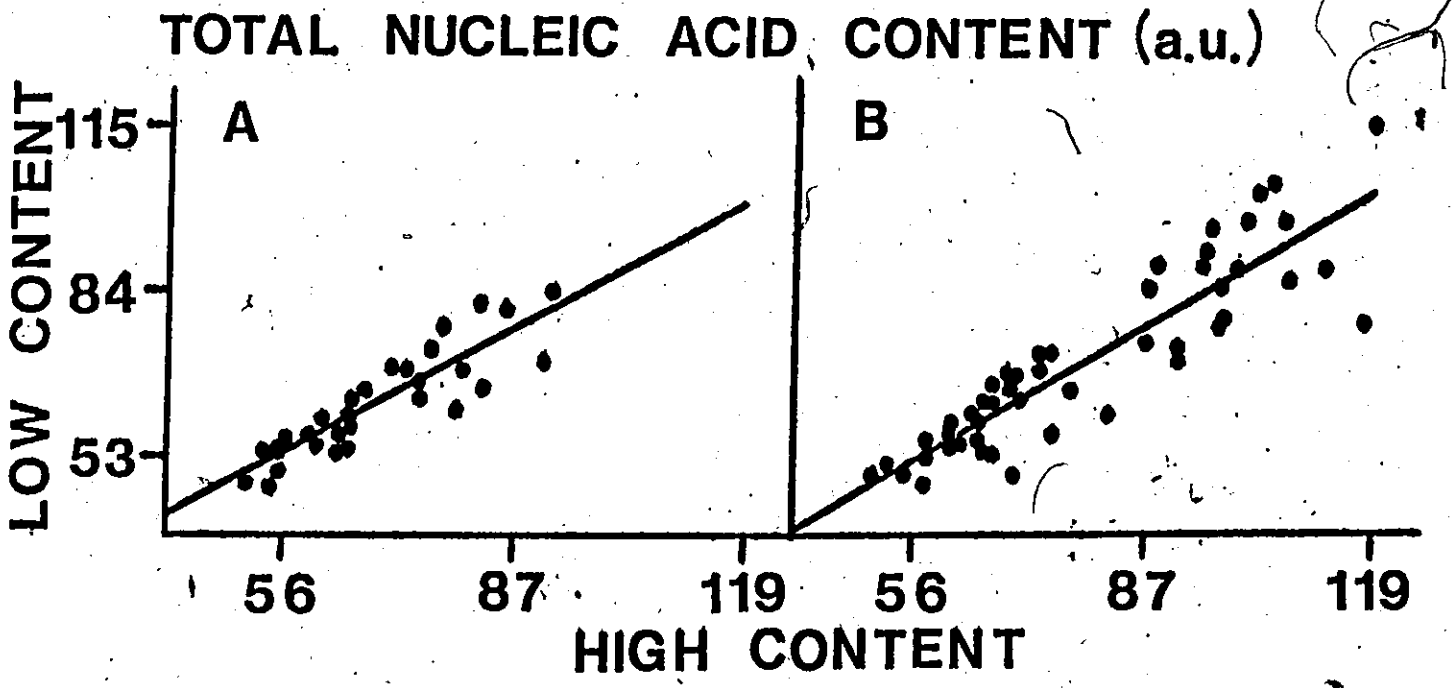
As with nuclear protein content, the RNA content of sister nuclei can be usefully compared in sister cells as well as in binucleate cells. From control preparations, stained with Gallocyanin-chrome alum, sister cells known to be in G1 were chosen and their nucleic acid contents were determined by microspectrophotometry. The mean nucleic acid content of the sister cells was 66.0 ± 11.3 a.u. From the estimated mean G1 DNA value of 57.7 ± 10.0 a.u. for Gallocyanin stained material, the mean RNA content for these cells was estimated to be 8.3 a.u. (Table 8). Comparison of the values from pairs of sister nuclei revealed that total nucleic acid content per nucleus was rarely the same in both nuclei. Since all these nuclei were in G1 their DNA content will be identical. The difference in total nucleic acid content is evidence of differences in RNA content between nuclei of sister cells.

The mean ratio of nucleic acid content, Higher to Lower (H/L), was 1.11:1 for the nuclei of sister cells and 1.14:1 for the sister nuclei of binucleate cells (Table 13). Since these ratios proved to be significantly different ($p = 0.05$), regression analyses of these two populations of cells were carried out. Plots of these regressions are shown in Fig. 11A and 11B; the two plots appeared to be similar, suggesting that the two populations of cells had similar distributions of RNA, higher to lower RNA content, for the pairs of sister nuclei.

