GROWTH DURING MICROSPOROGENESIS OF HEXAPLOID TRITICALE

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Richard L. White

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Abstract

During the 100 h period from quartet break-up until first pollen grain mitosis, mean cell, nuclear and nucleolar volumes were calculated at 10 h intervals in hexaploid triticale (Triticosecale Wittmack cv. Rosner). Changes in DNA content per microspore were also measured, to determine the time when DNA synthesis occurs. From 0-30 h (G_1); (1) cell volume increased at a constant rate from 9400 to 48,297 μm^3; (2) nuclear volume initially increased from 1674 to 3261 μm^3 but then returned to 1568 μm^3; and (3) nucleolar volume did not change. From 30-70 h (S) cellular volume increased to 79,090 μm^3, nuclear volume did not change, while nucleolar volume initially increased two-fold but then returned to G_1 value. From 70-100 h (G_2) there was no change in cell volume, while nuclear and nucleolar volumes increased to 4365 and 344 μm^3, respectively. Regression analysis throughout microsporogenesis indicated a constantly changing significant relationship between the volume of a cell and its nucleus and nucleolus. However, no significant regression coefficients were obtained during early prophase. These results are interpreted as indicating that critical cellular, nuclear or nucleolar volumes are not necessary for the development of a cell through interphase.

FOREWORD

This thesis has been prepared in manuscript style. The single manuscript "Growth during microsporogenesis of hexaploid triticale" will be submitted to the Canadian Jounal of Botany.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Cell division and growth are primary events in cellular differentiation and development. Thus an understanding of how cell division and growth are related is essential if man expects to manipulate plant development for human needs. Unfortunately, most models relating cell growth and division are based on studies of single-celled organisms in which development is synchronous and growth can be easily measured (Fantes, 1977 and Johnston, Pringle and Hartwell, 1977). The few higher eukaryotic cell lines that have been studied are cells in tissue culture, not in the the intact organism (Killander and Zetterberg, 1975; Fox and Pardee, 1970 and Fournier and Pardee, 1975). Relationships observed, and models postulated, in these systems may not be related to cell growth at the multi-cellular level.

The major problems presented by multi-cellular organisms are that proliferating tissues do not provide a synchronously dividing population of cells nor precise measurements of growth. However, in plants microsporogenesis, the period from quartet break up until pollen grain mitosis, is a relatively synchronous interphase in which growth can be measured in a large number of cells. Hexaploid triticale cv. Rosner was chosen for this study because (a) a method was available to estimate the stage of microsporogenesis a particular sample was at and (b) Bennet and Smith (1972) observed that almost sixty percent of the cells can be found in division at the same time i.e. development is synchronous.

Microsporogenesis is also of interest as this period especially just before division is the optimum time for the induction of embryoids

in pollen culture. A better understanding of cellular events of this period may be useful in increasing haploid plant production, an important aspect of all modern plant breeding programs. Therefore, in this study the relationship of cellular, nuclear and nucleolar growth during microsporogenesis was investigated.

LITERATURE REVIEW

The cell cycle is defined as the period between the formation of the cell by division of its mother cell and the time when the cell itself divides to form two daughters (Mitchison, 1971). The stages of the cell cycle as they are presently known were first described by Howard and Pelc (1953), who divided the cyclic process of cell division into mitosis(M), G_1, DNA synthesis(S) and G_2. The G_1 and G_2 phases are gaps between the well defined events of mitosis and DNA synthesis. Although the durations of the various stages vary considerably, the overall sequence holds true for almost every system subsequently studied. The exceptions are Chara vulgaris, Amoeba proteus and Schizosaccharomyces pombe which lack a G_1, while the last premeiotic interphase in Triticum aestivum L. var. Chinese Spring is without a G_2 (Bennett, 1973).

The cyclic process raises two questions: (1) What are the signals that determine the entrance of a cell into DNA synthesis or division; and (2) What are the events taking place in G_1 and G_2?

To maintain a specific cell type, a cell is required to double its constituent components. This is necessary if, following division, the two daughter cells are to be equal to each other and to the parental cell at its birth. Many of the investigations aimed at answering the two questions asked above, were therefore directed towards growth.

Growth can be measured as changes in volume, total dry mass or protein. Although the exact pattern of growth may differ depending on whether volume, dry mass or total protein is measured, the commonest pattern is one of continuous increase throughout the cycle.

In those systems which have been studied, cell growth is generally more prominent during the early stages of interphase with reduced amounts of growth prior to division. Nuclear growth, measured as dry mass or as volume, generally shows maximal increases during the later half of the cycle, especially just before division. Increases in nuclear growth also differed from that of whole cells in being mainly a net rather then a gross accumulation of proteins, since protein molecules are probably moving in both directions across the membrane.

Some of the major problems in interpreting growth curves are: (1) lack of precision in measurements;(2) variation from cell to cell so that only average patterns can be measured;(3) artifical treatments, necessary to produce a marked population or to induce synchrony, may have an effect on cellular metabolism, i.e. growth; and (4) lack of any general patterns.

GROWTH IN RELATION TO THE INITIATION OF DNA SYNTHESIS

CELLULAR

Evidence that cell growth controls the initiation of DNA synthesis has come from many systems. Killander and Zetterberg (1965) reported that in cultured mouse L cells the percent mass increase was related to the length of G_1. Thus the smaller the cell at birth, the more it grows and the longer the G_1. The variation in mass among newly formed cells was significantly greater then the variation in mass of cells at the beginning of the S period. This led to the conclusion that the initiation of DNA synthesis was regulated by a critical cell mass.

In Saccharomyces cerevisiae, Johnston, Pringle and Hartwell (1977) reported a coordination of growth and the cell cycle. Using several temperature sensitive mutants, which when shifted to the critical temperature,

arrested cells at various stages of the cell cycle, they demonstrated that growth continued even when the cell cycle was halted. When growth was limited due to nutritional deprivation, cells were able to complete their cycle but became arrested in G_1 of the next cycle. These results showed that growth was rate limiting to continued cell division. The cells arrested in G_1, were abnormally small, and when they were transferred to a complete nutritional medium again they did not bud (a G_1 event) until they grew to a critical volume. After this point was reached the rate of growth and progression through the cell cycle were both constant in a given medium. The observation that DNA synthesis is initiated when the cell reaches a critical volume would therefore appear to be the consequence of the cell having reached a critical size early in G_1.

In Schizosaccharomyces pombe, Nurse (1975) and Fantes (1977) found that a temperature sensitive mutant resulting from a single genetic lesion changed the volume of cells undergoing division. When grown at 25°C, the daughter cells immediately entered DNA synthesis after division, because they are greater then the critical volume required to enter DNA synthesis. At 35°C, the cells divided at a volume smaller than the controls; this necessitated a period of growth before these cells began DNA synthesis. These results are interpreted in the same manner as those for Saccharomyces cerevisiae.

Frazier (1973) in microsurgical experiments on Stentor showed that DNA synthesis ceased when the organism was grown on a deficient medium. However, by either grafting on extra cytoplasm from another starved Stentor, or by removing part of the polyploid macro-nucleus DNA synthesis could be initiated. These results suggested that a critical nuclear:cytoplasmic ratio is necessary for DNA synthesis.

There are, however, several reports in the literature which disagree with the experiments described above. Using Chinese hamster cells, Fox and Pardee (1970) found a slight correlation between the mass of new G_1 cells and the length of G_1. However, the correlation was too small to account for the high degree of variability observed in the duration of G_1. These results are in direct conflict with those of Killander and Zetterberg (1965) and were interpreted to indicate that a critical cell mass may not be necessary for the initiation of DNA synthesis.

