NUCLEAR VOLUMES: AN ANALYSIS DURING A CELL CYCLE

By

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A thesis

submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

July 1975

NUCLEAR VOLUMES: AN ANALYSIS DURING A CELL CYCLE

DOCTOR OF PHILOSOPHY (1975) McMASTER UNIVERSITY

Biology Hamilton, Ontario

TITLE: Nuclear Volumes: An Analysis During a Cell Cycle

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NUMBER OF PAGES: XV, 131

ABSTRACT

Cells in root meristems of Vicia faba are heterogeneous for cell cycle duration, nuclear volume and cell shape. The present study was made: (1) to determine volumes of meristem cell nuclei, (2) to analyze changes in nuclear volumes during a cell cycle in a marked population of tetraploid cells in the meristems of long lateral roots.

The results show that there is also heterogeneity in nuclear volume, growth rates of nuclei, protein content and shapes of nuclei. The degree of heterogeneity is different in meristems of roots at different developmental stages. The results also reveal that there is no definite correlation between the volume of a nucleus and its age in interphase. Furthermore, nuclei of non-cycling cells also appear to grow.

The data on the marked population of tetraploid cells provide specific information about changes in nuclear volumes during one cell cycle. The range in nuclear volumes is large even in early G_1. Nuclei grow at different rates: 1) within any one subphase of interphase, 2) between different subphases. Even the volumes of prophase nuclei from cells with

more or less the same cell cycle duration (i.e., fast cycling cells) appear to be variable. This indicates variability in nuclear growth rates even among the cells that take approximately the same time to complete a cell cycle.

From the volumes of metaphase chromosome complements, an estimate can be made of minimum possible volume of nuclei at the beginning of interphase. The variability of these values suggest that there is a range of volumes among the nuclei at the very beginning of interphase. Hence, one of the factors affecting nuclear growth could be the volumes and, therefore, the chemical composition of the chromosome complements from which the nuclei are formed.

Acknowledgements

I wish to thank Dr. D. Davidson for his supervision, interest and encouragement during the course of the present work; Dr. D. Carr, Dr. S. Mak and Dr. A. D. Dingle for their suggestions and helpful criticisms of the thesis; Dr. R. A. Morton, Dr. M. Stephens and Mr. J. Thomas for help in computer programming; Dr. J. Pasternak for the use of microspectrophotometer facilities and Dr. M. Haight for help in the protein determinations.

I am also grateful to many friends for discussions and encouragement: Mr. J. Thomas, Mrs. M. Eastman, Mr. G. Stallwood, Mr. T. Y. Wong, Ms. C. Wellwood, Miss A. Islam and Ms. R. Persaud.

I am thankful to Om Bansal and to my family in India for their interest and words of encouragement and to Miss J. Street for typing the thesis.

Finally, I would like to thank C.I.D.A. for financial support in the form of a Commonwealth Scholarship during the course of the work.

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I. INTRODUCTION

Meristems of roots of angiosperms provide a convenient system for the study of cell proliferation. The meristem, which includes the proliferating cells is localized at the apex of the root. Moreover meristems are steady state systems, i.e., cell number remains approximately constant because, in any period of time, the number of cells that are produced by cell division equals the number of cells that leave the meristem. Root meristems have been subjected to experimental manipulation in order to study cell proliferation. The agents used in such studies include radiations, temperature changes and chemicals.

In angiosperms, after seed germination, the radicle emerges and grows into the primary root. Later the primary root may develop lateral roots. The lateral roots are not present during embryogenesis. They arise from pericycle and/or endodermal cells of the primary roots sometime after radicle emergence. The group of cells derived from the pericycle and/or endodermal cells and which eventually give rise to a lateral root constitute a primordium.

Generally speaking the meristems of primary and lateral roots have the following parts:

(i) root cap: a protective structure around the tip of the meristem,

(ii) epidermis: a layer of cells that covers the meristem,

(iii) a group of cells that constitute the meristem proper, lie beneath the root cap and the epidermis; these cells undergo cell divisions and produce new cells.

A quiescent centre may or may not be present at the apex of the meristem. The cells of the quiescent centre divide rarely and have very long cell cycle durations (Clowes, 1961). The quiescent centre is not present in the early stages of development of primary roots (Clowes, 1958), or lateral roots; it develops later. In the lateral roots, for example, the quiescent centre develops after root emergence (Clowes, 1958; Macleod and Mclachlan, 1974).

In studies of the proliferating cells of meristems it has often been assumed that the cells are alike. But root meristems are not always homogeneous populations of cells (Jensen and Kavaljian, 1958; Clowes, 1961). Root meristem cells of Vicia faba are known to be heterogeneous for a number of characteristics. These include cell cycle duration, nuclear volume, chromosome volume and cell shape.

(1) Cell cycle duration: though the majority of the cells in the meristem divide actively, some cells divide very rarely (Howard and Dewey, 1960; Clowes, 1961; Murín, 1966; Rasch, Rasch and Woodard, 1967). There are two subpopulations of cells with different cell cycle durations:

(a) fast cycling cells: they constitute about 75% of the cells in mitosis at any time; their average cell cycle duration is 14 hours (Webster and Davidson, 1968). In the long laterals they constitute about 50% of the meristem cell population (Webster and Davidson, 1968). Macleod (1972) however, estimated that about 64% of the meristem cells are fast cycling in the long laterals.

(b) slow cycling cells: they constitute about 25% of the cells in mitosis at any time; their cell cycle duration is 30 hours or more. In the long laterals they constitute about 35% (Webster and Davidson, 1968) or 28% (Macleod, 1972) of the meristem cells of long laterals.

Macleod (1971) also reported that in the old primary root meristems of V. faba, 41% cells are fast cycling and 19% are slow cycling.

Besides these two subpopulations of proliferating cells there are also non-proliferating (non-cycling) cells in the meristems of primary and lateral roots. In the

long lateral meristems the frequency of non-cycling cells is estimated to be 15% (Webster and Davidson, 1968) or 8% (Macleod, 1971). Approximately 40% of the cells in the meristems of primary roots are non-cycling (Macleod, 1971). The small primordia, which is the earliest stage in the development of lateral roots, appear to be composed exclusively of proliferating cells and they do not have any non-cycling cells (Macleod, 1971); 76% of the cells are fast cycling and 24% are slow cycling.

(2) Nuclear volume: it has been suggested (Rasch et al., 1967) that primary root meristems consist of two subpopulations of cells each of which is log-normally distributed: (i) comprising 10% of the cells, which have smaller nuclear volumes and (ii) comprising 90% of the cells, having larger nuclear volumes. This was based on probit analyses of nuclear volumes. However, it was assumed by Rasch et al.; that nuclear volume is proportional to the time spent by the cell in interphase and that nuclear growth rate is uniform throughout the interphase. Hence, the values used for the probit analysis were selected, not taken at random. Continuous treatments with ^{3}H-labeled thymidine for 96-120 hours showed that the 10% of the meristem cells with smaller nuclear volumes did not incorporate any of the labeled precursor into their DNA, and therefore, did not enter S phase in the 96-120 hours. Rasch et al.

suggested that they were non-cycling cells. These cells were found to be dispersed throughout the meristem and therefore, were distinct from the quiescent centre cells. Rasch et al. suggested that presumably they represent additional loci of quiescent cells dispersed throughout the meristem.

The two assumptions made by Rasch et al. (1967) are important since they concern a critical aspect of the physiology of the nucleus, i.e., rate of growth. Rasch et al. (1967) did not provide data to support their assumptions and they cannot be accepted a priori. Evidence to be presented later shows that both assumptions of Rasch et al. (1967) are incorrect.

It has also been reported that nuclear volumes differ in primary and lateral root meristems of V. faba (Bennett, 1970; Bennett, Smith and Smith, 1972) and the various stages of the development of lateral roots of V. faba.

(3) Chromosome volume: Bennett (1970) and Bennett, Smith and Smith (1972) have reported differences in the volume of the chromosome complements of primary and lateral roots of V. faba. Bennett (1970) suggested that these variations are related to different levels of cellular metabolism and are accompanied by parallel changes in the amounts of total nuclear protein, DNA content of

chromosome complements of different volumes remained constant. Hence, there is evidence for the existence of variability both within and between meristems of roots at different developmental stages. The variability occurs both at the cellular level (cell cycle duration and cell shape) as well as the subcellular level (nuclei, chromosomes and nuclear proteins).

(4) Cell shape: the majority of the cells in the meristems appear to be square or rectangular in shape. Dissection of columns of cells from the primary and lateral roots has revealed the presence of odd-shaped cells. The frequency of these odd-shaped cells is associated with (a) variations in the orientation of the spindle, which is sometimes asymmetrical and (b) unequal growth of different parts of a cell (Davidson, 1972, 1975).

The presence of fast, slow and non-cycling cells within a meristem means that it is physiologically complex, since there are cells with different cell cycle durations. It is also structurally complex. Fast and slow cycling cells, for example, are present throughout the meristem. The results suggest that cells adjacent to one another along a column and in different columns may differ in cell cycle duration (Davidson, 1972). In addition to the various types of heterogeneity just described, meristems may differ from one another in other ways. A number of physiological changes take place during

the development of lateral roots from the primordia; i.e., changes in the proportion of cycling and non-cycling cells, changes in the cell cycle duration of fast cycling cells, and in the response to exogeneous thymidine, to colchicine, 5-aminouracil and indole acetic acid (Davidson, 1972).

Variability in cell or nuclear shape, in the volume of metaphase chromosome complements or protein contents of nuclei all provide evidence that the meristematic cells of a root are heterogeneous for more than cell cycle duration. Meristems appear to be physiologically and structurally complex populations of cells. One problem is to relate the heterogeneity of cell cycle duration to the other aspects of heterogeneity shown by meristems. Some evidence for heterogeneity for nuclear volume has been presented (Rasch et al., 1967; Lyndon, 1967; Bennett, 1970); but the results were not interpreted in terms of heterogeneity for cell cycle duration. It was therefore decided to study nuclear volumes, in lateral and primary roots and in primordia, and to attempt to correlate them with the time spent by a cell in interphase.

The analysis of the form of the distribution of nuclear volumes in lateral roots led to similar studies on young and old primaries and on primordia. The results yielded data on randomly selected nuclei that

represent all parts of interphase. For an estimate of nuclear volume in a specific part of the cell cycle, prophase nuclei were measured. Studies of interphase and prophase nuclei revealed that there is considerable variation in nuclear volume. To identify the time when this variability is generated in interphase, a marked population of tetraploid cells was induced in the meristems of long lateral roots by treating them with 0.002% solution of colchicine for one hour. Volumes of interphase nuclei were determined at 4, 10 and 14 hours from the end of treatment. Tetraploid prophase nuclear volumes were determined at 13 and 14 hours from the end of treatment. These results provide data on nuclear volumes in early (G_1), middle (S) and late (G_2) stages of interphase and in prophase for the tetraploid cell population.

Volumes of total chromosome complement at metaphase were determined. These values give an estimate of the minimum possible nuclear volumes in early G_1 and also the degree of variation that would be expected among nuclei in early G_1.

From these results, we can: (1) define the variability and the form of distribution of nuclear volumes in an unselected and hence, heterogeneous population of interphase cells; moreover, this also provides data bearing on the variability of growth rates

of nuclei of meristem cells.

(2) define the variability of volumes of nuclei in prophase; this also indicates variations in growth rates among the nuclei in interphase as well as a lack of correlation between nuclear volumes and the time spent in interphase.

(3) define the variations in the amount of nuclear proteins and its relationship to nuclear volume.

(4) define the changes in nuclear volumes of tetraploid cells as they progress through interphase and when some of them enter prophase.

(5) use the estimates of minimum possible nuclear volumes in early G_1 to define the variation in volumes of nuclei at the beginning of interphase.

The results, reported here, provide substantial evidence for heterogeneity in nuclear volumes and growth rates of nuclei in populations of proliferating cells, in general, as well as within one cell cycle, in particular. Hence they extend the evidence for the heterogeneous nature of the meristem cell populations.