Figure 11

Total Nucleic Acid Contents of Sister Nuclei

Linear regression plots for the total nucleic acid contents are given for the sister nuclei of sister cells (A) and of binucleate cells (B). Since all cells were in G1 the DNA content of both nuclei, in pairs of sister cells and in binucleate cells was identical, any differences indicated by the regression analysis must be due to differences in the RNA content of the nuclei. Total nucleic acid content was measured by microspectrophotometry of Gallocyanin stained material; 50 binucleate cells at 1 + 2 hr and 30 pairs of sister cells from triplet analysis of control material were scored. The correlation coefficient (r) and slope (m) are also given.


TOTAL NUCLEIC ACID CONTENT (a.u.)
A
B
LOW CONTENT
115
84
53
56
87
119
56
87
119
HIGH CONTENT
A: m=0.768
r=0.887
B: m=0.824
r=0.907

However, statistical analysis of the two regressions showed them to be different ($p = 0.05$). Since the regression coefficient for the binucleate cells was greater than that of the sister cells, 0.824 cf. 0.768, the RNA contents of the sister nuclei of binucleate cells tended to be closer to each other than the RNA contents of nuclei in sister cells. This was confirmed by the estimates of the RNA content of the nuclei; this is shown below.

Mean RNA contents of the sister nuclei in binucleate cells were estimated to be 21.7 a.u. in the nucleus with the higher RNA content and 12.5 a.u. in the nucleus with the lower RNA content (Table 13). For nuclei of sister cells, mean RNA contents were estimated to be 11.5 a.u. for the nucleus with the higher RNA content and 5.0 a.u. for the nucleus with the lower RNA content (Table 13). Thus, even though in absolute terms the amount of RNA per nucleus was less in the sister cells than in the binucleate cells, the ratio of nuclear RNA content, H/L, was 2.30:1 for sister cells as compared to 1.74:1 for binucleate cells. Therefore, on a relative basis, sister nuclei of binucleate cells were more alike in RNA content than nuclei of sister cells. Nevertheless, it is obvious that there are differences between sister nuclei and these differences persist whether the sister nuclei share a common cytoplasm or are in their own separate cytoplasm.

III.5 Cytoplasmic Gradients

The mechanism underlying the differential behaviour of sister nuclei may involve cytoplasmic gradients. Cytoplasmic gradients in

mitotic cells have long been regarded as a possible mechanism for producing differences between daughter cells (Jaffe, 1979; Sinnott and Bloch, 1939; Woodruff and Telfer, 1980; Zeiger, 1971). Since the nucleus sheds its RNA and non-histone proteins during mitosis and these macromolecules migrate back into the daughter nuclei, near or at the completion of karyokinesis, (Goldstein, 1976; Phillips, 1972; Rao and Prescott, 1970), it is possible that cytoplasmic gradients involving an unequal distribution of these macromolecules could result in the differential behaviour of sister nuclei. This section, therefore, deals with the distribution of protein and RNA within the cytoplasm during mitosis.

Microspectrophotometric determinations were carried out on metaphase cells; using a constant measuring area, the amount of protein or RNA was determined at the 2 poles of these metaphase cells. Protein content was determined from control cells stained with DNFB and RNA was determined from control cells stained with Gallocyanin chrome alum.

III.5.1 Cytoplasmic Distributions of Protein and RNA

Mean protein content of cytoplasm differed at the two poles of the cell. The mean cytoplasmic protein content for the pole of the cell with the higher amount of protein was 23.1 $\pm$ 4.5 a.u. and for the opposite pole, the side with the lower amount of protein, the mean cytoplasmic protein content was 21.3 $\pm$ 4.5 a.u.; these values were significantly different ($p = 0.05$, Table 14). The mean ratio, H/L, of protein was 1.09:1 (Table 14) and analysis of the distributions of protein content at the two poles indicated that they came from dif-

Table 14

Protein and RNA Content in Metaphase Cells

Mean values for cytoplasmic protein and RNA at the spindle poles of metaphase cells are given. Protein and RNA content were measured microspectrophotometrically on DNFB and Gallocyanin-chrome alum stained material, respectively. A fixed plug size was used for all determinations; 100 cells for each macromolecule were scored. The ratio of macromolecular content (High/Low) is also given. All values are in arbitrary units (a.u.)

	Macromolecular Content (a.u.)		
	High ± S.D.	Low ± S.D.	Ratio (H/L)
Protein Content	23.1 ± 4.5	21.3 ± 4.5	1.09:1
RNA Content	20.2 ± 6.8	17.9 ± 6.6	1.15:1

ferent populations ($p = 0.05$). Thus the amount of protein found at opposite ends of a dividing cell, i.e. at the spindle poles of a metaphase cell, are different.