Fournier and Pardee (1975) studied the relationship between cell mass and initiation of DNA synthesis in baby hamster kidney cells using another procedure. Serum deprivation arrested cells that were in G_1. Additions of serum made it possible to induce the cells back into division. However, when the cell re-entered the cell cycle but were prevented from entering S by the addition of hydroxyurea, cell growth was not halted. Thus in this tissue the initiation of DNA synthesis and a critical cell mass are not tightly coupled.

Graham (1966) showed that haploid and diploid frog cells had similar G_1, S and G_2 durations, despite differences in their volume. Since haploid cells initiated DNA synthesis at a smaller volume than the diploid, the attainment of a critical volume *per se* could not be the signal for DNA synthesis but the nuclear:cytoplasmic ratio might be.

Several studies have shown that cell growth is under nuclear control. In *Micrasterias*, Selmon(1966) demonstrated that treatment with ultraviolet light which affected the nucleic acids also resulted in abnormally large cells. In embryos of cereals, gamma irrdiation arrests cellular division but cells continue to grow to abnormally large volumes (Haber, 1963). Furthermore, the addition of analogues of nucleic acids, which interfere

with normal nucleic acid synthesis, have been shown to result in cell growth at a high rate over a long period (Heyes, 1963).

Satina (1959) and Nagal(1973) described nuclear doubling which was accompanied by an increase in cell size. It is thought that an increase in the number of identical DNA templates allows a higher rate of transcription leading to an increased metabolic activity which can support a large volume of cytoplasm.

A positive relationship has been reported between nuclear DNA content and mean cell volume using root-tip cells (Skult, 1969), stomata guard cells (Greenleaf, 1938) and pollen mother cells at first metaphase of meiosis for unrelated species (Bennett, 1974). The relationship, however, may be quite complex. In Tradescantia paludosa anthers containing both haploid and diploid microspores, cell volumes paralleled the two-fold difference in DNA content. Nevertheless, haploid and diploid cells did not double their volume during DNA synthesis when DNA content was doubled (White and Davidson, 1977). Baetcke et al. (1967), Paroda and Rees (1971) and Bennett (1973), showed that for many related and unrelated plant species the mean volume of the interphase nucleus increased proportionately to the increase in nuclear DNA content.

NUCLEAR

Following the published reports that cell volume, nuclear volume and nuclear DNA content are all interrelated and that the nuclear:cytoplasmic ratio may be the trigger for DNA synthesis, nuclear growth was investigated. Increases in volume as the nucleus progresses through interphase have been documented for Amoeba (Prescott, 1955). The results indicated that nuclear volume changed very little during the first eighty percent of the cell cycle but that it increased rapidly during the last twenty percent.

However, no critical volume was present after division when DNA synthesis began.

Nevertheless, Woodard, Rasch and Swift (1967) reported that in Vicia faba roots nuclear volume increased progressively throughout interphase. In fact the authors suggests that nuclear volume could be used to determine the position of the cell within the cell cycle i.e. that there are characteristic nuclear volumes at each point in the cell cycle, including the beginning of DNA synthesis. However, a large heterogeneity exists in the volume of nuclei from the root meristem of Vicia faba. The degree of heterogeneity is large enough that nuclei positively identified as being in G_1 have volumes which overlap with those nuclei which had been identified as being in G_2 (Davidson, 1975 and Bansal and Davidson, 1977).

In microsporogenesis of Tradescantia paludosa (White and Davidson 1976) where a naturally synchronous interphase occurs, a minimum eight-fold variation in the volume of nuclei with the same DNA content was reported. Furthermore, the amount of variation changed during interphase reaching a maximum during DNA synthesis. This is contrary to the results on cell mass that Killander and Zetterberg (1965) used to conclude that a critical cell mass was necessary to initiate DNA synthesis in mouse L cells.

Similary in meristematic cells of Pisum sativum a considerable overlap existed in the size of the individual nuclei of different DNA classes (Lyndon, 1967). These results show that there is no particular nuclear volume at which DNA synthesis begins.

GROWTH IN RELATION TO CELL DIVISION

A coordination between cellular growth and division was implied by the fact that growing cells tend to maintain a constant size (Prescott, 1976). Furthermore, since on average a cell doubles its size before it divides it was supposed that a prerequisite for division was a doubling or at least cell growth to a particular size.

As previously discussed, in Saccharomyces cerevisiae entry into the cell cycle was dependent on reaching a critical cell size during early G_1 (Johnston et al. 1977). As a consequence of a constant rate of growth and cell cycle duration from this point, all cells reaching division were of the same size. Furthermore, abnormally large cells prepared by blocking division produced daughter cells which did not double their size. Thus after several generations the cells would return to their normal size. It was therefore concluded that cell growth and cell division are normally coupled.

Using Schizosaccharomyces pombe, Nurse (1975) and Fantes (1977) reported that mutants which divided at a smaller volume than the wild type also had a different cell cycle duration. Small deviations from the division volume, due to artificial treatments were corrected within a single cycle while larger deviations required more generations because the cycle could not be shortened by more than one quarter. These observations were best explained by hypothesizing that a critical cell volume governs entry into division.

The relationship between cell growth and division in Amoeba has been summarized by Prescott (1976). Since Amoeba is negatively phototropic, amoeboid movement away from the light resulted in an unequal distribution of the cytoplasm between the two daughter cells. During the next cycle,

the smaller cell grew more rapidly while the larger grew slower than a normal amoeba. The net result was that each cell attained the same weight before dividing. The second approach involved the induction of binucleate cells using albumin solution. Removal by microsurgery, of one nucleus, resulted in division size cells containing a nucleus at the beginning of the cycle. Even when these cells were deprived of nutrients, they divided with a shorter than normal cycle. Finally, cell growth and division were investigated by halting growth of a normal cell at various points along the cell cycle by nutrient deprivation. Although the generation time was longer, these amoeba were observed to divide regardless of growth. Moreover, the generation time increased with the degree of growth limitation. Thus it was concluded that cell mass or volume is important for the rate at which a cell progresses towards cell division, however, attainment of a particular cell size is not an absolute requirement for division.

Increases in cell volume may also be important to the development of cells into meiotic divisions. In one variety of Triticum aestivum, the volume of archesporial cells increased from 6.2×10^3 to 9.1×10^3 and 13.6×10^3 as the number of cells increased from 12 to 50 and finally 100. The final volume of premeiotic cells was 31.3×10^3 (Bennett et al. 1973). Similar increases in cell volume prior to the meiotic division were reported in Zea mays (Moss and Heslop-Harrison, 1967) and Saccharomyces cerevisease (Simchen et al., 1972). However, the attainment of a critical size as a necessary prerequisite for entering meiotic division has not been invoked.

Unlike cell growth, which takes place mostly during the early

part of the cell cycle, nuclear growth predominates in the late stages of the cell cycle; this suggests that nuclear growth may influence entry into division. However, in Pisum sativum, prophase nuclei ranged from 260 to 860 um^3 while telophase nuclei ranged from 30 to 300 um^3, i.e. the largest telophase nucleus was equivalent to the smallest prophase (Lyndon, 1967). These data are difficult to reconcile with the idea that a particular nuclear volume is critical to division.

In their study with Tradescantia paludosa microspores, White and Davidson (1976) found that although nuclear volume increased rapidly during G_2, no critical volume was necessary for entry into division. They concluded that growth may be associated with division but the two are not absolutely coupled.