II. MATERIAL AND METHODS

1. Germination and culturing of beans

Seeds of Vicia faba were soaked in distilled water for 24 hours. After removal of testae, they were planted in moist sterilized sand and grown for three days. They were washed with distilled water and suspended over plexiglass tanks containing distilled water; pH was adjusted to 7.0. They were kept in darkness at 21 ± 1°C. The water was continuously aerated and changed twice daily. The seeds were grown for 6 to 7 days, i.e., until the lateral roots were about 2 cms long.

2. Treatment with colchicine

2.1 Induction of tetraploid cells

A population of tetraploid cells was induced by treatment with 0.002% colchicine for one hour. At the end of the treatment the beans were washed carefully with distilled water, transferred to tanks containing fresh distilled water, and whole root systems were fixed 4, 10, 13, 14, 15 and 16 hours from the end of the colchicine treatment.

2.2 Pretreatment of metaphases

Whole root systems were treated for 2 hours with a 0.002% solution of colchicine. This treatment prevents spindle formation and facilitates separation of metaphase chromosomes. With such a low concentration, any

effects colchicine might have on chromosome volume should be minimal. Long lateral roots were fixed immediately after treatment.

3. Fixation and staining

Apices of young primary roots (3 days after germination) and whole root systems, with fully developed lateral roots, were fixed in a chilled mixture of 3 parts absolute alcohol and 1 part glacial acetic acid (v/v) containing a few drops of formaldehyde. Fixed roots were washed for about one hour in 3 or 4 changes of water and hydrolyzed in 1 N HCl at 60°C for 10 minutes and stained in Feulgen. Permanent preparations were made of individual root meristems or primordia.

For determination of nuclear volumes, columns of cells were used. The columns of cells were separated from one another using fine needles. Fast green was used as a counterstain in order to identify the boundaries of cells. The use of columns, in place of squashes or sections, for the study of nuclear volumes has two advantages:

(a) cells are not subjected to pressure, as they are in the preparation of squashes, and therefore the method of preparation does not alter nuclear volumes.

(b) the preparation of sections involve fixation of tissues in formalin which can lead to marked shrinkage of tissues; column separation avoids this distortion of

nuclear volumes.

For determinations of total chromosome complement volumes, squashes were made from the meristems of long lateral roots.

4. Nuclear volumes

Nuclei were measured along two axes using a Zeiss ocular micrometer. In all the developmental stages of the roots that were studied, i.e., small primordia, 1 mm lateral, long laterals, young primary and old primary root meristems, 650 interphase nuclei and 250 prophase nuclei were measured. In the analysis of colchicine-induced tetraploid cells, 650 interphase nuclei were measured 4, 10 and 14 hours from the end of colchicine treatment; 100 prophase nuclei were also measured, 13 and 14 hours from the end of treatment. The tetraploid nuclei were studied only in the long lateral root meristems.

Calculation of Nuclear Volumes

The formulae used for the calculation of nuclear volume (v) are as follows:

spherical nuclei (v) = $4/3\ \pi r^3$

r = 1/2 diameter

nearly spherical nuclei (v) = $4/3\ \pi ab^2$

a = 1/2 length

b = 1/2 width

oval nuclei (v) = $4/3\ \pi ab\ (a+b)/2$

a = 1/2 length (i.e., long axis)

b = 1/2 width (i.e., narrow axis)

elongated nuclei (v) = $4/3\ \pi r^3 + \pi r^2 h$

$= \pi r^2\ (4/3r + h)$

r = 1/2 width (i.e., narrow axis)

h = length (i.e., long axis)

5. Total chromosome complement volumes

Metaphase chromosome volumes were determined only in long lateral root meristems. The lengths of all chromosomes were measured in 50 metaphase complements. Width of five chromatids were measured at random within each cell. Two methods were used for the determination of the volume of the metaphase complement of chromosomes.

Method 1:

Volumes were calculated on the assumption that chromosomes are cylindrical in form.

$v = \pi x\ h\ x\ r^2$

v = total chromosome complement volume

h = total length of all the chromosomes in the complement

r = chromatid width

Method 2:

(i) volumes of chromatids calculated on the assumption that chromatids are cylindrical in form

(ii) volume of total chromosome complement is taken to be double of the total chromatid volume.

total chromatid volume = $\pi \times h \times (r/2)^2$

total chromosome complement volume

= $2_d [\pi \times h \times (r/2)^2]^2$

r = chromatid width

r/2 = chromatid radius

h = total chromatid length

In each method two different average measurements of chromatid width were used: (i) mean of width of five chromatids per cell for each of the 50 cells, (ii) mean of the width of all the chromatids from 50 cells, i.e., 250 chromatids, was used for each of the 50 cells.

6. Calculation cumulative percentage frequency of nuclear volumes

The percentage cumulative frequency of nuclear volumes was also computed. The volumes were at first ranked into an ascending order and the cumulative percentage frequency associated with a particular volume (P_{xi}) was computed as follows:

$$P_{xi} = \frac{2i-1}{2N} \times 100$$

i = rank

xi = nuclear volume at rank i

P_{xi} = cumulative percentage frequency associated with xi

N = number of observations.

7. Probit analysis of nuclear volumes

Cumulative percentage frequencies of nuclear volumes were plotted as a function of linear and log values of nuclear volumes. These plots were done on two kinds of scales: (i) probability scale and (ii) normal equivalent deviate scale. If such a plot generates a straight line, it indicates that the sample being plotted comes from a normally distributed population (Sokal and Rohlf, 1969).

7.1 Probability Scale

Plots of cumulative percentage frequencies of nuclear volumes on a probability scale can be used to estimate the mean and standard deviation of a sample. The mean is approximated by a graphic estimation of the median; the values of mean and median will be close if the distribution is more or less normal. The median value is estimated by dropping a perpendicular from the intersection of the 50% point on the ordinate and the cumulative frequency curve to the abscissa. By dropping similar perpendiculars from the intersections of 15.9 and 84.1% points, and 2.3% and 97.7% points respectively, estimates of $\pm$ 1 and $\pm$ 2 standard deviations are obtained.

The probability scale contracts the scale around the median and expands it at low and high cumulative percentages.

7.2 Normal Equivalent Deviate Scale

This is similar to the probability scale except that it is graduated in units of standard deviation units, and is very much contracted around the median and expanded at the ends. Thus the 50% point becomes 0 standard deviation, the 2.3%, 15.9%, 84.1% and 97.7% point become respectively -2, -1, +1 and +2 standard deviation. Such standard deviations are called normal equivalent deviates.

The cumulative percentage frequencies of values of nuclear volumes were plotted as a function of linear or log values of nuclear volumes on a normal equivalent deviate scale with the help of a computer. The computer also put 95% confidence limits to the cumulative percentage points (see page 17). However, these plots do not show every datum point because the data were grouped to facilitate plotting within the limits set by the computer program. The 95% confidence intervals predict that if the result is repeated under the same or as close as possible conditions, the results will probably be within these limits.

Plots of nuclear volumes on either scale (probability or normal equivalent deviate), may not generate a straight line; they may show a point, or points of inflexion where

the direction of the curve changes. This indicates the presence of more than one population. In a probability scale the point or points of inflexion can be used as break points to separate the subpopulations and the data can be replotted. Upon replotting there should be as many straight lines as there are subpopulations.

8. Calculation of 95% confidence limits for Normal Equivalent Deviate (N.E.D.) plots

This was done according to Colquhoun (1971). If we have ideal, infinitely large population of cells, we take a sample N. The ideal or true proportion of cells with nuclear volume $\leq$ v is P_v. In a finite sample P_v is estimated as:

$$P_v \simeq \frac{r}{N}$$

r = number of cells with nuclear volume $\leq$ v in the sample of N

If it is assumed that r is distributed binomially (because r's are independent of one another), then the estimate of variance of p (= measured value of $\frac{r}{N}$) will be:

$$S^2(p) \simeq \frac{p(1-p)}{N}$$

The 95% confidence limits for $p = \frac{r}{N}$, are therefore:

$$t_{95} S(p)$$

Where t_{95} = value of "Students t" for 95% probability and N degrees of freedom.

Since for N > 30 the t distribution is approximately normal: $t_{95} \approx 1.96$.

Therefore, it is estimated that in 95 out of 100 samples of size N from the ideal population P will be between

$$p + 1.96\sqrt{\frac{p(1-p)}{95}} \quad \text{and} \quad p - 1.96\sqrt{\frac{p(1-p)}{N}}$$

9. Frequency distribution diagrams (histograms) of nuclear volumes

Frequency distribution diagrams (histograms) of nuclei belonging to different volume classes were made. The values of nuclear volumes were grouped into classes in the interval of 100 μm^3 e.g. 100 - 199 μm^3, 200 - 299 μm^3 etc. The number of nuclei belonging to the different volume classes were plotted against the nuclear volume classes.

10. Nuclear morphology

Nuclei of meristem cells and primordial cells were assigned to four different morphological classes, depending upon their shape.

class 1	spherical
class 2	nearly spherical
class 3	oval
class 4	elongated

That the nuclei are only of these four shapes and are not doughnut shaped is supported by the following observations:

(i) before applying the coverslip, when the columns of cells are rotated on all sides, nuclei appear to be spherical, nearly spherical, oval and elongated.

(ii) in transverse sections of roots the nuclei do not appear as rod-shaped as would be expected for nuclei that are doughnut shaped; the nuclei always appear more or less spherical.

11. Statistical analyses

11.1 Empirical Distribution Function Test

A test of goodness of fit of values of nuclear volumes to normal distribution was made using statistics based on the empirical distribution function, EDF (Stephens, 1974). The statistics that were used in this test are modified statistics, D, V, W^2, U^2 and A^2. They were modified in order to ignore the sample size, otherwise one would need to use different tables for different sample sizes. Percentage points for a test of normality (when μ and σ^2 are unknown) based on empirical distribution function are given in Table 2.1. The commonly accepted level of significance for this test is 5%.

11.2 Chi-square Test

Goodness of fit tests of values of nuclear volumes were made using the Chi-square test. The test

was done on grouped data with the help of the computer (CDC 6400).

12. Isolation of nuclei

The method of isolating nuclei from the root meristems is that of McLeish (1963) with some modifications. Meristems of long lateral roots were collected in ice-cold water. They were fixed for 25 - 30 minutes in ice-cold 2% formaldehyde solution (w/v) in a mixture of 0.066 M Na_2HPO_4 and 0.066 M KH_2PO_4 at pH = 7. After washing with the same buffer, the meristems were crushed in the buffer solution, using a hand homogenizer. Thereafter the solution was filtered through a clean cheese-cloth. One to two drops of the filtrate containing the isolated nuclei were put on pre-cooled slides. The suspension of nuclei was then allowed to adhere to slides. Dry slides were stored until required.

13. Estimation of total nuclear protein

Slides of isolated nuclei were first fixed in acetone-alcohol (50:50, v/v) for 30 minutes and then stained in 2:4 dinitro-1 fluorobenzene (DNFB) solution for one hour. The solution contained 0.15 ml DNFB in 100 ml ethanol, 1 ml M sodium bicarbonate and 5 ml water. The slides were then washed in 70% ethanol (15 minutes) and warm water (~30°C; 15 minutes) to remove excess sodium bicarbonate.

They were further washed in 70% ethanol (5 minutes), 95% (10 minutes) and 100% ethanol (2 washes of 10 minutes each); coverslips were applied over a drop of glycerol. DNFB stains protein by forming a yellow complex with the ε-NH_2 group of lysine and the α-NH_2 group of N-terminal amino acids, the sulphhydryl groups of cysteine, the OH group of tyrosine and the imidazole ring of histidine (Sanger, 1949; and Maddy, 1961). This stain gives a good measure of total protein content of nuclei (Mitchell, 1966). Total protein content of nuclei was estimated as absorption at 400 mμ (Mitchell, 1966, 1967). All the slides used for measurements were stained at the same time because (i) this avoids the effects of random dye binding and (ii) it is valid to make quantitative comparisons on such a sample. Photometric measurements were made with a Leitz MPV microscope photometer equipped with an in-line monochromator and photometer designed by Koch, Pasternak and Kruv (1973). The diameters of the nuclei were measured with an ocular micrometer.