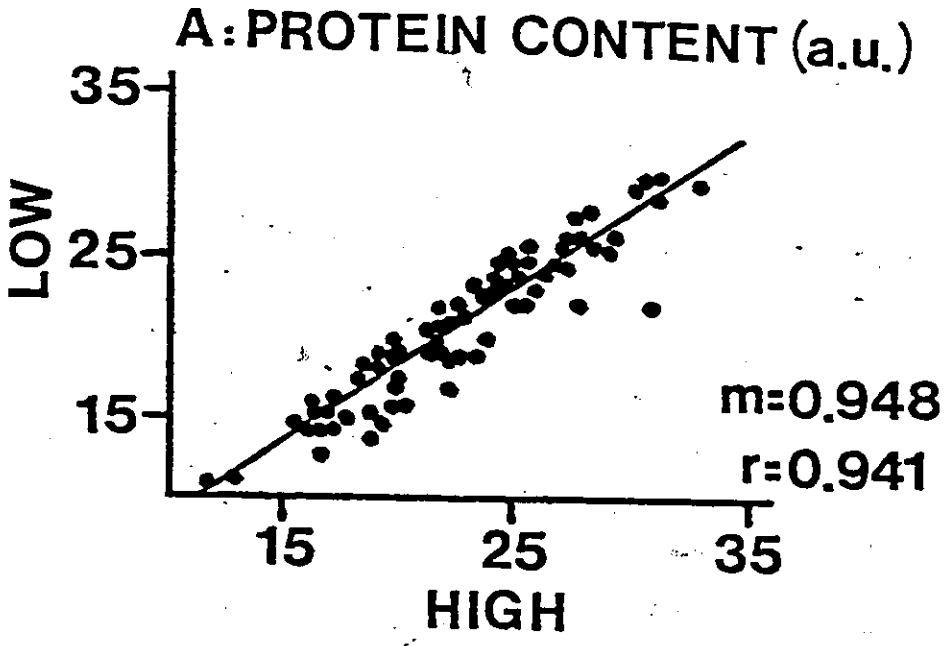
RNA showed a similar distribution pattern. There was a markedly asymmetrical distribution of RNA between the two poles of the cells. Analysis of the distribution of RNA content at the two poles of the cells, high content compared to low content, indicated that the RNA contents at the poles came from populations having different distributions ($p = 0.05$). Mean RNA content at the pole with the higher RNA content was 20.2 ± 6.8 a.u. and at the pole with the lower RNA content the mean value was 17.9 ± 6.6 a.u. (Table 14); these values were significantly different ($p = 0.05$). Thus, RNA within the cytoplasm of metaphase cells is asymmetrically distributed with a mean ratio, H/L, of 1.15:1 (Table 14).

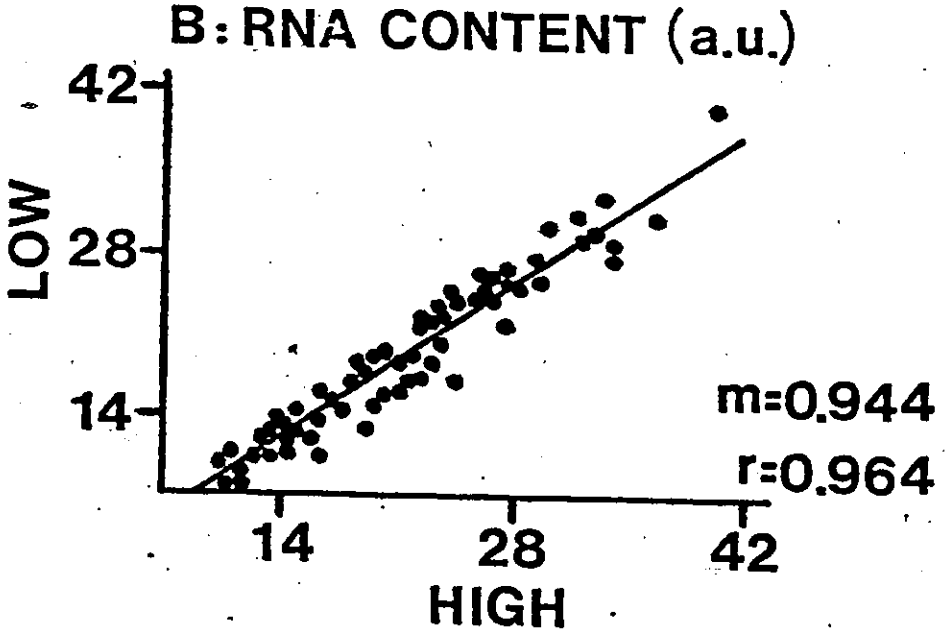
Since both RNA and protein showed an asymmetric distribution, an analysis of regression was carried out on the distribution of these two macromolecules; Figure 12A and 12B shows the linear regression for protein and RNA respectively. The slopes for both the regression curves were identical ($p = 0.05$), this suggests that the relative distributions of these two macromolecules, over the population of metaphase cells, were the same. As stated above, if key regulatory molecules were distributed in this fashion, it could lead to the differential behaviour observed in sister nuclei.