OVERVIEW

The existence of a close correlation between some parameters of cellular or nuclear growth and the cell cycle in some organisms but not in others, indicates that there are no simple universal rules. This finding is not surprising if one considers that the coupling of growth to the cell cycle, especially in eukaryotic systems, may take place within critical limits rather than at some absolute quantities. It is only those systems which are operating at their absolute minimum or maximum growth rates which indicate that the critical values control development through the cell cycle. Most sytems operate at some intermediate region between their limits and consequently give results which are variable and inconsistent with the theory of critical values.

Mitchison (1971) proposed a model which is consistent with this idea. His theory is the cell cycle is composed of two coupled cycles, the "growth" cycle and the "DNA-division" cycle. Evidence for the

presence of the two cycles came from the observations that (1) inhibition of the "DNA-division cycle causes cells to be blocked at S or division without halting growth and (2) when cells are grown under starved conditions DNA synthesis and division may proceed in the absence of growth.

The degree to which these two cycles are coupled was discussed in two recent articles on Saccharomyces cerevisiae (Johnston et al., 1977) and Schizosaccharomyces pombe (Fantes, 1977). Both, however, are consistent with a model containing two cyclic processes, which may be coupled to varying degrees within the maximum and minimum limits defined by the organism, cell type and the external conditions.

MANUSCRIPT

" GROWTH DURING MICROSPOROGENESIS OF HEXAPLOID TRITICALE "

Abstract

During the 100 h period from quartet break-up until first pollen grain mitosis, mean cell, nuclear and nucleolar volumes were calculated at 10 h intervals in hexaploid triticale (Triticosecale Wittmack cv. Rosner). Changes in DNA content per microspore were also measured, to determine the time when DNA synthesis occurs. From 0-30 h (G_1); (1) cell volume increased at a constant rate from 9400 to 48,297 μm^3; (2) nuclear volume initially increased from 1674 to 3261 μm^3 but then returned to 1568 μm^3; and (3) nucleolar volume did not change. From 30-70 h (S) cellular volume increased to 79,090 μm^3, nuclear volume did not change, while nucleolar volume initially increased two-fold but then returned to G_1 value. From 70-100 h (G_2) there was no change in cell volume, while nuclear and nucleolar volumes increased to 4365 and 344 μm^3, respectively. Regression analysis throughout microsporogenesis indicated a constantly changing significant relationship between the volume of a cell and its nucleus and nucleolus. However, no significant regression coefficients were obtained during early prophase. These results are interpreted as indicating that critical cellular, nuclear or nucleolar volumes are not necessary for the development of a cell through interphase.

Introduction

Howard and Pelc (1953) first defined the cyclic stages of the cell cycle as being G_1, DNA synthesis (S), G_2, and mitosis (M). Since then, considerable interest has been generated towards the events of the cell cycle and what controls them. G_1 and G_2 represent gaps in our knowledge of cellular events between the two discernible stages, DNA synthesis and division.

Growth of both the nucleus and cytoplasm are an integral part of any cell cycle if cell type is to be maintained. Organized growth must exist to enable each of the two daughter cells to be equal to each other and the parental cells. Growth and the cell cycle are so closely intertwined in some systems that researchers have proposed that growth to a critical point controls a cell's progression through the cell cycle. However, no general rules have been found, since results are variable depending on the growth parameter measured and organism studied. Mitchison (1971) and Prescott (1976) have summarized the most current findings in this area.

One of the major difficulties of studying the cell cycle has been that most eukaryotic systems are asynchronous; this necessitates the use of artificial treatments for the production of marked populations or synchrony. These treatments may also alter normal cellular metabolism thereby introducing possible artifacts when studying growth in relationship to the cell cycle. A system that lends itself to the study of the events of cell division because of its high degree of natural synchrony is pollen maturation. In hexaploid triticale cv. Rosner, all cells of the quartets break up and enter interphase within the span of 2 h. At the end of interphase, four days later at 20°C, a mitotic index of 58 (containing 13 percent of

the cells at metaphase) was observed (Bennett and Smith 1972). This period, microsporogenesis, provides us with a means of studying a large, naturally homogenous population of cells developing through a single cell cycle. We, therefore, undertook to study cell, nuclear and nucleolar volumes at 10 h intervals during this interphase. The results are discussed in terms of the importance of critical volumes in controlling a cell's progression through the cell cycle.

Methods and Materials

I. Preparation of Slides and Calculation of Volumes

Hexaploid triticale cv. Rosner (X Triticosecale Wittmack) plants were kept in a growth chamber at 20°C ± 1°C under continuous illumination. Natroka (1968) reported that pollen size differs among primary, secondary and tertiary tillers; for this reason only primary florets from the center of primary tillers were used for this study. No difference was found between the volumes of anthers from within a particular floret. Light intensity has also been found to influence cell size: high energy treatments increased cell volume four or five times over those of low energy treatments (Murray 1968; Dale and Murray 1968). Spikelets were fixed in freshly mixed acetic acid alcohol (1:3) before microspores were stained with acetocarmine (2%). To avoid distortion of shapes and volumes, slides were not squashed and cell, nuclear and nucleolar measurements were taken immediately without removing the coverslip. One hundred microspores were measured at 10 h intervals from quartet break-up (0 h) until first pollen grain mitosis (100 h). Twenty-five prophases were also measured. Classification of shapes and calculation of cell, nuclear and nucleolar volume were as previously described by White and Davidson (1976).

the linear volumes facilitate comparison with other data in the literature. Unfortunately, the linear values necessary for testing differences in slope or intercept of regression coefficients between samples results in numbers with too many digits for calculation. For this reason the regression analysis was performed on the log volumes.

Results

I. Changes in DNA Content during Microsporogenesis

The mean DNA content was in arbitrary units 78, 85, 99, 153 and 187 at 0, 30, 40, 50, and 70 h respectively (Fig. 1). Moreover, it was found that although the mean DNA content at 30 h was not significantly different ($P < 0.05$) from that at 0 h the value at 40 h was. The change in DNA content from 50 to 70 h was again not significantly different. These results suggest that DNA synthesis began between 30 and 40 h and was completed during the interval from 50 to 70 h. To determine how many of the microspores were in each stage of the cell cycle for any particular population the following assumptions were made:

(1) The maximum DNA value of a G_1 microspore was approximated by the largest value at 0 h, i.e. 101.

(2) The minimum 2C value was twice the mean value of the 0 h sample, i.e. 156.

If this were the case, the proportion of cells at any given stage of the cell cycle can be calculated (Table 1). From these data it can be seen that by 30 h DNA synthesis had started in 25 percent of the microspores. However, because the S values did not lie far above the assumed limit of G_1 (Fig. 1) and since the mean value of this population was not significantly

Figure 1: Changes in DNA content during microsporogenesis of the hexaploid triticale cv. Rosner

Abscissa, indicates hours from quartet break-up. "$\bar{x}$" indicates the mean of the population.