Two methods of estimation of the amount of absorbing material i.e. protein per nucleus were used:

(1) $\mathcal{U} = AxE$

Where $\mathcal{U}$ = amount of dye bound

A = area of the nucleus

E = extinction coefficient

Area (A) was calculated according to Garcia and Iorio (1966).

$$A = \frac{\text{background reading of a nucleus}}{\text{reading for the reference ares}} \times \text{actual area of reference area}$$

$$E = \text{Log}_{10}\left(I_i/I_t\right)$$

I_i = intensity of incident light i.e. background reading for a nucleus

I_t = intensity of transmitted light i.e. reading for a nucleus.

(2) $\delta = ExV$

Where δ = amount of dye bound

E = extinction coefficient, calculated in the same way as in method 1.

V = actual volume of the nucleus; was directly computed from the nuclear diameters.

The total amount of protein per nucleus determined by both of these methods was expressed in arbitrary units (AU).

III. RESULTS

1. Interphase and prophase

1.1 Interphase

1.1.1 Long lateral meristems

Nuclei of long lateral root meristems show a large range in volume; the range is 14 fold, 169 to 2381 μm^3. Mean nuclear volume ± S.D. is 766 ± 408 μm^3. A frequency histogram of nuclear volumes (Figure 1 c) reveals a positively skewed distribution and when a probit plot (Material and Methods, p. 15) was made, the values did not yield a straight line. Thus, it appears that nuclear volumes do not form a single population whose values follow one linear normal distribution. In particular, when the probit plot of the cumulative percentage frequency was made on a normal equivalent deviate scale (Figure 2), it revealed that the central part of the population, i.e., between the 25^{th} and 75^{th} percentiles does not fall on a single straight line. The actual data points fall within the 95% confidence limits for most of the population (Figure 2); only in the top 2% of the values do the points lie at the edge of the 95% confidence limits. Furthermore, using the X^2-test reveals that the distribution of nuclear volumes is significantly different ($p = 0.001$; Table 1) from a linear normal distribution. From both

Figure 1

Frequency histograms of volumes of interphase nuclei in *V. faba*.

1 a. Small primordium: 650 nuclei were scored.

1 b. 1 mm lateral root meristem: 650 nuclei were scored.

1 c. Long lateral root meristem: 650 nuclei were scored.

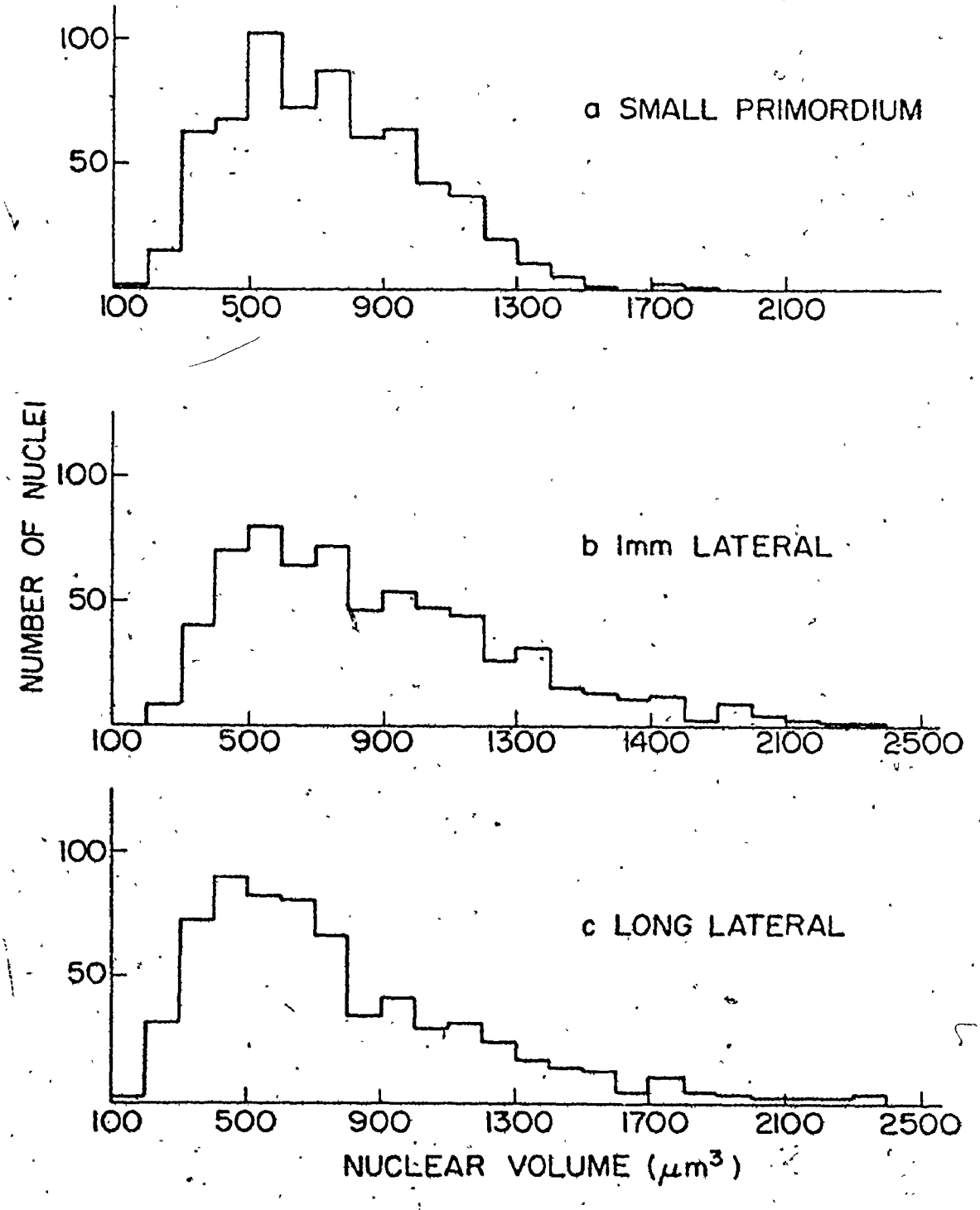

a SMALL PRIMORDIUM
b 1mm LATERAL
c LONG LATERAL
NUMBER OF NUCLEI
100
50
100
500
900
1300
1400
1700
2100
2500
NUCLEAR VOLUME (μm³)

Figure 2

V. faba long lateral root meristems. Plot of cumulative percentage frequencies of nuclear volumes on normal equivalent deviate scale. Ordinate - volume x 1000 (μm^3). Based on 650 nuclei.

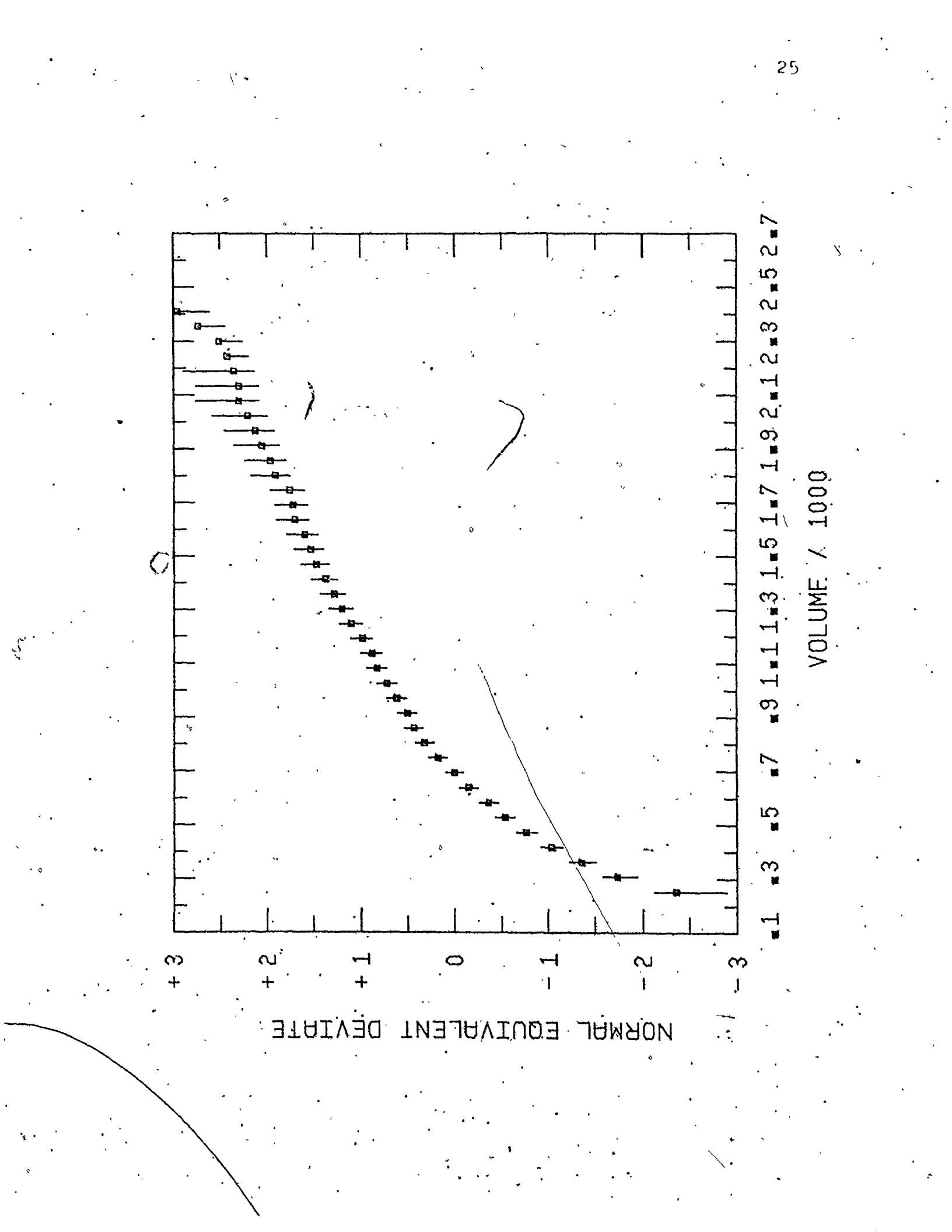

NORMAL EQUIVALENT DEVIATE
+3
+2
+1
0
-1
-2
-3
VOLUME / 1000
1
3
5
7
9
11
13
15
17
19
21
23
25
27

TABLE 1

^{2}-test of the fit of volumes of interphase nuclei in V. faba root meristems. Goodness of fit to inear and log normal distribution were tested. Meristems of roots at different developmental stages were studied. 650 nuclei were measured from each meristem. X^2-test was done on grouped data.

Interphase Nuclear Volume

	Linear				Log			
Develop-mental Stage	Observed X^2-sum	X^2-sum expected at p=0.001	Degrees of freedom	Do values fit a normal distribution	Observed X^2-sum	X^2-sum expected at p=0.001	Degrees of freedom	Do values fit a normal distribution
mall rimordium	90.12	46.80	21	no; significantly different at p=0.001	81.20	48.27	22	no: significantly different at p=0.001
1 mm Lateral	178.14	48.27	22	no: significantly different at p=0.001	56.74	51.18	24	no: significantly different at p=0.001
ong ateral	681.44	48.27	22	no: significantly different at p=0.001	36.54	51.18	24	yes:not significantly different at p=<0.025
oung rimary	1353.02	45.32	20	no: significantly different at p=0.001	65.81	48.27	22	no: significantly different at p=0.001
ld rimary	1526.94	42.31	18	no: significantly different at p=0.001	91.58	51.18	24	no: significantly different at p=0.001

graphical analyses, using probit plots, and from the X^2-test it is concluded that nuclear volumes do not follow one linear normal distribution.