Figure 12

Cytoplasmic Protein and RNA Content in Metaphase Cells

Computer generated linear regressions are given for the plots of the differences in protein (A) and in RNA (B) content on either side of the metaphase plate. Protein and RNA were measured by microspectrophotometry of DNFB and Gallocyanin-chrome alum stained material, respectively. A fixed plug size was used for all determinations; 100 cells were scored for each macromolecule. The correlation coefficient (r) and the slope (m) are also given.


A: PROTEIN CONTENT (a.u.)
35
25
15
LOW
15
25
35
HIGH
m=0.948
r=0.941


B: RNA CONTENT (a.u.)
42
28
14
LOW
14
28
42
HIGH
m=0.944
r=0.964

DISCUSSION

IV.1 Induction and Use of Binucleate Cells

The origin of this study lies in an attempt to determine how heterogeneity of cell cycle duration arises in root meristems. Methylxanthines prevent cytokinesis in cells undergoing mitosis; the resulting sub-population of binucleate cells promised to be an almost ideal system for the study of variation in cell cycle duration. This estimate of the potential in the use of a methylxanthine treatment was based on a number of studies of binucleate cells induced in roots of Allium cepa (Giménez-Martín et al., 1965; González-Fernández et al., 1977; Sacristan-Garate et al., 1974). Unfortunately we have not been able to repeat the results reported by the Spanish workers. From the outset (Stallwood and Davidson, 1977; Wellwood and Davidson, 1977) the methylxanthines have proved to be potent inhibitors of cell growth and proliferation; these observations agree with those of many other workers that methylxanthines inhibit RNA and protein synthesis (Putrament et al., 1972; Sakaguçhi and Tanifuji, 1968; Zuk and Swietlinska, 1973), reduce cell proliferation (Timson, 1972; Weinstein et al., 1975) and affect cell differentiation (Ahmad, Russell and Ahmad, 1979; Kreider et al., 1975; Yoshimi and Yasumasu, 1978). But though methylxanthines did not facilitate the analysis of cell cycle heterogeneity in meristems, further study of the binucleate cells they induce revealed inherent differences between sister nuclei; these differences are considered

to be inherent since they occur even though the two sister nuclei share a common cytoplasm. The present study developed, therefore, out of the initial observations of the differential behaviour of the sister nuclei.

The use of binucleate cells to study differences between sister nuclei has several advantages; first the time at which they were formed and thus the time spent in interphase is known. Secondly, differences between sister nuclei can be determined without the complication of having to take into account the different sizes of mononucleate cells. The results from the binucleate cells (Table 15) led to a study of the behaviour of nuclei of sister cells; these results are also summarized in Table 15. The results show, for all of the parameters measured, that sister nuclei are distinctly different, whether they are sister nuclei from a binucleate cell or from sister cells.

The implication of the results from the binucleate cells on the process of cell division is two-fold. First, differences between sister nuclei must arise as a result of mitosis and not of events that occur after cell division, when daughter nuclei no longer share a common cytoplasm. Secondly, these differences in sister nuclei once established are stable, inherent differences exist even when sister nuclei share a common cytoplasm. The importance of these findings for meristematic cells is that mitosis must be viewed as a highly differential event which not only leads to differences in the size of sister cells but also to differences in behaviour between sister cells; i.e. it is not just a means of segregating DNA into two more or less identical cells. In

light of these findings we will discuss the mechanisms which may be involved in generating these differences between sister nuclei during mitosis and the result of these differences on cell cycle duration, nuclear size and rates of growth in both sister nuclei and sister cells.

IV.2 Asymmetrical Mitoses: The Role of Cytoplasmic Gradients

The differences found between sister nuclei must arise as a result of events in mitosis, since they are exhibited both in binucleate and sister cells. Given the quantitative and qualitative equality of DNA separation at mitosis, the differences in the behaviour of sister nuclei can not be due to genetic differences. The differential behaviour of sister nuclei in the initial part of G1 confirms that mitosis results in a differential distribution of some key molecules to the groups of chromatids present at the two poles of a spindle at the end of telophase.

Such a differential distribution could be achieved if these molecules were carried, as structural components, by the chromatids themselves or, alternatively, by a gradient around the spindle. In metaphase cells we have shown that protein and RNA are unequally distributed on opposite sides of the metaphase plate (Table 14). Thus, at the end of karyokinesis, as the nuclei reform, the two sister nuclei occupy different cytoplasmic environments. If regulatory molecules were present in the cytoplasm and were distributed along the lines of the gradients exhibited by protein and RNA, then as the sister nuclei reformed the number of regulatory molecules taken in by the sister nuclei could be different.