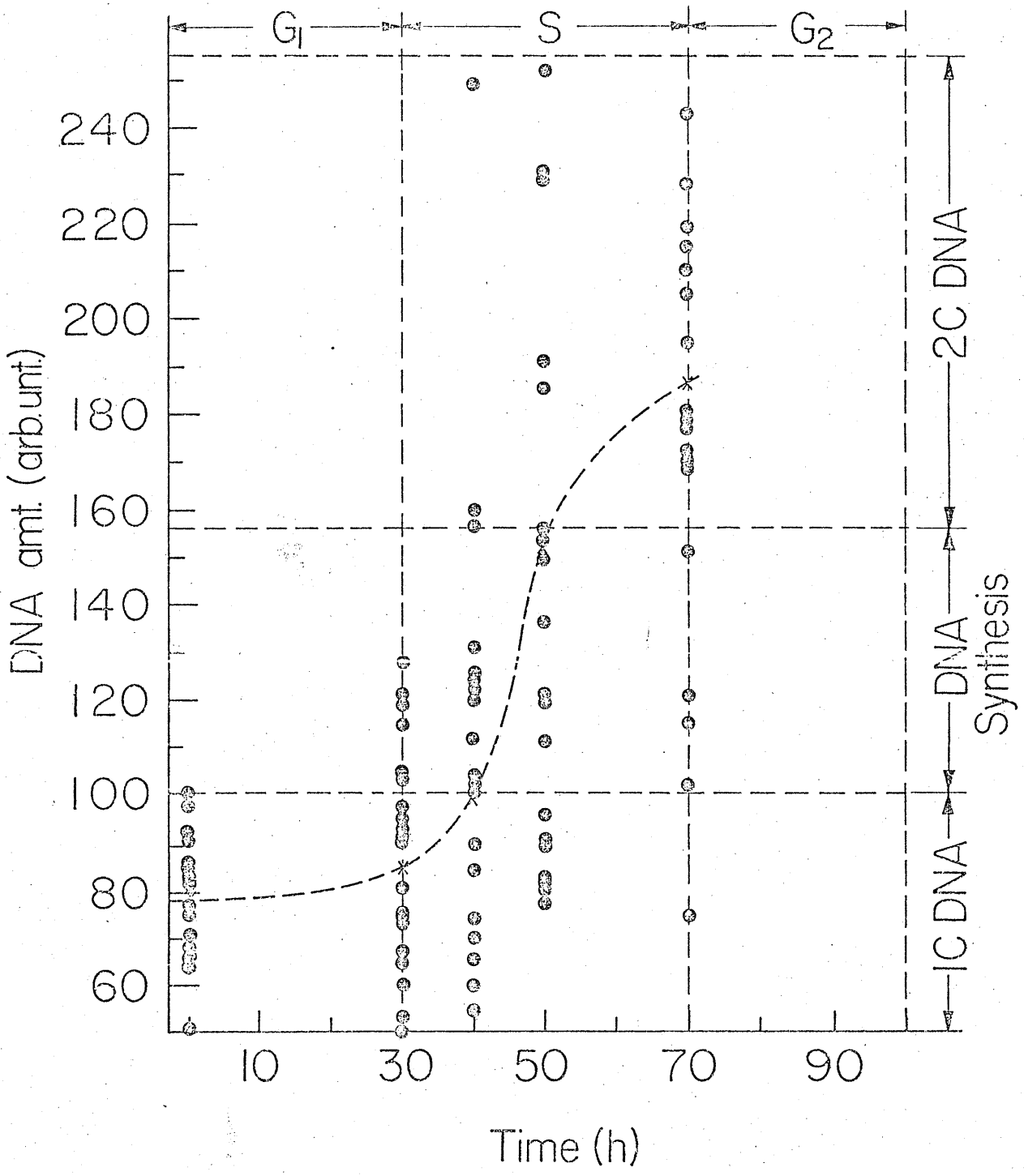

G1
S
G2
240
220
200
180
160
140
120
100
80
60
DNA amt. (arb.unt.)
10
30
50
70
90
Time (h)
2C DNA
DNA Synthesis
IC DNA

TABLE 1

Percent of microspores in the three stages of the cell cycle as determined by microspectophotometry

Time after quartet breakup (h)	G_1	S	G_2
0	100	-	-
30	75	25	-
40	50	40	10
50	35	25	40
70	5	15	80

different from the value at 10 h, it was estimated that S began at 30 h. DNA synthesis was assumed to be completed in the majority of cells by 70 h for the following reasons:

(1) The mean DNA value did not change significantly ($P < 0.05$) between 50 and 70 h.

(2) Eighty percent of the population was in G_2.

(3) The mean value of 187 was more than double the value at 0 h.

(4) The values not at a G_2 level were either close to a G_2 value or very low (Fig. 1). The latter was thought to represent microspores which were aborting.

It was decided, therefore, that the three stages of cell cycle lasted as follows: G_1 from 0 to 30 h, S from 30 to 70 h and G_2 from 70 to 100 h.

II. Changes in Number of Nucleoli per Microspore during Microsporogenesis

Because microspores of hexaploid triticale cv. Rosner are haploid, they have three nucleolar organizing chromosomes (Darvey 1973). However, in this study the maximum number of nucleoli observed was two. It was found that from 0 to 90 h the number of microspores with two nucleoli fluctuated randomly around 50 percent (Fig. 2). As the microspores entered prophase, however, this number dropped to 10 percent. This may result from the microspores with two nucleoli developing faster and entering into division sooner or it may be caused by nucleolar fusion. For this reason, it was decided to ascertain whether those microspores with one or two nucleoli were functionally equivalent.

Microspores with one or two nucleoli were analyzed separately with respect to mean cell, nuclear and nucleolar volume (Tables 2, 3, 4). At each stage sampled mean cell and nuclear volumes for microspores with one or two nucleoli were not significantly different. Mean nucleolar volumes

216

Figure 2: Changes in the number of microspores with two nucleoli during microsporogenesis of the hexaploid triticale cv. Rosner.

Abscissa, indicates hours from quartet break-up.