The log values of nuclear volumes were analyzed in the same way; a probit plot on a probability scale (Figure 3) yielded a single straight line. Similar results were obtained when the plot was made on a normal equivalent deviate scale, which somewhat contracts the central part of the distribution relative to a probit plot. Except at the extremes of the distribution, the data points fall within 95% confidence limits (Figure 4). Thus, within the range ± 2 standard deviations log nuclear volumes form a single straight line. This implies that the values of the majority of the population closely approximate a log-normal distribution. The goodness of fit of the log values to a log normal distribution was tested using the X^2-test and EDF, empirical distribution function, statistics (Material and Methods, p.19). The X^2-test, df = 24, gives a X^2 value of 36.54 ($p = >0.025 <0.05$; Table 1); the nuclear volumes are significantly different from one log normal distribution, only at a borderline level. Analysis using EDF statistics indicates that the distribution of log values is not significantly different from one log normal distribution at the 5% level, i.e., the acceptable level of significance for these statistics. The 5% level of acceptance was found applying the V, W^2,

Figure 3

V. faba long lateral root meristems. Plot of cumulative percentage frequencies of log values of nuclear volumes on probability scale. Based on 650 nuclei.

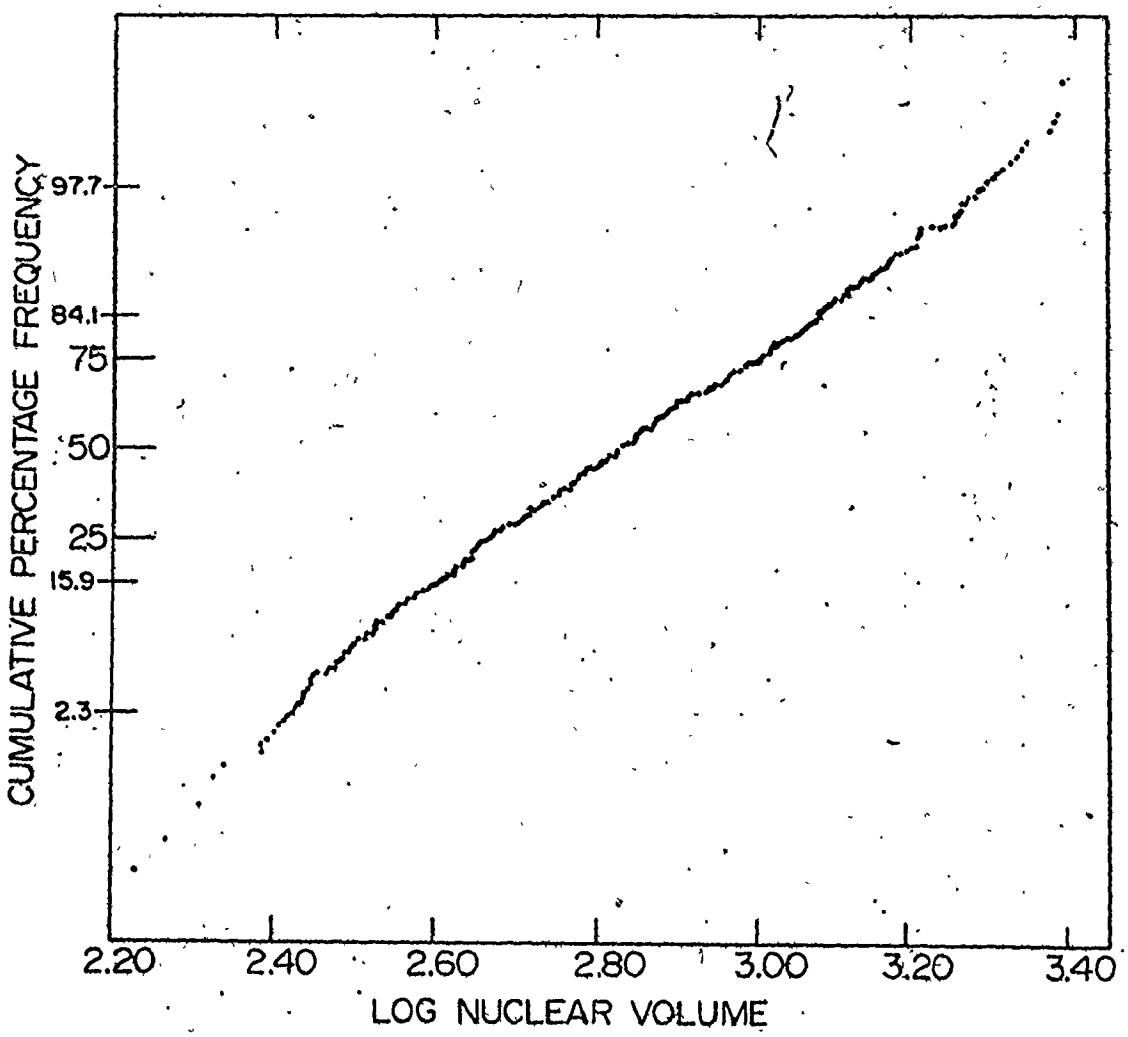
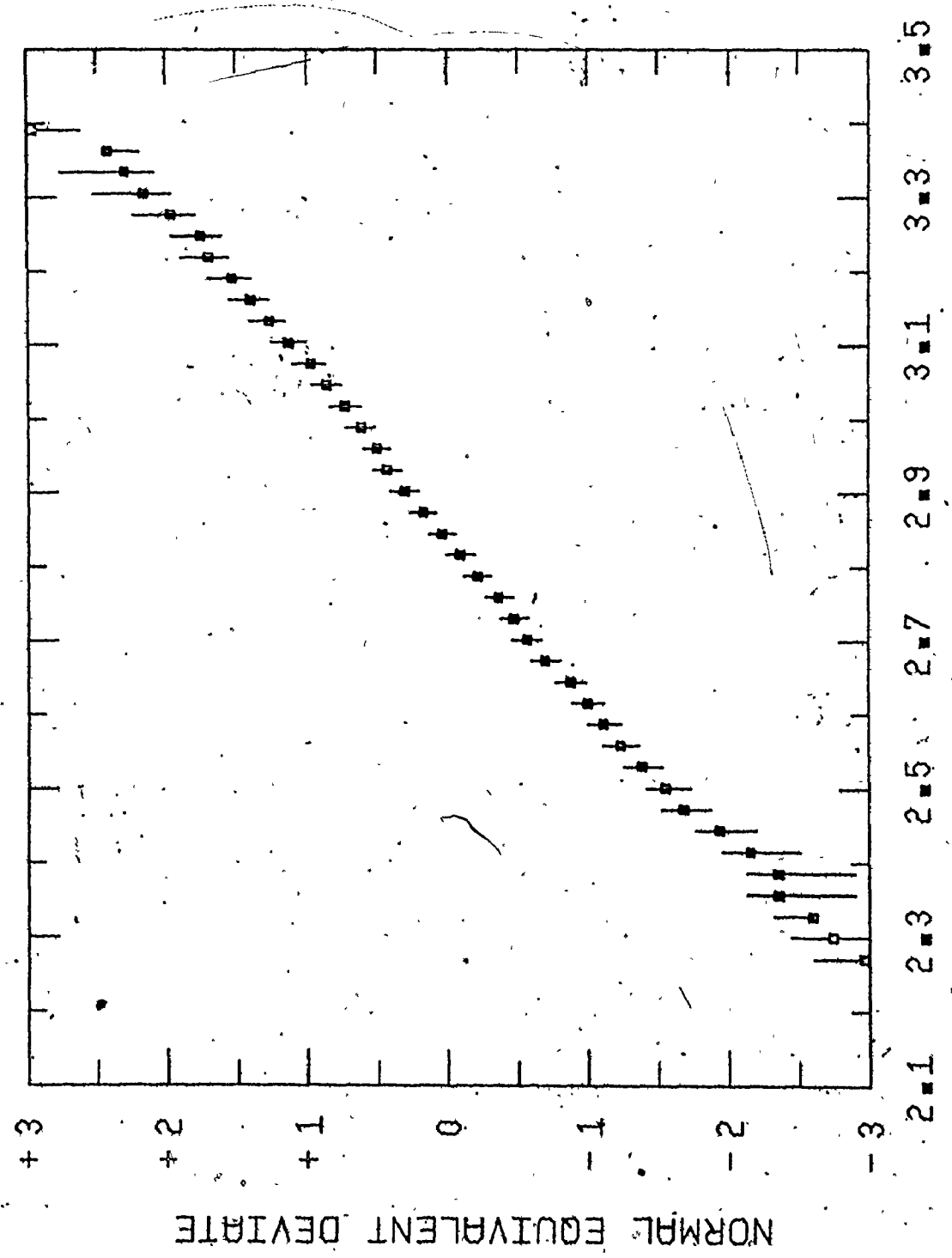

CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
2.20
2.40
2.60
2.80
3.00
3.20
3.40
LOG NUCLEAR VOLUME

Figure 4

V. faba long lateral root meristems. Plot of cumulative percentage frequencies of log values of nuclear volume on normal equivalent deviate scale. Based on 650 nuclei.


NORMAL EQUIVALENT DEVIATE
+3
+2
+1
0
-1
-2
-3
2.1
2.3
2.5
2.7
2.9
3.1
3.3
3.5
LOG 10 (VOLUME)

U^2 and A^2 statistics (Table 2); using the D statistic, the distribution was highly acceptable as being log normal, i.e., at the 15% level (Table 2). It is concluded, therefore, that nuclear volumes in long laterals follow one log normal distribution. The values that do not lie on the line generated by the majority of the nuclear volumes are the extremes of the distribution; these values form the lower and upper 1-2 percentiles of the population.

From the probit plot, we obtain a median nuclear volume of 668 μm^3, which is the value at the 50th percentile (Figure 3) and zero normal equivalent deviate (Figure 4). The large difference between the median and mean, 766 μm^3, nuclear volume is further evidence that the distribution of these values is not symmetrical about the mean value, and is therefore not linear normal.

The lower 50% of the values (Table 3) cover a range of only 499 μm^3 (169 to 668 μm^3), while the upper 50% cover a range of 1613 μm^3 (668 to 2381 μm^3). The range in nuclear volumes indicates, that, in some cells the nuclei grow disproportionately large, relative to the rest of the population. Such a wide range in volume was unexpected in a population of cells that must be very closely, if not absolutely identical in genotype. It seems important, therefore, to define how such a condition could arise. We know that the population (see Introduction,

TABLE 2

2.1. Percentage points for a test of normality (μ and σ^2 unknown) based on the empirical distribution function. The statistics used in the test are those commonly labelled D, W^2, V, U^2 and A^2 (5% level is the common accepted level of significance).

Test Statistic	Percentage Points				
	15.0	10.0	5.0	2.5	1.0
D	0.775	0.819	0.895	0.955	1.035
V	1.320	1.386	1.489	1.585	1.693
W^2	0.091	0.104	0.126	0.148	0.178
U^2	0.085	0.096	0.116	0.136	0.163
A^2	0.576	0.656	0.787	0.918	1.092

TABLE 2

2.2. Results of the empirical distribution function (EDF) test of goodness of fit to one log normal distribution for the interphase nuclear volumes of meristems of V. faba roots at different stages of development. The calculated statistics were suitably modified according to the size of the various samples.

Stages in Development	Modified Statistics				
	D	V	W^2	U^2	A^2
Long lateral	0.751	1.445	0.113	0.109	0.765
Small Primordium	1.487	2.280	0.403	0.386	3.056
1 mm Lateral	0.964	1.880	0.257	0.253	1.612
Young Primary	1.288	2.495	0.392	0.391	2.208
Old Primary	1.773	2.850	0.808	0.666	4.863

TABLE 3

Mean, median and range of volumes of interphase nuclei in V. faba root meristems. Five different meristems were studied; 650 nuclei were measured from each meristem. The median value is taken from the 50 percent point on a probit plot of log values of nuclear volumes. The range ± 1 S.D. covers the values between the 15.9 and 84.1 percent points; ± 2 S.D. covers the values between 2.3 and 97.7 percent points.