Table 15

Summary of the Ratios Between Sister Nuclei in Binucleate Cells and in Sister Mononucleate Cells

The ratios of values from sister nuclei, high to low or large to small, are given in this table. The values are a summary of the data reported in Table 1-13.

		Binucleate Cells		Sister Cells	
Parameter	Species	Age[a] (hr)	Ratio	Phase	Ratio
Nuclear Volume	V. faba	1 + 1	1.27:1	-	-
	P. sativum	1 + 2	1.19:1	G1	1.20:1
Nucleolar Volume	P. sativum	1 + 2	1.25:1	G1	1.35:1
Nuclear Protein Content	P. sativum	1 + 2	1.14:1	G1	1.09:1
Nuclear RNA Content	P. sativum	1 + 2	1.74:1	G1	2.30:1
Preformed ^{3}H-RNA per nucleus	V. faba	1	1.71:1	-	-
		1 + 1	1.55:1	-	-
		1 + 3	1.31:1	-	-
Amount of ^{3}H-RNA per nucleus	V. faba	1	1.80:1	G1,S,G2	1.52:1
		1 + 1	2.14:1	-	-
		1 + 3	1.57:1	-	-
Net Rate of RNA Synthesis	V. faba	b	1.44:1	-	-

[a] All binucleate cells at the times given are known to be in G1

[b] The rates of RNA synthesis were calculated over a 3 hr period, i.e. from 1 to 1 + 3 hr.

It is known that the nucleus sheds its RNA and non-histone proteins during mitosis and that these macromolecules migrate back into the daughter nuclei near or at the completion of division (Goldstein, 1976; Phillips, 1972; Rao and Prescott, 1970), therefore, it was felt unlikely that regulatory molecules were carried along as structural components of the chromatids (Goldstein, 1976). There is evidence, however, which suggests cytoplasmic gradients may be involved. The mitoses which give rise to root hair cells and guard mother cells are asymmetric divisions (Sinnott and Bloch, 1939; Zetger, 1971); furthermore, in both of these mitoses the cytoplasmic organelles are polarized along a gradient so that the end of the cell destined to give rise to the more highly differentiated cell i.e. the root hair cell or the guard mother cell, receives the higher density of organelles. Not only do cells have the ability to differentially distribute organelles within their cytoplasm, they also possess the capability to distribute macromolecules within the cytoplasm (Capco and Jeffery, 1981; Woodruff and Telfer, 1980). Exogenous vegetal pole poly $(A)^+$ RNA, when injected into Xenopus laevis zygotes, accumulated along a concentration gradient from the vegetal hemisphere to the animal hemisphere (Capco and Jeffery, 1981). Similarly, in the oocyte-nurse cell syncytium of Hyalophora cecropia the distribution of proteins, of known electrical charge, is affected by an endogenously generated gradient of electrical potential (Woodruff and Telfer, 1980); in this system the exogenous proteins carrying a negative charge accumulated within the oocyte while those proteins with positive charges accumulated within the nurse cells. These findings support the theory that gradients of macromolecules can

be used as mechanisms for the distribution of regulatory molecules to the spindle poles on either side of the metaphase plate and thus ultimately to the sister nuclei.

Two examples of molecules, postulated to have regulatory roles in the reformation of nuclei at the end of mitosis, which are shed from the nucleus during mitosis, are the lamins of the nuclear envelope (Gerace and Blobel, 1980; Gerace, Blum and Blobel, 1978) and the NuMa protein (Lydersen and Pettijohn, 1980). During interphase both of these protein classes are found within the nucleus; the lamins are the major polypeptides making up the lamina structure of the nuclear envelope (Gerace and Blobel, 1980; Gerace, Blum and Blobel, 1978) while NuMa is found scattered throughout the nucleus in distinct clumps (Lydersen and Pettijohn, 1980). During mitosis the lamins are found distributed throughout the cytoplasm (Gerace and Blobel, 1980; Gerace, Blum and Blobel, 1978) while NuMa is localized at the spindle poles (Lydersen and Pettijohn, 1980); during late anaphase-telophase, however, these proteins return to the reforming nucleus. These proteins, together with other matrix proteins and nuclear envelope proteins, are thought to play an important role in the regulation of nuclear reformation at the end of telophase. Whether or not they are responsible for controlling the differential uptake of macromolecules, by sister nuclei during early G1, is not known. Nevertheless, if these regulatory molecules were distributed unequally between the two sister nuclei it could lead to the kind of differences in behaviour observed between sister nuclei in this study.