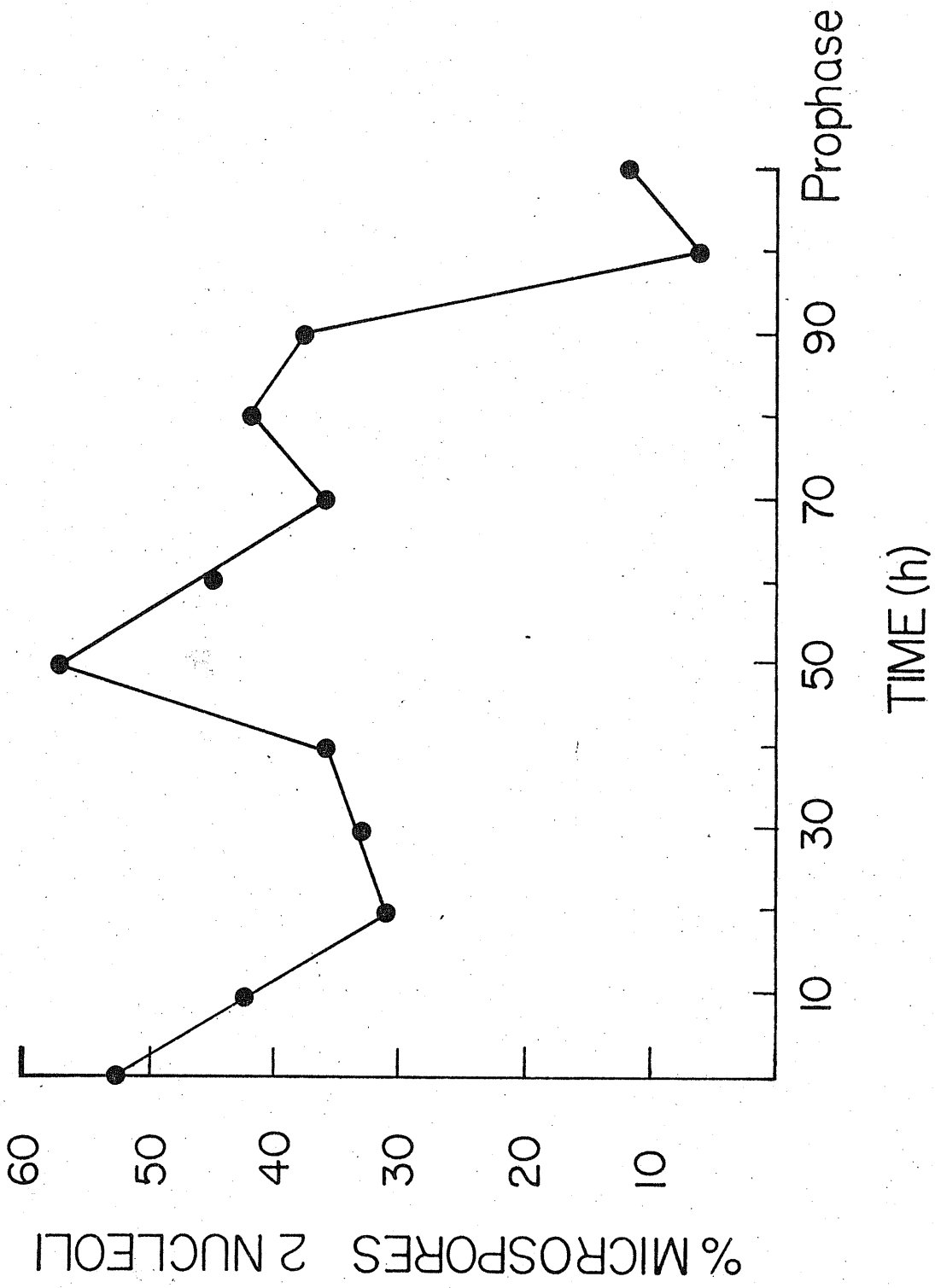

% MICROSPORES 2 NUCLEOLI
60
50
40
30
20
10
10
30
50
70
90
Prophase
TIME (h)

TABLE 2

Mean linear cell volumes and standard deviations of successive populations during microsporogenesis of hexaploid triticale cv. Rosner

Time after quartet breakup (h)	Microspores with 1 nucleolus	Microspores with 2 nucleoli	Combined
0	9,900 ± 3,284	8,937 ± 2,053	9,401 ± 2,715
10	15,796 ± 6,152	14,765 ± 3,662	15,353 ± 5,231
20	36,610 ± 8,245	38,508 ± 7,682	37,198 ± 8,084
30	49,315 ± 13,517	46,231 ± 8,774	48,297 ± 12,199
40	56,287 ± 8,199	55,572 ± 8,790	56,029 ± 8,586
50	68,175 ± 10,183	69,395 ± 9,955	68,870 ± 10,021
60	75,832 ± 15,682	74,083 ± 12,115	75,045 ± 14,988
70	78,010 ± 16,492	81,010 ± 17,472	79,090 ± 16,845
80	75,975 ± 13,597	77,388 ± 14,842	76,568 ± 14,077
90	75,053 ± 12,915	72,132 ± 11,757	74,001 ± 12,530
100	71,942 ± 9,944	76,575 ± 15,782	72,220 ± 10,294
prophase	76,289 ± 18,182	78,321 ± 20,172	76,533 ± 18,471

TABLE 3

Mean linear nuclear volumes and standard deviations of successive populations during microsporogenesis of hexaploid triticale cv. Rosner

Time after quartet breakup (h)	Microspores with 1 nucleolus	Microspores with 2 nucleoli	Combined
0	1,774 ± 767	1,612 ± 467	1,674 ± 622
10	3,476 ± 1,895	2,956 ± 1,302	3,261 ± 1,677
20	2,552 ± 877	2,866 ± 806	2,649 ± 864
30	1,582 ± 559	1,541 ± 402	1,568 ± 511
40	1,452 ± 678	1,538 ± 647	1,483 ± 680
50	1,623 ± 718	1,671 ± 810	1,650 ± 768
60	1,352 ± 601	1,467 ± 519	1,404 ± 566
70	1,425 ± 491	1,401 ± 311	1,417 ± 435
80	2,170 ± 1,158	2,263 ± 833	2,209 ± 1,030
90	2,727 ± 1,335	2,225 ± 1,161	2,546 ± 1,297
100	3,552 ± 1,157	3,988 ± 2,367	3,579 ± 1,246
prophase	4,347 998	4,499 ± 754	4,364 ± 960

TABLE 4

Mean linear nucleolar volumes and standard deviations of successive populations during microsporogenesis of hexaploid triticale cv. Rosner

Time after quartet breakup (h)	Microspores with 1 nucleolus	Microspores with 2 nucleoli	Combined
0	46 ± 23	49 ± 25	48 ± 24
10	50 ± 23	54 ± 19	52 ± 22
20	40 ± 25	50 ± 17	43 ± 23
30	29 ± 16	38 ± 26	32 ± 20*
40	61 ± 25	58 ± 29	60 ± 25
50	114 ± 42	123 ± 49	119 ± 46
60	84 ± 40	76 ± 30	81 ± 36
70	58 ± 21	49 ± 16	55 ± 19*
80	91 ± 64	98 ± 66	94 ± 64
90	317 ± 120	299 ± 170	310 ± 139
100	313 ± 108	270 ± 63	311 ± 106
prophase	353 ± 129	280 ± 35	344 ± 123

*significantly different at $p < 0.05$

for microspores with one or two nucleoli did not differ significantly except at 30 and 70 h. These results indicate that the number of nucleoli did not significantly alter the rate of growth.

III. Changes in Mean Volumes during Microsporogenesis

(i) Cell

Mean cell volume increased linearly until 60 h (Fig. 3a). During G_1, cell volume increased five-fold while during the first half of S, cell volume increased by 1.6 times. From 60 h until first pollen grain mitosis mean cell volume did not change significantly. Thus, cell growth is restricted to the first half of the cell cycle.

(ii) Nucleus

Nuclear growth showed a two-fold increase during the first 10 h, as the DNA unwound after meiosis (Fig. 3b). The initial swelling of the nucleus was followed by a similar two-fold decline in volume from 10 to 30 h.

During S no significant change was detected in the size of the nucleus. However, during G_2 mean nuclear volume increased three-fold without any change in DNA content. These results indicate that nuclear volume is not very sensitive to change in DNA amount *per se*.

(iii) Nucleolar

Nucleolar volume did not change during G_1, however, upon entry into S a two-fold increase was observed (Fig. 3c). This initial increase was followed by a similar two-fold decline from 50 to 70 h. During G_2, nucleolar volume increased dramatically from 50 μm^3 to 310 μm^3, i.e. six-fold.