Developmental Stage	Nuclear Volume (μm^3)				
	Range	Arithmetic Mean±S.D.	Median	Range Between ± 1 S.D.	Range Between ± 2. S.D.
Small Primordium	193-1845	733±238	706	432-1022	297-1348
1 mm Lateral	258-2340	875±404	789	466-1276	317-1904
Long Lateral	169-2381	766±408	668	398-1177	261-1852
Young Primary	184-2566	803±389	718	429-1165	306-1783
Old Primary	202-1873	691±255	681	418-920	272-1264

p. 3) (a) is asynchronous (b) varies in cell cycle duration and (c) may also vary in the volume of the interphase nucleus in very early G_1. Given this heterogeneity in the population, variation in nuclear volumes would occur:

(i) if growth rate was constant and identical in all nuclei: the wide range in nuclear volumes would then result from the variability in the cell cycle duration,

(ii) if growth rate of the nucleus was different in different cells of each subpopulation, i.e., fast cycling, slow cycling and non-cycling cells,

(iii) even if growth rate was different in different cells of a meristem but was directly proportional to the duration of interphase.

With (iii) we would expect all nuclei at the same stage of the cell cycle to have identical volumes. Mitotic index in lateral roots is 8-10. Thus, the 8-10% nuclei in the cells in late G_2, should, on this basis have closely identical volumes. A discrete group of volumes, making up 8-10% of the population and lying at the upper end of the distribution was never observed (Figure 1 c; Figure 3). Also there should be a discrete group of 16-20% nuclei at the lower end of the distribution; these would be nuclei in early G_1 and would be formed by the division of the 8-10% cells that are in mitosis at any time. This discrete subpopulation was never found.

Furthermore, volumes of nuclei in prophase cover a 6 fold range (Table 5); this is additional evidence that nuclei at one stage in the cell cycle do not have identical volumes. It appears that nuclear volume is not directly proportional to the age of a cell in the cell cycle. This conclusion together with the wide range in nuclear volume in lateral root meristems, suggests that the growth rates of nuclei within a meristem are highly variable. In the analysis of nuclear volumes that follows, this variability in growth rate has been related to the heterogeneous nature of the population.

The nuclei of the cells of long lateral root meristems were found to be of four morphological types: spherical, nearly spherical, oval and elongate (cylindrical). For calculation of nuclear volumes the appropriate formula (Material and Methods, p.12) was used for each nuclear type. In the long lateral root meristems, relatively few cells have spherical nuclei (2.8%); there are 23.7% nearly spherical and 21% elongate nuclei, while 52.6% of cells have an oval nucleus (Table 4). Thus, meristems of long lateral roots are also heterogeneous with respect to nuclear morphology. Since meristems of long laterals differ physiologically from other meristems e.g., small primordium, 1 mm lateral (a little bigger than a just emerged lateral) young and old primary roots, the next step in the analysis was to determine whether or not

TABLE 4

Percentage frequencies of interphase cells with nuclei of different morphological classes in the root meristems of V. faba at different developmental stages. Number of cells scored at each stage = 650.

Developmental Stage	Class-1 Spherical	Class-2 Nearly Spherical	Class-3 Oval	Class-4 Elongated
Small Primordium	10.2	50.6	38.0	1.2
1 mm Lateral	5.8	38.6	44.9	10.6
Long Lateral	2.8	23.7	52.6	21
Young Primary	6.2	51.2	38.3	4.3
Old Primary	4.5	31.4	54.0	10.2

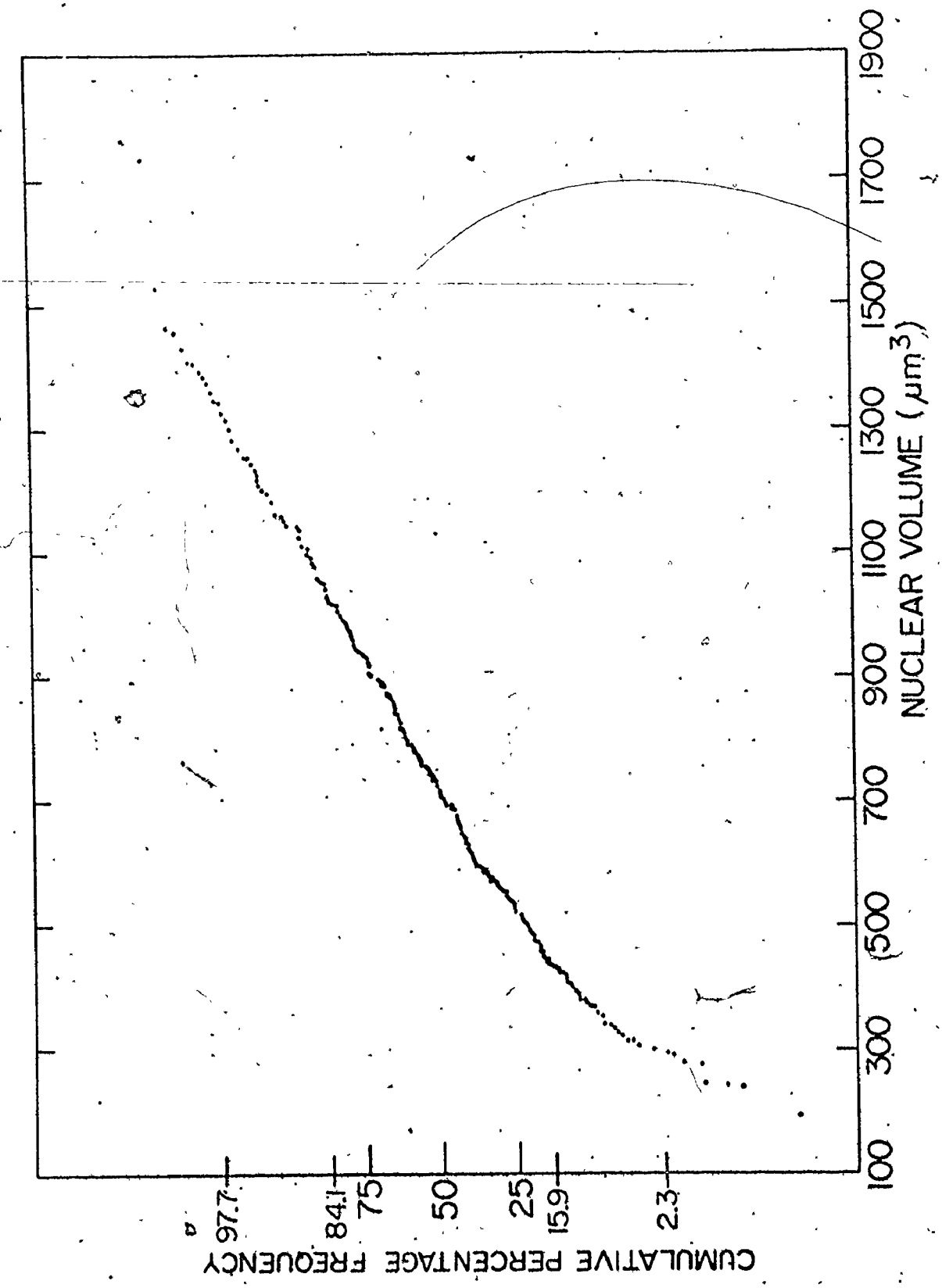
nuclear morphology differed in different stages of root growth. The main features of these results are summarized in Tables 3 and 4. The specific features of each meristem are described in the following sections.

1.1.2 Small Primordium

A small primordium is the earliest stage in the development of a lateral root. In the small primordium, there is about a 10 fold range in nuclear volume, i.e., from 193 to 1845 μm^3. The mean nuclear volume $\pm$ S.D. is 733 $\pm$ 238 μm^3. Both the range of nuclear volumes and the mean nuclear volume are somewhat smaller than in the long lateral root meristems (Table 3). The frequency histogram of nuclear volumes approximates a linear normal distribution more closely than those of a long lateral (Figure 1 a, cf. Figure 1 c). However, a probit plot of nuclear volumes, on a probability scale (Figure 5), generates a straight line only for the upper 80% of the values - 20^{th} to 99.9^{th} percentiles. Thus, about 80% of the nuclei have volumes that follow one linear normal distribution. The lower 20% of the nuclei have volumes that do not fall onto one straight line and are, therefore, distinct from the majority of the nuclei. The volumes of these nuclei do not seem to approximate a linear normal distribution. A probit plot of the cumulative percentage frequencies of nuclear volumes on a normal equivalent

Figure 5

V. faba small primordium. Plot of cumulative percentage frequencies of nuclear volumes on probability scale. Based on 650 nuclei.


CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
100
300
500
700
900
1100
1300
1500
1700
1900
NUCLEAR VOLUME (μm³)

deviate scale (Figure 6) yields a similar result: the population appears to consist of 2 or 3 subpopulations but only the central group of values falls on a straight line. As expected from the graphical analysis, the X^2-test (Table 1) shows that nuclear volumes in small primordia are not distributed linear-normally ($p = 0.001$).

A probit plot of log values of nuclear volumes (Figure 7) reveals the presence of two subpopulations that appear to be log-normally distributed; one subpopulation makes up about 75% of the values, the second subpopulation makes up 25% of the values. The breakdown of the values into subpopulations is slightly different on a probit plot of log values on a normal equivalent deviate scale (Figure 8) but this plot suggests that nuclear volumes do not fit one log normal distribution. This is confirmed by tests of goodness of fit to one log normal distribution.

The X^2-test shows that nuclear volumes deviate significantly ($p = 0.001$) from a log-normal distribution (Table 1). A similar conclusion was reached from the application of EDF statistics (Table 2); all EDF statistics reject a log-normal distribution for these nuclear volumes at a highly significant level (1%).

Since both statistical and graphical analyses of nuclear volumes in small primordia agree that the values do not follow linear or log normal distribution, it

Figure 6

V. faba small primordium. Plot of cumulative percentage frequencies of nuclear volumes on normal equivalent deviate scale. Ordinate - volume x 1000 (μm^3). Based on 650 nuclei.

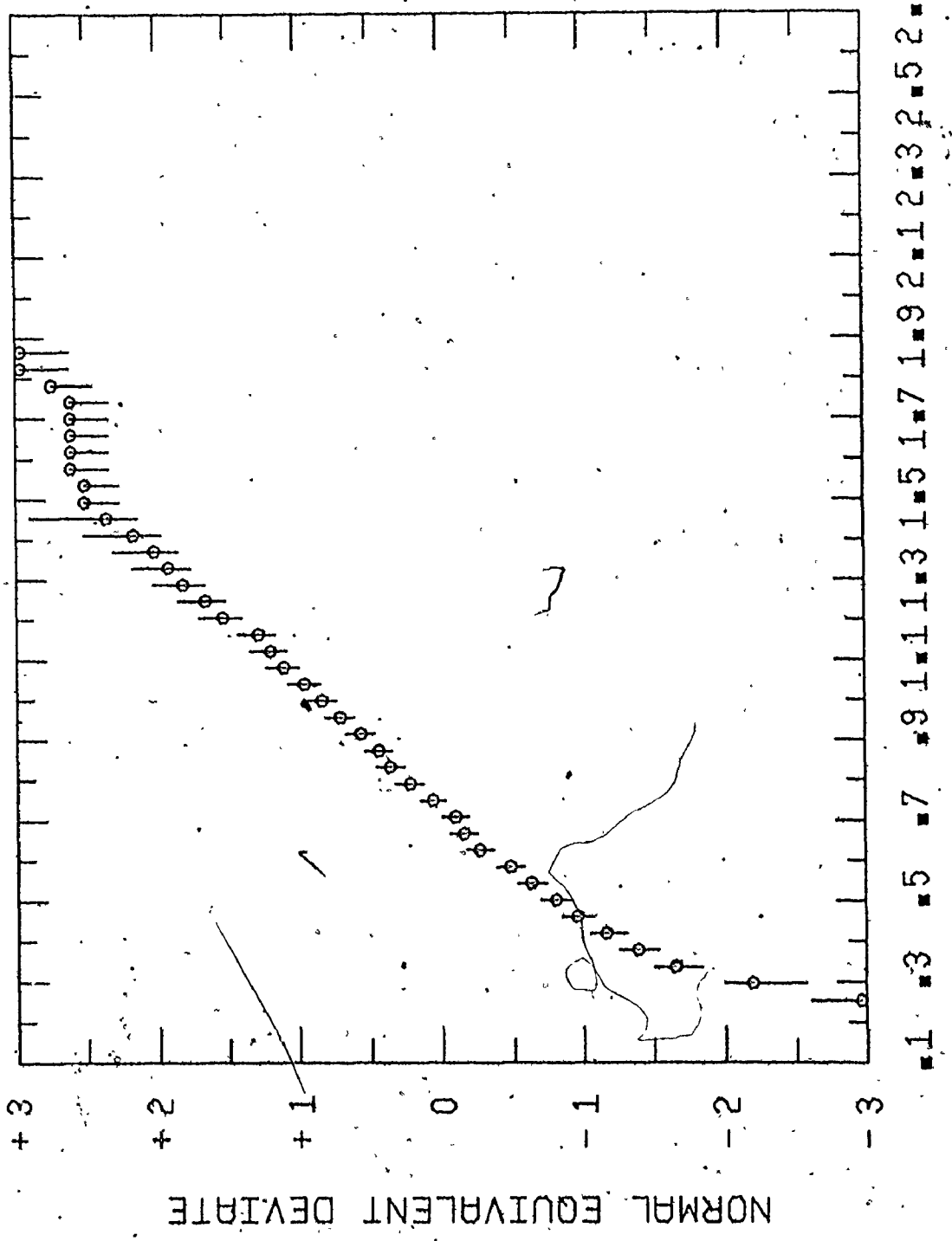

NORMAL EQUIVALENT DEVIATE
+3
+2
+1
0
-1
-2
-3
VOLUME / 1000
1.3 1.5 1.7 1.9 2.1 2.3 2.5 2.7

Figure 7

V. faba small primordium. Plot of cumulative percentage frequencies of log values of nuclear volumes on a probability scale. Based on 650 nuclei.