IV.3 Cell Cycle Differences Between Sister Cells

The asymmetric distribution of macromolecules, i.e. protein and RNA, within the cytoplasm of mitotic cells (Table 14) also has implications for the duration of the cell cycle in the daughter cells. Because of the asymmetric distribution of macromolecules the resulting daughter cells, which we know can differ in size as a result of the position of the crosswall (Davidson, Pertens and Eastman, 1978; Davidson and Pertens, 1981a; Ivanov, 1971), differ in the concentration of macromolecules per unit cytoplasm. It has been shown in a number of plant species that, 1) the concentration of RNA per unit of cytoplasm of a tissue is negatively correlated with the mean duration of the cell cycle for the cells in that tissue (Hallet, 1972, 1978; Michaux, 1971; Michaux-Ferrière, 1981; Nougarède and Rembur, 1976, 1978), 2) the rate of RNA synthesis as calculated per unit cytoplasm is also negatively correlated with the duration of a cell cycle (Hallet, 1972, 1978; Michaux, 1971; Michaux-Ferrière, 1981). Extrapolating these results to the situation found between sister cells, the variation seen in cell cycle duration of sister cells (Davidson and Pertens, 1981a; Ivanov, 1971; López-Saéz, Giménez-Martín and González-Fernández, 1966; Webster, 1979a) would correlate with differences in the concentration of RNA within the cytoplasm of the sister cells. Our results on the distribution of RNA during mitosis indicate that there is a difference in the concentration of RNA on either side of the metaphase plate (Table 14). These results parallel the results from studies on animal cells grown in culture which showed that mitosis results in an unequal distribution of RNA to

daughter cells (Darzynkiewicz et al., 1982; Killander and Zetterberg, 1965a, 1965b) and which led to the conclusion that the ability of the cell to go through a cell cycle is negatively correlated to its RNA content (Castor, 1980; Cleffman, Reuter and Seyfert, 1979; Darzynkiewicz et al., 1979, 1982; Johnston and Singer, 1978; Zetterberg and Killander, 1965a, 1965b). Since 80% of a cell's RNA content is ribosomal RNA it would appear that the ability to traverse a cell cycle may lie in the metabolic capability, or more precisely, the ability to synthesize protein within the cell.

IV.4 Differences in Size and Growth of Sister Nuclei

The asymmetric distribution of RNA between daughter cells not only correlates with differences in cell cycle duration in sister cells but also effects the size of sister nuclei. Nuclear size, in part, is a reflection of the amount of material available to the nucleus in the cytoplasmic environment in which it forms at the end of mitosis and it would be expected that the larger nucleus of the pair of sisters will form in the part of the cytoplasm with the higher density of macromolecules. However, whether the uptake of material into the nucleus is due to regulatory molecules or is merely a passive response to the material present in the cytoplasm is not known.. Regardless of the mechanism involved a differential uptake of material does occur, resulting in different protein and RNA contents in sister nuclei of sister cells (Table 15); and sister nuclei, whether they are in binucleate or sister cells, are rarely identical in size.

The case of the binucleate cells is particularly interesting. While caffeine blocks cytokinesis, the difference in size of the sister nuclei of the binucleate cells indicates that it does not destroy the gradient that existed in the mother cell at the time of mitosis. Furthermore, as the binucleate cells proceed through a cell cycle the differences in nuclear size (Tables 4 and 10; Davidson and Pertens, 1978, 1981a; Wellwood and Davidson, 1977) and protein content (Table 11) are maintained. This data from the binucleate cells suggests two things; first that the regulation of nuclear growth, once the initial differential uptake of material at the end of mitosis has occurred, resides in the nucleus and not the cytoplasm. Secondly the molecules involved in the regulation of nuclear growth are not exchanged between the two nuclei of a binucleate cell.

This second point is in contrast to the situation found in other binucleate systems. In binucleate cells, constructed by nuclear transplantation, exchanges of specific classes of proteins and RNAs between the 2 nuclei have been reported (Goldstein, 1976; Goldstein and Ko, 1981; Legname and Goldstein, 1972; Prescott and Goldstein, 1968; Wise and Goldstein, 1973). From these results from binucleate cells as well as the observation that these proteins and RNAs are shed from the nucleus during mitosis only to reassociate with the reforming daughter nuclei at the end of mitosis (Goldstein, 1976), it has been postulated that these shuttling proteins and small nuclear RNAs play a key role in gene expression by acting as regulatory molecules (Goldstein, 1976). Furthermore, in binucleate HeLa cells, formed by

Table 16

Correlation Coefficients Between Nucleus and Nucleolus or Nucleus and RNA Content

The correlation coefficients showing the relationship between the size of the nucleus and the size of the nucleolus or the RNA content of the nucleus are cited from the literature and this study.