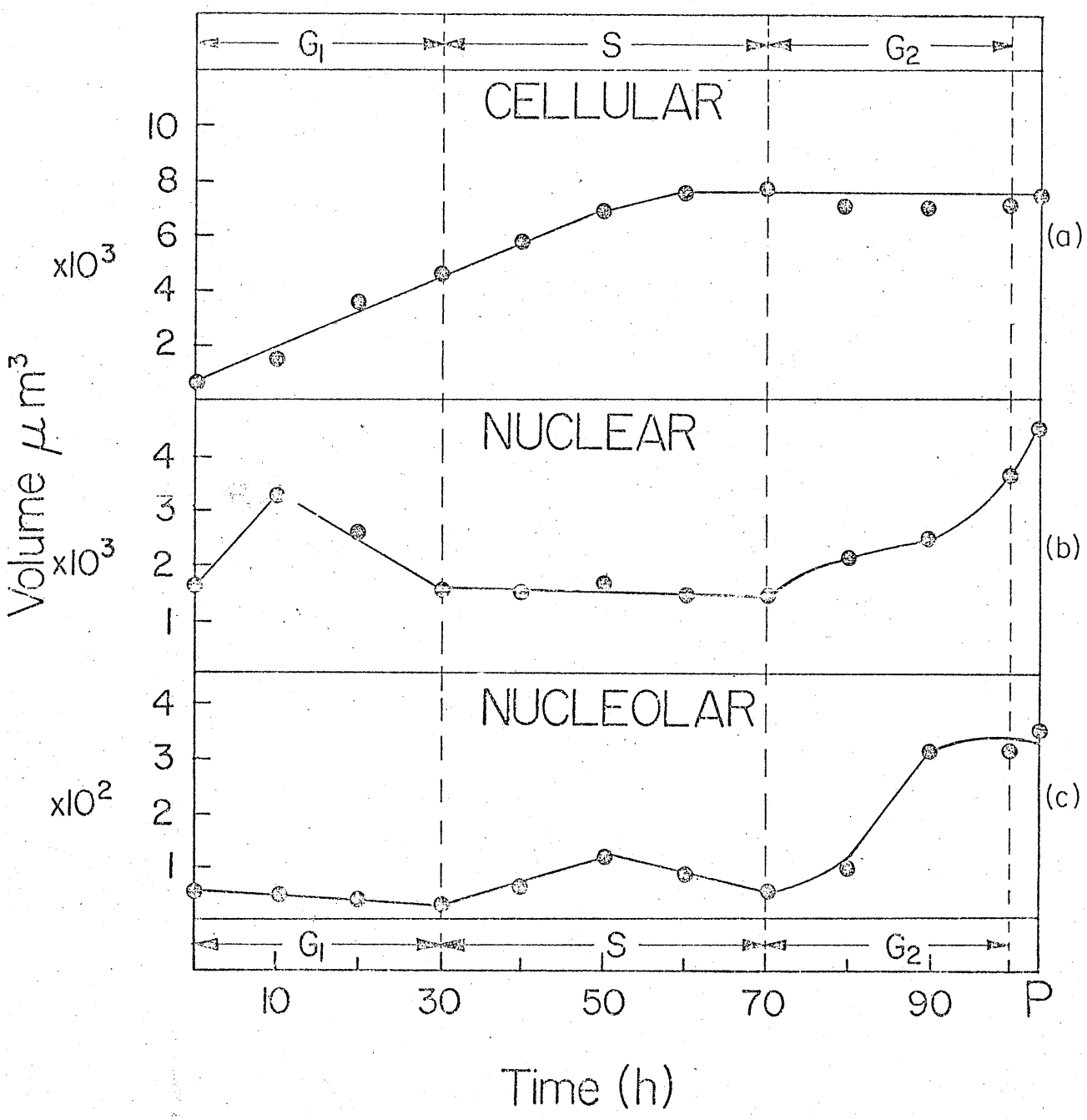
IV. Changes in Regression Coefficients during Microsporogenesis

Regression analysis allowed the study of the dependence of one parameter on another within the individual microspores of a population. It was

Figure 3: Changes in mean volume during microsporogenesis of the hexaploid triticale cv. Rosner.

Abscissa, indicates hours from quartet break-up.

"P" indicates that the value is of a prophase population.


G1
S
G2
CELLULAR
NUCLEAR
NUCLEOLAR
Volume μm³
x10³
x10³
x10²
(a)
(b)
(c)
10
30
50
70
90
P
Time (h)

also possible to compare the slopes and the intercepts of successive stages of microsporogenesis to check if the same relationship was maintained. To illustrate this, regression coefficients of cell vs. nuclear volume, nucleus vs. nucleolar volume and cell vs. nucleolar volume were plotted throughout microsporogenesis (Fig. 4).

(i) Volume of Cell vs. Nucleus

A highly significant regression coefficient was found between cell and nuclear volume as the microspores were released from the quartets (Fig. 4a). Subsequently, regression coefficients steadily declined until by 40 h cell vs. nuclear volume no longer had a significant relationship. However, a significant relationship was restored at 50 h and maintained for the remainder of interphase. The regression coefficient values for G_2 did not change in slope but did change in intercept. At division, there was a fall in the regression coefficient to a level where it was not longer significant.

(ii) Volume of Nucleus vs. Nucleolus

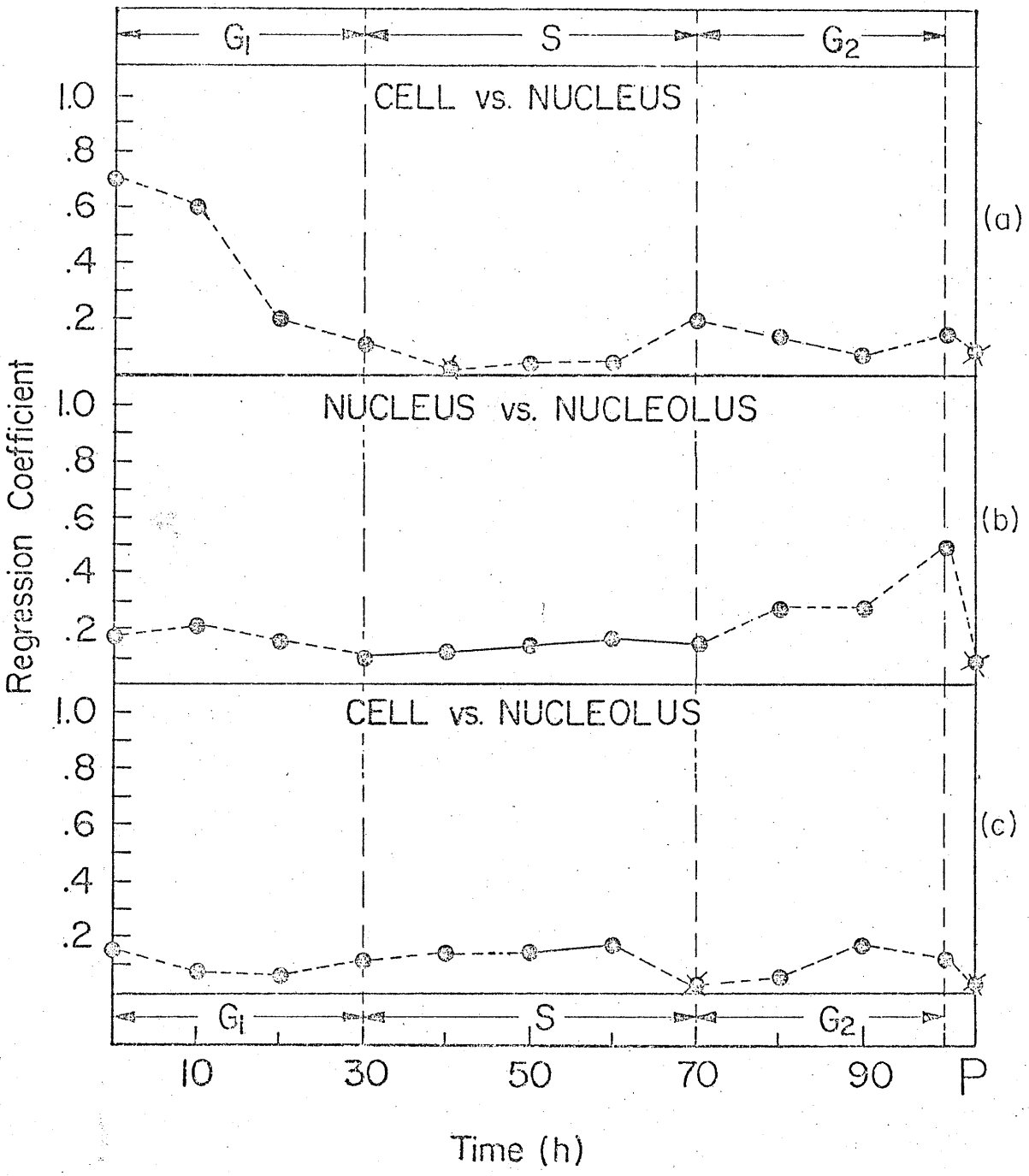
A similar analysis of nuclear vs. nucleolar regression coefficients showed that all values were significant except during pollen grain mitosis (Fig. 4b). It was also found that from 20 to 70 h all regression values had the same slope. Furthermore, from 30 to 70 h the samples had the same intercept. Thus, during DNA synthesis, the microspores maintained a constant relationship between the size of the nucleus and its nucleolus.