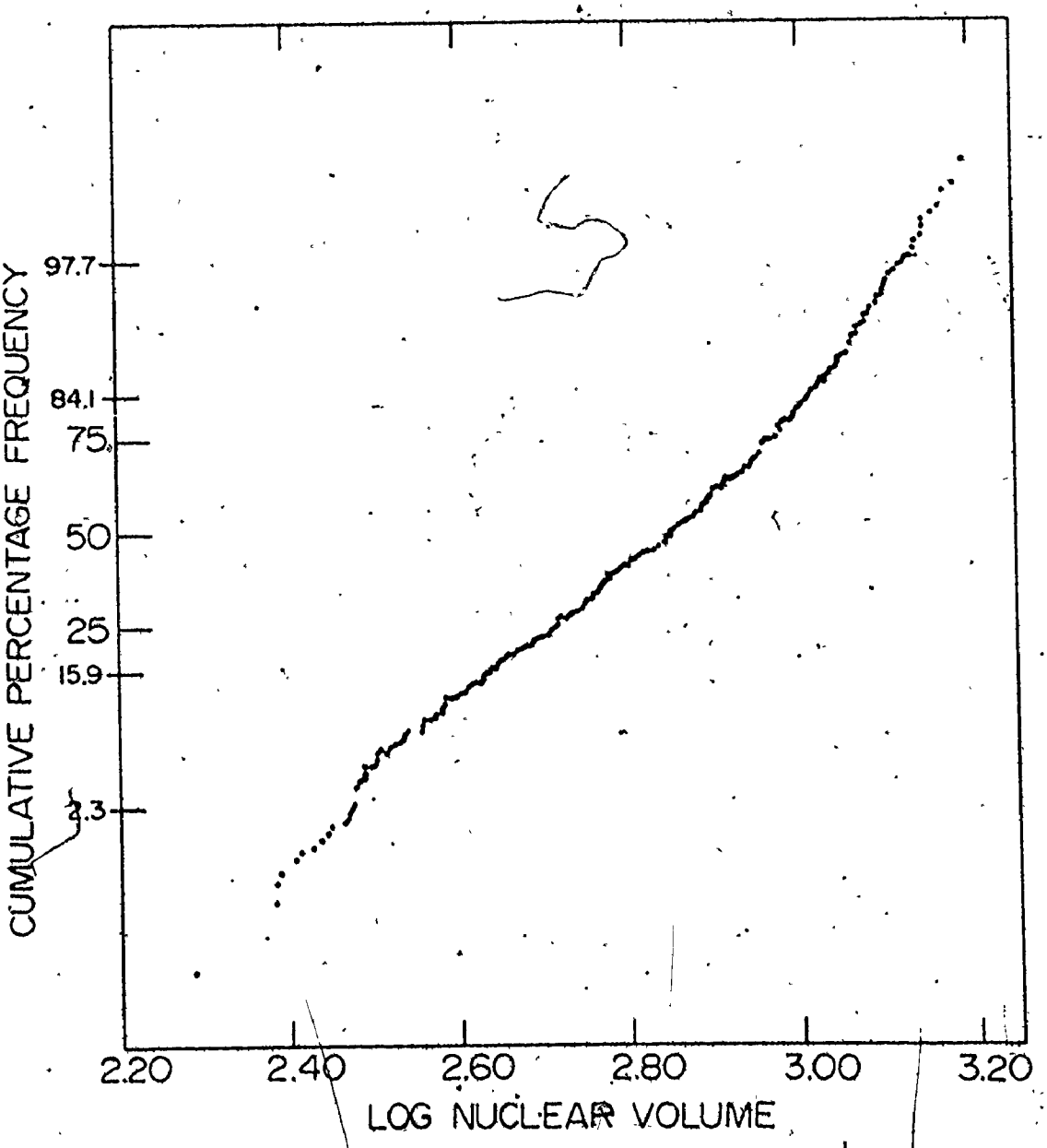
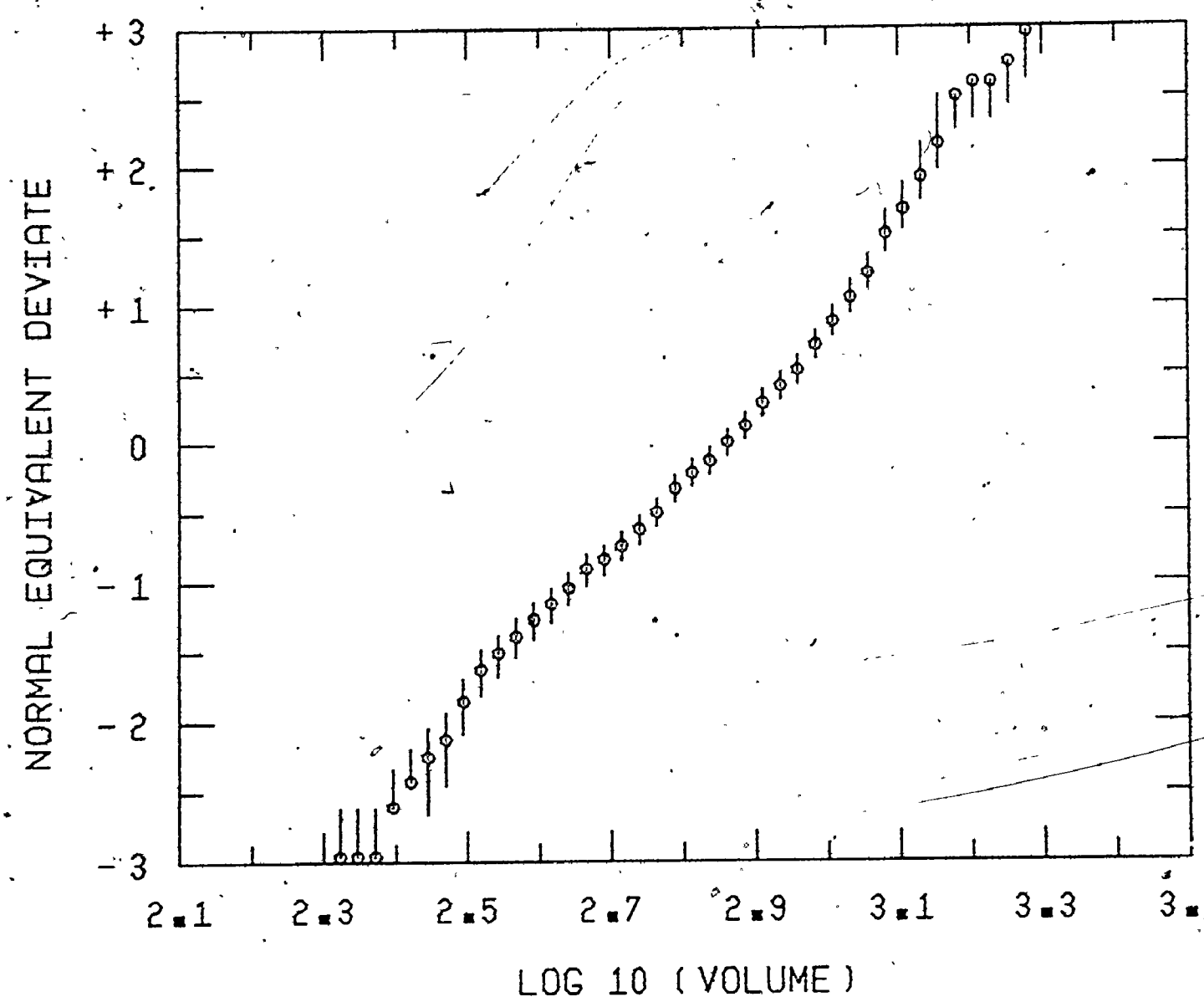

CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
2.20
2.40
2.60
2.80
3.00
3.20
LOG NUCLEAR VOLUME

Figure 8

V. faba small primordium. Plot of cumulative percentage frequencies of log values of nuclear volumes on normal equivalent deviate scale. Based on 650 nuclei.


+ 3
+ 2
+ 1
0
- 1
- 2
- 3
NORMAL EQUIVALENT DEVIATE
2.1
2.3
2.5
2.7
2.9
3.1
3.3
3.5
LOG 10 (VOLUME)

appears that the nuclei are even more heterogeneous with respect to rates of nuclear growth than the nuclei of long lateral root meristems. These results from small primordia confirm that increases in nuclear volume are not uniform throughout a meristem.

The cells of the small primordia are also heterogeneous with respect to nuclear morphology; about half the cells (50.6%) have nearly spherical nuclei, 10.2% have spherical nuclei, 38% have oval nuclei and a remarkably low proportion of cells (1.2%) have elongate nuclei. Compared to the long laterals, relatively higher proportions of cells of small primordium have spherical and nearly spherical nuclei (Table 4), while the proportion of cells with oval and elongate nuclei are lower. This is responsible, in part, for the smaller range of volumes in the small primordium relative to the long lateral (Table 3). Differences in the range of nuclear volumes and perhaps also in the growth rates of nuclei in long laterals and small primordia are reflected in the different patterns of distribution of nuclear volumes (Figure 1 a, cf. 1 e; Figures 5 and 7, cf. Figure 3; Figures 6 and 8, cf. Figures 2 and 4).