Type of Measurement[a]	Source	Correlation Coefficients: Nucleus vs Nucleolus	Correlation Coefficients: Nucleus vs RNA Content
Volume	Armstrong (present study)	0.992	0.866
	Bennett (1970)	-	0.919
	Dosier & Riopel (1978)[b]	0.941	-
	Hallet (1978)	0.998	-
	Lepoint & Goessens (1982)	0.833	-
	Maher & Fox (1973)	0.847	-
	Sacristan-Garate et al. (1974)	0.990-0.997	-
	White & Kaltsikes (1978)	0.624	-
Diameter	Kohlenbach & Meusser 1971)[b]	0.932	-
Mass	Ayonoadu & Rees (1971)	0.952	0.967
	Bennett & Rees (1969)	0.918	-

[a] Refers to how the nucleus and nucleolus were measured, i.e. either by volume, diameter or mass measurements; all RNA measurements were by microscopectrophotometry

[b] These values were estimated from graphed results

fusion of 2 mononucleate cells, there is an interaction between the 2 nuclei (Rao and Johnson, 1970); when cells in different phases of the cell cycle were fused together the two nuclei became synchronized and enter mitosis together. Therefore, it would seem that components responsible for the differential behaviour of sister nuclei in our system, i.e. the differential growth (Table 10), the differences in protein and RNA contents (Table 11 and 13) and the differential rate of RNA synthesis (Table 5), must belong to a different class of molecules than the shuttling proteins and small nuclear RNAs or the substances responsible for the synchronization of nuclei in binucleate cells of HeLa.

Since, as we have stated above, the differences in cell cycle duration of sister cells are related to the metabolic capabilities of the cells, it appears that the differences in the sister nuclei may also be related to their metabolic abilities. Nucleolar volume, for example, can be used as an estimate of rRNA production (Maher and Fox, 1973); this was confirmed by Hallet (1978), who showed that after labelling with ^{3}H-UR the amount of labelled RNA found in the cytoplasm of a cell was highly correlated with its nucleolar volume. Since a larger nucleolus implies greater activity of rDNA, producing higher quantities of rRNA, and since nuclear and nucleolar size are highly correlated (Table 16), a larger nucleus must represent a more metabolically active nucleus than a smaller nucleus. Our results from binucleate cells, induced in *V. faba*, confirm that sister nuclei, even when occupying the same cytoplasm, differ in their ability to synthesize

RNA (Table 15). Thus, nuclear volume is not merely a reflection of the amount of material present within the nucleus but is also a reflection of its metabolic state.

IV.5 Consequences of Asymmetrical Mitoses

It has been suggested that the asymmetry of division, whether it produces cells of different sizes or of different macromolecular contents, is merely a "random error in division" (Killander and Zetterberg, 1965a). It has also been suggested that the volume differences observed between sister nuclei is also a random event of mitosis (Webster, 1979b). We feel that this is not the case. Given the overall structure of the root and the precise patterns of cell division and growth that occur to give rise to this structure, it seems to us, that any asymmetry arising between components within this system must be carefully regulated and integrated into the overall pattern. This means that the cytoplasmic gradients, the differences in nuclear growth rates and cell cycle durations in sister cells must be regulated in such a way that the overall growth and development of the root is unimpaired. In this section we will be examining how the differences in sister cells are compensated for within the root; that is, how these differences are made part of overall root growth and development.

It must be kept in mind that the meristems we are looking at are mature, steady state systems in which: 1) meristematic cell number is kept constant; 2) cells appear, on average, to double in size over a cell cycle; 3) mean cell cycle duration and mean cell size remain

constant and 4) the range in values for both cell cycle duration and cell size also remain constant. While the exact value at which these parameters are set may vary from root to root and which are most certainly affected by the environmental conditions under which the roots are grown (Thomas, 1980; Thomas and Davidson, 1981, 1982), it is obvious that within any meristem these four points hold true. This means that differences between sister cells, which are generated at every division, must be compensated for by some subtle balance between rates of cell growth and cell cycle rates or else the differences between the largest and smallest cells in the population would become greater and greater.

Compensating mechanisms, that correct for inequalities of cell division, have been studied in a few species. For animal cells, grown in culture, the differences between sister cells are compensated for during G1 of the cell cycle (Darzynkiewicz et al., 1982; Killander and Zetterberg, 1965a); measurements of RNA, protein and mass show the highest coefficients of variation immediately after mitosis, i.e. early in G1, and the lowest c.v's occur in S phase cell. From this work it has been postulated that differences in sister cells, with respect to RNA and protein, are compensated for during the phase or compartment of equalization ($G1_A$) of G1 which is then followed by a prereplicative compartment ($G1_B$); the heterogeneity in cell cycle duration is a reflection of the time spent in $G1_A$ (Darzynkiewicz et al., 1982).