(iii) Volume of Cell vs. Nucleolus

All regression coefficients of cell vs. nucleolus were significant except at 70 h and prophase of pollen grain mitosis (Fig. 4c). Again during DNA synthesis from 30 to 60 h the slope was constant; however, the intercept was constant only from 50 to 60 h.

Figure 4: Changes in volume regression coefficients during microsporogenesis of the hexaploid triticale cv. Rosner.

Abscissa, indicates hours from quartet break-up. The dashed lines indicate that linked values are not significantly different, solid lines indicate that the values are not significantly different at p = 0.05. "*" indicates that the regression coefficient at that time were not significant at p = 0.05 level. "P" indicates that the value is of a prophase population.


G1
S
G2
CELL vs. NUCLEUS
NUCLEUS vs. NUCLEOLUS
CELL vs. NUCLEOLUS
(a)
(b)
(c)
Regression Coefficient
1.0
.8
.6
.4
.2
10
30
50
70
90
P
Time (h)

Discussion

I. Duration of DNA Synthesis

Although it is not possible to determine the time required for an individual microspore to complete DNA synthesis the average individual duration can be approximated. From Table 1, it can be seen that between 30 and 40 h, 10 percent of the cells had completed DNA synthesis while 25 percent had entered DNA synthesis. Between 40 and 50 h, another 30 percent of the cells finished DNA synthesis and 15 percent entered DNA synthesis. This means that 40 percent of the cells had completed DNA synthesis during the first half of S, with the remainder either completing DNA synthesis in the second half or aborting. These results suggest that although cells can be found in the process of DNA synthesis during the 40 h period from 40 to 70 h most individual cells require about half or 20 h to complete DNA synthesis.

This value is longer than the 6.2 h estimated for the root meristem of hexaploid triticale cv. Rosner (Kaltsikes 1972), although they are not strictly comparable since the microspores at 20 ± 0.05°C develop slower than the roots at 25 ± 0.05°C. Nevertheless, it can be seen that the total duration of S in the gametophytic cycle is only 40 percent of the total duration of interphase, while it comprises at least 50 percent in the sporophytic cycle. Furthermore, the duration of S for individual microspores is only 20 percent of the total interphase duration. Although the rate of DNA synthesis is considerably slower during the haplophase, it does not comprise a larger portion of the total interphase. This situation has been reported even more dramatically in Tradescantia by Satô et al. (1967) who estimated the duration of S during microsporogenesis to be 9 to 18 h out of a total interphase of 120 h. Similarly, the duration of S in the root meristem of Tradescantia has been shown to be 10.5 h with the total interphase duration being only 20 h

(Wimber 1960). In this case, the rates of DNA synthesis were comparable and the relative rate was six-fold faster in the haplophase.

The difference between Tradescantia and the triticale used in this study may result from differences in their degree of synchrony. However, the results do indicate that the extended interphase of microsporogenesis does not result from an equal lengthening of all interphase stages. In fact the major relative difference appears to be in the duration of G_1 which comprises only 6 percent of the diplophase's (root meristem's) interphase while occupying 30 percent of the haplophase's (microspore's). This suggests that after meiosis the microspores require an extended period of time to adjust to the haplophase generation. During this same interval a pronounced enlargement and contraction in nuclear volume has been observed (Rasch and Woodward, 1959; White and Davidson, 1977; see also discussion III). These results suggest that the cell requires this extended period to unwind and possibly alter the organization of DNA.

II. Nucleolar Organization

The observation that only two of the potential three nucleoli were ever expressed agrees with the findings in the root meristem where the maximum number of nucleoli observed, four, was less than the six possible (Thomas and Kaltsikes 1977). Furthermore, it was the nucleolar organizer on 1R that was always suppressed (Darvey 1973). Amphiplasty has been reported in many other systems where more than one genome is present (Bennett et al. 1976). The present observation demonstrated that both the haplo- and diplophase organized their nucleoli in the same way.

It was also observed that at the beginning and end of DNA synthesis, total nucleolar volume was significantly different between microspores with

one or two nuclei. This difference, however, was not related to a difference in the entry or exit of cells from DNA synthesis for the following reason: if the presence of a particular number of nucleoli changed the rate of cell development one would expect those cells to exhibit an earlier increase and decrease in nucleolar volume (Fig. 3c) than observed for the overall population during S. However, microspores with one or two nucleoli both showed a similar pattern of changes at the beginning and end of S (Table 4) although not to the same extent. This further supports the previous claim that the presence of one or two nucleoli does not affect cell, nuclear or total nucleolar volumes and their subsequent growth.

Since (1) there is no difference in the growth of microspores with one or two nucleoli and (2) prophase nuclei also have a reduced number of nucleoli, the observed decrease in the number of nucleoli immediately prior to division is interpreted as representing a period of nucleolar fusion. This fusion may be related to the entry of cells into division, even though it may not be absolutely necessary since not all prophase nuclei have one nucleolus. A similar period of nucleolar fusion is observed upon entry into the meiotic division (Roupakias and Kaltsikes 1977 a, b, c), but no reason for this has been reported.

III. Critical Volumes during the Cell Cycle

(i) Cellular

Cell growth was restricted to the beginning of interphase; this agrees with the generalized pattern reported by Mitchison (1971). No critical volume appears to be associated with the initiation of DNA synthesis, as the spread of values is the same at the beginning of G_1 and DNA synthesis. Furthermore, the 1.6 fold spread with ± 1.S.D. of the mean (Table 2), indicates a highly heterogenous population of cell volumes at the initiation

of DNA synthesis. This does not coincide with the theory that a critical cell volume is capable of triggering DNA synthesis.

Similarly during G_2, the variation in cell volume does not change, even when at division. If a critical cell volume was necessary for cells to enter division, one would expect the spread of volumes to decrease at division but this was not the case. Thus, cell volumes do not appear to be critical to the development of a microspore through the cell cycle. This disagrees with the findings of Fantes (1977) in Schizosaccharomyces pombe and Johnston et al. (1977) in Saccharomyces cerevisiae but agrees with the conclusion of Prescott (1976) who reviewed several systems. The contradictory findings using different systems indicate that uni-cellular and multi-cellular organisms differ in the degree of co-ordination between cell growth and division.