The median and mean nuclear volumes are similar in small primordia, 706 and 733 μm^3 (Table 3) unlike the situation in long laterals, where they are quite different, 668 and 766 μm^3. Since small primordia and long laterals

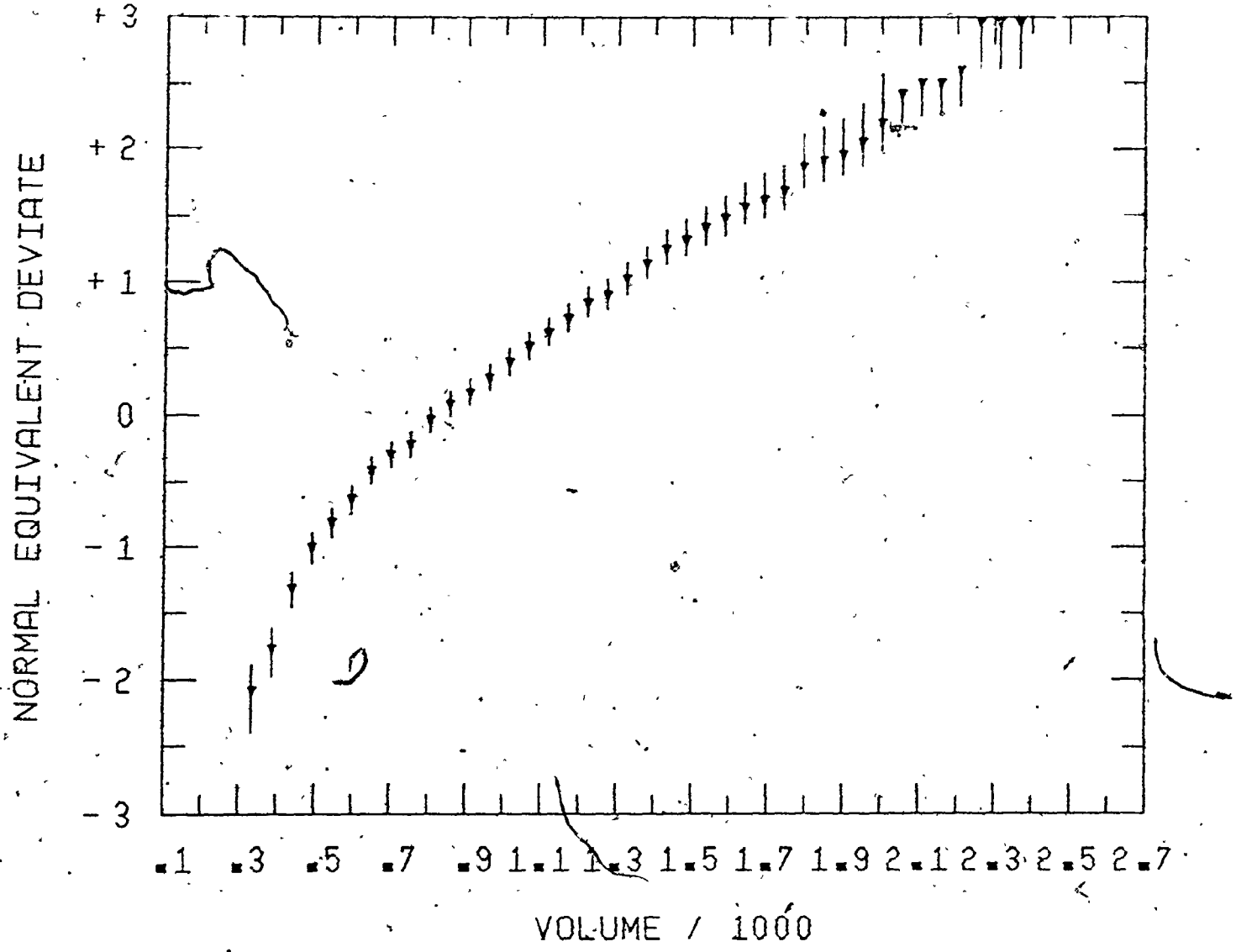
also differ in the form of distribution of their nuclear volumes (Figure 1 a, cf. Figure 1 c; Figures 5 and 7, cf. Figure 3; Figures 6 and 8, cf. Figures 2 and 4), it is clear that there are significant differences in the overall pattern of nuclear growth between two meristems that are part of a single developmental pathway. This is further evidence of heterogeneity in meristematic cells of V. faba.

1.1.3 1 mm lateral meristems

Laterals that are 1 mm long have just emerged from the cortex of the main root. In these meristems there is about a 10 fold range in nuclear volumes, i.e., 258 to 2340 μm^3 and the mean nuclear volume $\pm$ S.D. is 875 $\pm$ 404 μm^3. A frequency histogram of nuclear volumes (Figure 1 b) yields a positively skewed distribution. A probit plot of cumulative percentage frequencies of nuclear volumes on a normal equivalent deviate scale (Figure 9) shows that the values of nuclear volumes do not fall into a straight line, i.e., these values do not appear to follow one linear normal distribution and a X^2-test of nuclear volumes is significantly different ($p = 0.001$) from one linear normal distribution (Table 1). A normal equivalent deviate transformation of the cumulative percentage frequency curve of log nuclear volumes yielded one straight line; but this is confined to the central

Figure 9

V. faba 1 mm lateral root meristems. Plot of cumulative percentage frequencies of nuclear volumes on normal equivalent deviate scale. Ordinate - volume x 1000 (μm^3).


NORMAL EQUIVALENT DEVIATE
+ 3
+ 2
+ 1
0
- 1
- 2
- 3
.1
.3
.5
.7
.9
1.1
1.3
1.5
1.7
1.9
2.1
2.3
2.5
2.7
VOLUME / 1000

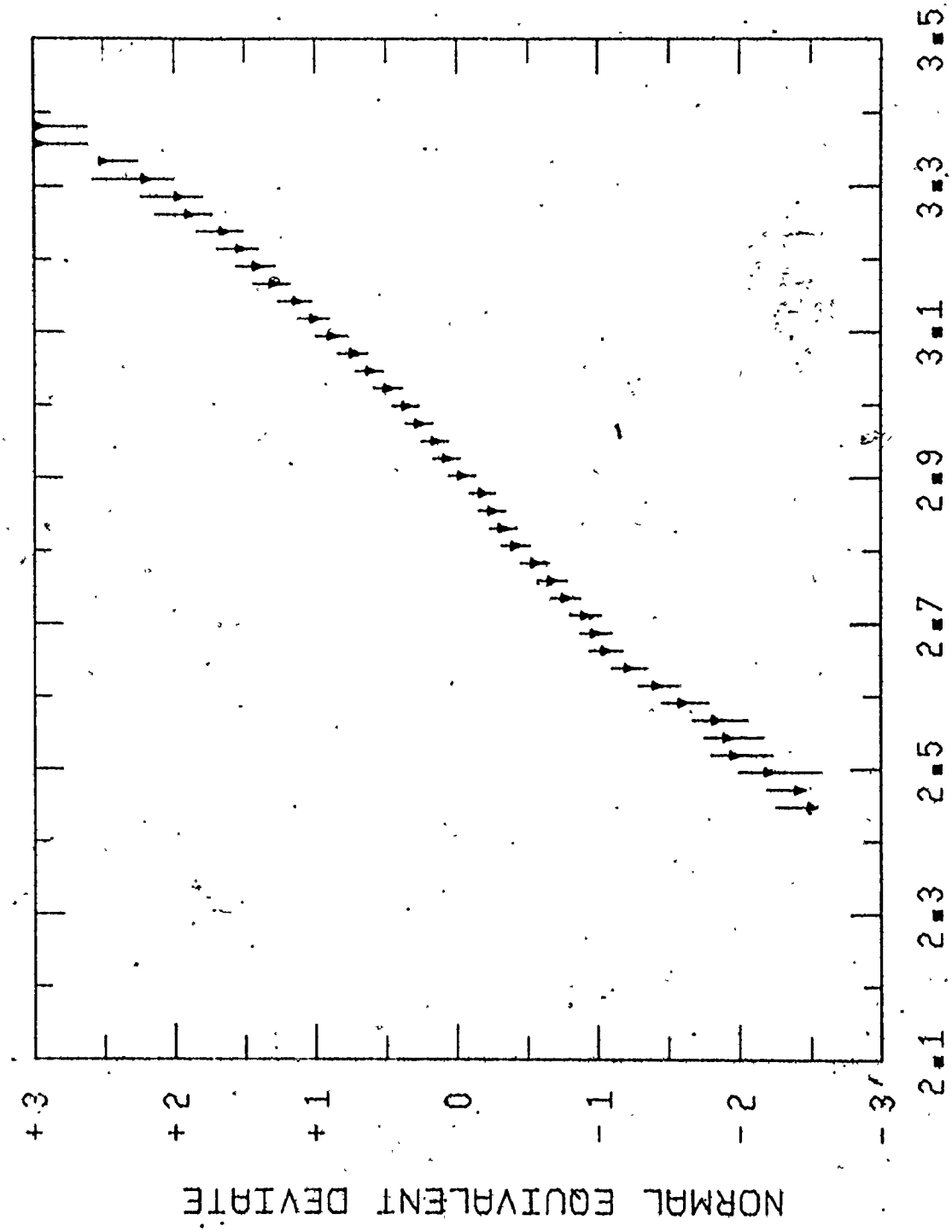
group of values, i.e., -0.7 to + 1.1 S.D. (Figure 10). The deviation from one log-normally distributed population was significant using the X^2-test ($p = 0.001$; Table 1) or EDF statistics (Table 2); all EDF statistics except D were significantly different at the 1% level. The statistic D was not significantly different at 1% (5% is the acceptable significance level).

A 1 mm lateral is an intermediate stage in the development of a long lateral root from a small primordium. During the growth of a small primordium into 1 mm lateral the form of the distribution of nuclear volumes changes (Figure 8, cf. Figure 10). This change continues as a 1 mm lateral becomes a long lateral; the complex form of the distribution of nuclear volumes in 1 mm laterals is resolved into one log-normally distributed population (cf. Figure 4).

The proportion of cells with spherical, nearly spherical, oval and elongate nuclei in 1 mm laterals are 5.8%, 38.6%, 44.9% and 10.6%. In these values too, 1 mm laterals are intermediate between long laterals and small primordia (Table 4). Lateral root development is accompanied by a number of changes in nuclear form and growth. Parallel changes in the growth of the primary root were also looked for by examining nuclei in 3 and 9 day old primaries.

Figure 10

V. faba 1 mm lateral root meristems. Plot of cumulative percentage frequencies of log values of nuclear volumes on normal equivalent deviate scale. Based on 650 nuclei.


NORMAL EQUIVALENT DEVIATE
+3
+2
+1
0
-1
-2
-3
LOG 10 (VOLUME)
2.1
2.3
2.5
2.7
2.9
3.1
3.3
3.5

1.1.4 Young primary meristems

The young primary root meristem cells, like those of the long lateral root meristems, show an approximately 14 fold range in nuclear volumes, 184 to 2566 μm^3. Mean nuclear volume ± S.D. is 803 ± 389 μm^3, higher than in the small primordium (733 ± 238 μm^3) or long laterals (766 ± 408 μm^3), but lower than that in the 1 mm lateral root meristem (875 ± 404 μm^3). In the young primary root meristem, there is a high proportion of cells with nearly spherical (51.2%) nuclei; 6.2% have spherical nuclei, 38.3% have oval and 4.3% have elongate nuclei. This situation is very similar to that in small primordia (cf. Table 4). Both in the young primary and small primordium, the proportion of cells with spherical and nearly spherical nuclei is higher than in other meristems (Table 4).

A frequency histogram of nuclear volumes appears to be positively skewed (Figure 11 a). A probit plot of cumulative percentage frequencies of nuclear volumes on a normal equivalent deviate scale (Figure 12) shows that these values do not fall into one straight line. This plot gives 95% confidence limits. Both linear (Figure 12) and log (Figure 13) plots of nuclear volumes fail to yield straight lines that include more than 50% of the data points. Moreover, a X^2-test showed

Figure 11

Frequencies histograms of volumes of interphase nuclei in V. faba.

11 a. Young primary root meristems: 650 nuclei were scored.

11 b. Old primary root meristems: 650 nuclei were scored.