In the conical mutant of Tetrahymena thermophilia, cells divide asymmetrically to produce a larger anterior cell and a small

posterior cell. Furthermore, more than one cell cycle is required to compensate for differences between the pairs of sister cells (Schäfer and Cleffmann, 1982). In this case it was found that the difference in the size of sister cells was eliminated exclusively by changes in cell cycle duration; it was not eliminated by differences in growth rates. Also, not only did G1 vary in duration but S and G2 were also found to be variable (Schäfer and Cleffmann, 1982). Therefore, for both of these examples the result is that in the cell cycle that follows an asymmetrical mitosis, the larger cell undergoes less than a doubling in size while the smaller cell more than doubles in size.

In plant cells there is an analogous situation. However, while the growth rate of individual cells can differ in single-celled organisms (Schäfer and Cleffmann, 1982), the sister cells of plant meristems are forced to grow at the same rate (Green, 1976; Webster, 1980). The rate of cell growth, or more precisely the rate of cell wall expansion must be the same in sister cells since within the meristem all the cells are interlocked into the overall structure and furthermore, the rate of cell wall expansion is governed by the meristem as a whole not by the individual cells (Green, 1976; Webster, 1980). Thus, in plants, the only method of compensating for differences in cell size is an alteration in cell cycle duration in sister cells. The result of this is that it is the larger cell of a sister pair which has the shorter cell cycle (Davidson and Pertens, 1981a; Ivanov, 1971; Webster, 1979a); and in general, the larger nucleus of a sister pair is located in the larger of the two sister cells (Davidson, Pertens and

Eastman, 1978; Ivanov, 1979). Thus the visible asymmetry of cell division is directly parallelled by the asymmetrical distribution of macromolecules (Table 14) in such a way that it is the end of the mother cell destined to become the larger daughter cell of a sister pair that possesses the cytoplasm with the higher density of macromolecules. This process insures that in the subsequent cell cycle the larger cell is provided with macromolecules that enable it to complete a cell cycle faster than its smaller sister. Furthermore, the size of the larger cell when it begins G1 is more than half the size of the mother cell: thus it does not need to double in size before reaching the size of the mother cell at mitosis. This means that, on average, the larger cell of a sister pair does not double in size, but it grows until it reaches the mean size for mitotic cells and then divides. Conversely, the smaller cell of a sister pair must grow more than twice its original size if it is to approach the mean mitotic size for the population-failure to do so before it divided would result in a progressive drop in mean mitotic size with time. However, mean mitotic size in mature roots remains constant, thus this compensatory mechanism insures that in the root meristem cells divide at a size which tends towards a constant mean value regardless of their size at birth. On this basis, the asymmetry of cell division and the resulting difference in nuclear size and nuclear macromolecular content, i.e. RNA and protein, are functionally related to the difference in cell cycle duration of sister cells.

CONCLUSIONS

1. Caffeine treatment of meristematic cells results in a population of cells which have altered cell cycle kinetics. In V. faba 85% of the binucleate cells induced by caffeine were still in G1 14 hours after the end of treatment; these cells have been changed from fast cycling cells to either slow cycling or non-cycling cells. In P. sativum the binucleate cells had an extended G1 period, however, there was a reduction in the duration of the S + G2 period resulting in an overall decrease in cell cycle duration.

2. Caffeine treatment also results in a decrease in nuclear volume with time. In V. faba the decrease in nuclear volume was continuous over the 14 hour period studied and was paralleled by a loss of ^{3}H-labelled RNA from the nucleus and a reduction in the ability to synthesize RNA. In contrast to the situation in V. faba, the decrease in nuclear volume of binucleate cells in P. sativum was only temporary and coincided with the extended G1 period. Microspectrophotometric determination of nuclear protein content revealed that the changes in nuclear volume were accompanied by changes in nuclear protein content.

3. While caffeine induced binucleate cells cannot be considered to be physiologically normal, the behaviour of sister nuclei, which share a common cytoplasm, showed interesting parallels to nuclei of sister cells, which are housed in their own separate cytoplasm.

4. Mitosis results in sister nuclei which differ in size, protein content and RNA content whether the nuclei are in a binucleate cell or in sister cells.

5. The differences between sister nuclei are established, in part, by a differential distribution of macromolecules within the cytoplasm of the mitotic cells. This results in sister nuclei forming in cytoplasmic environments with different amounts of material which may be taken into the nuclei as they form at the end of mitosis. This was confirmed by a differential uptake of preformed ^{3}H-RNA into sister nuclei in binucleate cells.

6. The differential mitosis results in sister nuclei with different rates of RNA synthesis and differential growth patterns.

7. We propose that, in general, mitosis is an asymmetrical event and that the asymmetry of cell division and the resulting difference in nuclear size and nuclear macromolecular content, i.e. RNA and protein content, are functionally related to the difference in cell cycle duration of sister cells. Furthermore, it is this asymmetry of division which is a major contributor to the striking degree of heterogeneity which is observed in root meristems.

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