(ii) Nuclear

The dramatic two-fold fluctuation in nuclear size during G_1 is thought to reflect a change in the programming of gene expression as the cell passes from the diplophase to the haplophase. As previously discussed, an extended G_1 is necessary to facilitate this fluctuation. Gurdon (1972) also reported changes in nuclear volume when nuclei, transplanted into a foreign cytoplasm were forced to reprogramme their gene expression.

Similar patterns of nuclear growth, i.e. a decrease during early microsporogenesis, were found in diploid and tetraploid Tradescantia paludosa (Rasch and Woodard 1959; White and Davidson 1977b).

During DNA synthesis, mean nuclear volume did not change significantly, even though there were changes in the spread of values. The nuclear samples at the beginning and end of S varied by two-fold but during mid-S the spread was 2.7 fold (Table 3). Similarly, at the end of G_2, the range of values

fell from 3.1 to two-fold. These results may suggest that the populations are more homogeneouswhen transferring from one stage of the cell cycle to another. Nevertheless, the minimum two-fold range indicates that considerable heterogeneity still exists throughout interphase. The heterogeneity was too large to consider critical nuclear volumes as an important criterion for controlling a cell's progression through a cell cycle.

The three-fold increase in mean nuclear volume during G_2 does, however, suggest that nuclear growth is associated with division. Mitchison (1971) also reported that nuclear growth is confined to the latter stages of interphase and that this may be related to division. White and Davidson (1976) demonstrated that the G_2 phase in Tradescantia paludosa also is a period of active nuclear growth. This growth coincided with the period of active synthesis of 5S RNA and protein (Bryan 1971; Pedda and Masarenhas 1975).

Changes in nuclear volume are limited to G_1 and G_2 when there is no change in DNA content. These data clearly demonstrate that nuclear volume does not reflect changes in the amount of DNA it contains. Nevertheless, Baetcke et al. (1967), Paroda and Rees (1971) and Bennett (1973), report a positive correlation between mean volume of interphase nuclei and nuclear DNA content for many related and unrelated plant species. However, Bryan (1951), Swift (1953), Sisken (1959) and White and Davidson (1976) all indicate that a doubling of the DNA content does not result in a simultaneous doubling in nuclear volume. Furthermore, Lyndon (1967) showed in Pisum sativum and Davidson (1975) and Davidson and Basal (1977) in Vicia faba root meristems and White and Davidson (1976, 1977 a, b) in Tradescantia, have reported that there is no correlation between nuclear DNA content and mean nuclear volume.

(iii) Nucleolar

Brown and Gurdon (1964) showed that anucleolate mutants of Xenopus laevis lacked rRNA synthesis. This and several other studies indicate that the nucleolus is associated with rRNA synthesis. Furthermore, it has been postulated that there is a relationship between the size of the nucleolus and the synthetic activity of the cell (De Robertis et al. 1975). If this is true, there is no increase in cellular activity during G_1, since nucleolar volume does not change.

During the first half of S, nucleolar volume increased three-fold; this may reflect the increased cellular activity which is necessary for the DNA synthesis machinery. The latter half of S is characterized by a return in nucleolar volume to the G_1 level; this may reflect the decreasd cellular activity as the pollen wall is completed.

G_2 is a period of rapid increase in nucleolar volume. This rapid increase coincides with the period of large increases in nuclear volume. Both of these observations suggest that G_2 in microsporogenesis of triticales is an intense period of gene activity, similar to that found in Tradescantia paludosa (Bryan 1951; Pedda and Mascarentias 1975).

At any point during interphase there is a minimum two-fold spread in values within ±1 S.D. fo the mean (Table 4). Critical nucleolar volumes, therefore, do not appear to be co-ordinated with particular stages of the cell cycle. However, nucleolar volumes fluctuated during interphase in a manner that may reflect changes in the gene activity. Similarly, de la Torre et al. (1975) postulated that nucleogenesis is a measure of gene activity. Whether nucleolar volume bears a direct or casual relationship to gene activity has not yet been determined.

IV. Organization of Growth and the Cell Cycle

Mitchison (1971) proposed that the cell cycle is composed of two cycles; the "growth" cycle and the "DNA-division" cycle. Evidence for the presence of the two cycles came from the observation that (1) inhibition of "DNA-division" cycle causes cells to be blocked at S or division without halting growth; and (2) when cells are grown under conditions of starvation, DNA synthesis and division may proceed in the absence of growth. The question then was how are these two cycles inter-related?

Campbell (1957) defined balanced growth as the situation when over a given time interval every property of the growing system increases by the same factor. In the present study, comparison of the changes in mean volumes of the three parameters measured during each stage of the cell cycle indicated the following:

G_1 (1) Cell volume increased linearly

(2) Nuclear volume increased initially but then decreased

(3) Nucleolar volume did not change

S (1) Cell volume increased linearly for the first half and then it did not change

(2) Nuclear volume did not change

(3) Nucleolar volume increased initially then decreased

G_2 (1) Cell volume did not increase

(2) Nuclear volume increased three-fold

(3) Nucleolar volume increased six-fold.

This indicates that during microsporogenesis mean cell, nuclear and nucleolar volumes change independently.

The organization of growth within individual microspores was studied by determining the volume regression coefficients of cell vs. nucleus, cell

vs. nucleolus and nucleus vs. nucleolus. Fig. 4 shows that significant regression coefficients were obtained throughout most of interphase. This indicates that a large cell usually contains a large nucleus and nucleolus. However, this significant relationship between the volume of a cell, its nucleus and nucleoli constantly changed during interphase. Thus, growth over the period of microsporogenesis is not balanced at the level of mean volumes of cell, nucleus and nucleolus or within the individual microspore.

Furthermore, regression analysis demonstrated no significant relationship between the three parameters at division. This strengthens the previous conclusion that critical volumes do not determine entry into first pollen grain mitosis. The literature contains contradicting reports about whether the "DNA-division" cycle is dependent on the "growth" cycle reaching critical levels (Killander and Zetterberg 1965; Fox and Pardee 1970; Fournier and Pardee 1975; Nurse 1975; Johnston et al. 1977 and Fantes 1977). Many of the reported differences probably relate to the fact that the organization of growth during interphase is cell type specific. The absence of critical volumes and the fluctuation in the relationship between the volume of an individual cell and its nucleus and nucleolus during the microsporogenic cell cycle suggests that changes in the rate of growth of a cell, nucleus and nucleolus are independent of one another.

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CONTRIBUTIONS TO KNOWLEDGE

(1) During the G_1 period, 0-30h, nuclear volume underwent a large increse and decrease; this was thought to reflect the change of the cells from the diplophase to the haplophase. To see if this indeed true, an E.M. study of the chromatin during this period would be useful.

(2) Similarly, an E.M. study of the density of pores in the nuclear membrane at various stages of microsporogenesis may be related to changes in nuclear volume or shape and metabolic activity.

(3) The G_2 period, 70 to 100h, displayed a large increase in both nuclear and nucleolar volume, suggesting a high metabolic rate at this point. It would be interesting to see how protein and RNA contents per cell change with respect to the changes in volumes.

(4) Determination of cell and nuclear mass, throughout microsporogenesis, would also be useful to better define growth during microspore differentiation.

SUMMARY AND CONCLUSIONS

(1) The approximate durations of the cell cycle stages are:

G_1	0 - 30 h
S	30 - 70 h
G_2	70 -100 h

(2) Cell, nuclear and nucleolar volumes change independently of one another, in both individual and mean valus. This indicates that growth at this level is not balanced during microsporogenesis.

(3) No significant relationship was found between the three parameters at division, suggesting that size relationships are not an important criterion for division.

(4) The presence of one or two nucleoli did not affect the rate of development or growth.

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