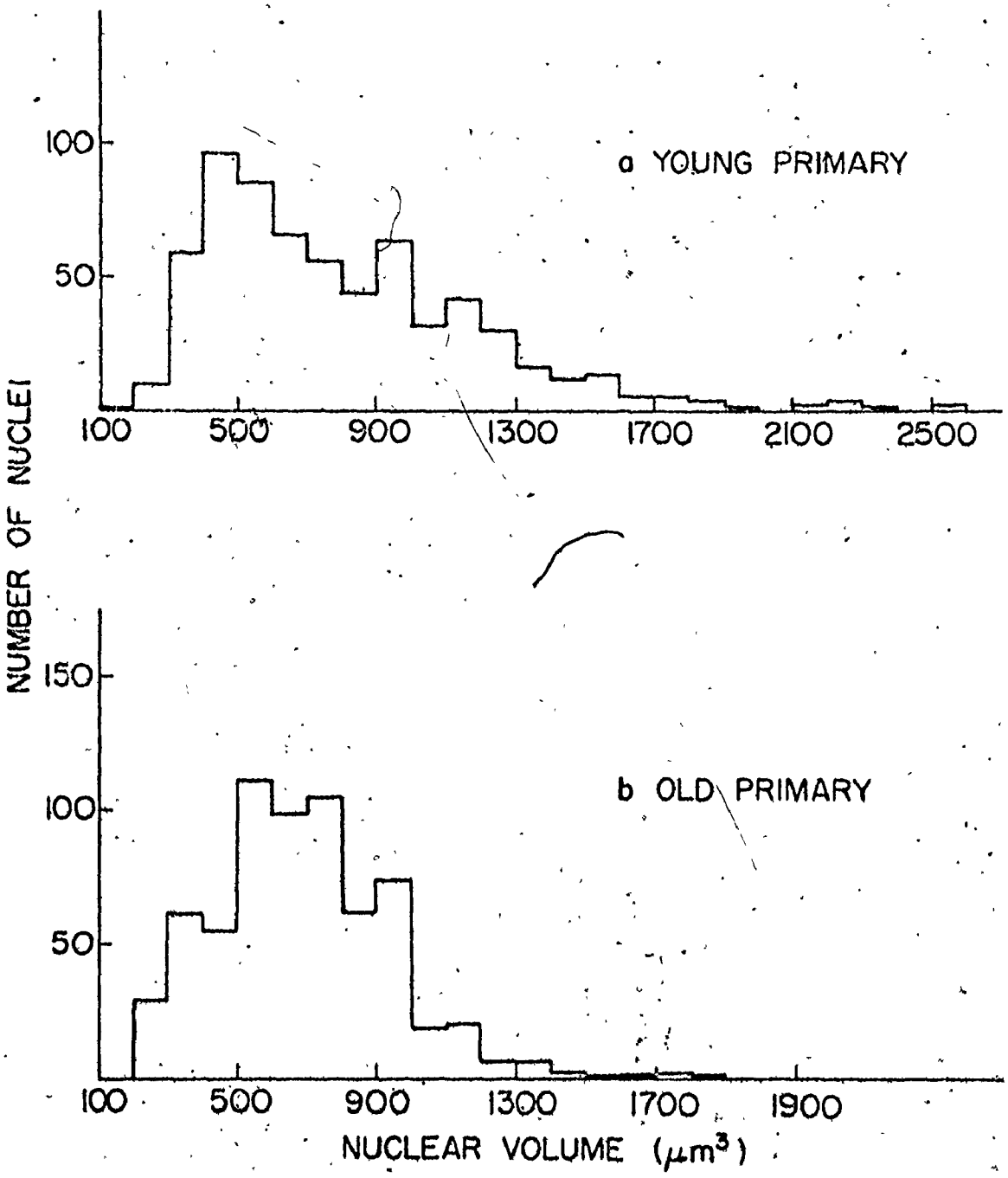

a YOUNG PRIMARY
b OLD PRIMARY
NUMBER OF NUCLEI
100
50
150
100
50
100
500
900
1300
1700
2100
2500
1900
NUCLEAR VOLUME (μm³)

Figure 12

V. faba young primary root meristems.

Plot of cumulative percentage frequencies of nuclear volumes on normal equivalent deviate scale. Ordinate - volume x 1000 (μm^3). Based on 650 nuclei.

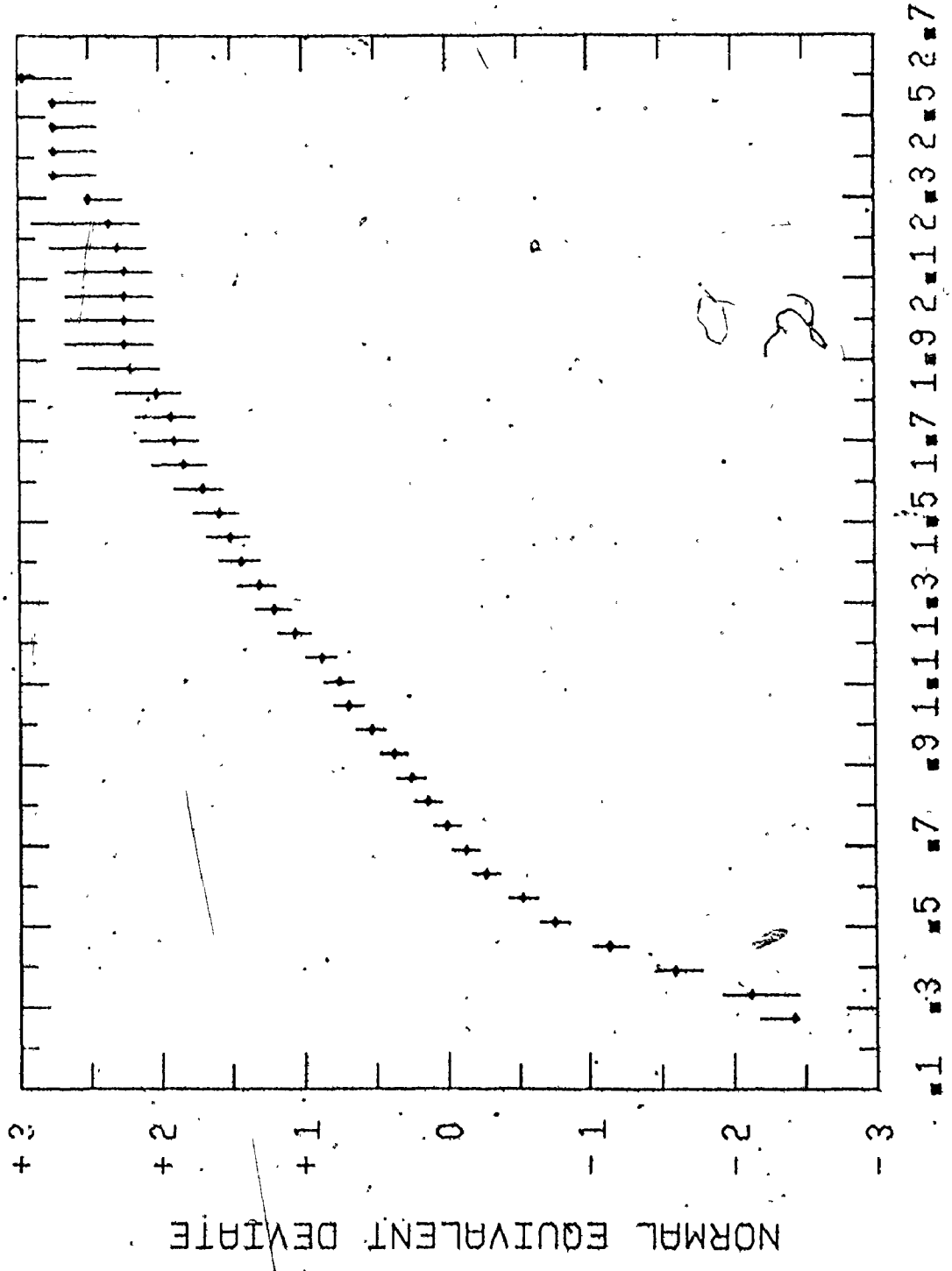
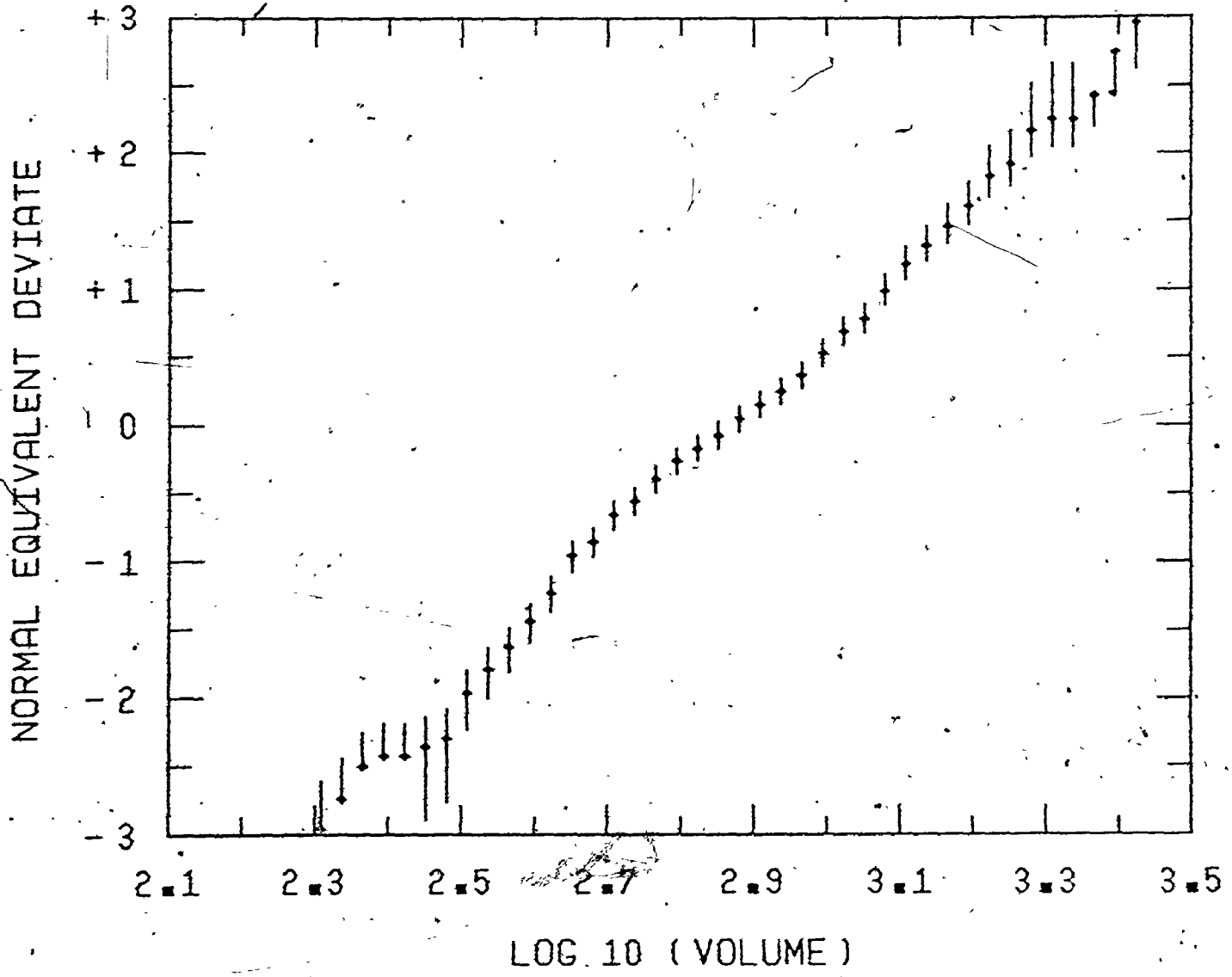

NORMAL EQUIVALENT DEVIATE
+2
+2
+1
0
-1
-2
-3
1
3
5
7
9
11
13
15
17
19
21
23
25
27
VOLUME / 1000

Figure 13

V. faba young primary root meristems. Plot of cumulative percentage frequencies of log values of nuclear volumes on normal equivalent deviate scale. Based on 650 nuclei.


NORMAL EQUIVALENT DEVIATE
+3
+2
+1
0
-1
-2
-3
2.1
2.3
2.5
2.7
2.9
3.1
3.3
3.5
LOG 10 (VOLUME)

that both linear and log values differed significantly from a normal distribution ($p = 0.001$; Table 1). Using the EDF statistics, it appeared that the deviation from one log-normally distributed population was significantly different at 1% level (Table 2). The normal equivalent transformation of the cumulative percentage frequency curve of log values of nuclear volumes (Figure 13) shows that the central group of nuclei, i.e., those lying between -1 to +0.8 standard deviation units, fall onto one straight line; values outside these limits, at both upper and lower ends of the distribution are distinct from the central group. This indicates that the sample is heterogeneous. Even where there appears, in the centre of the distribution, a group of nuclei whose volumes are distributed log-normally (Figure 13), it cannot be concluded that this group is all part of a single population. Particularly, in young primaries, small primordia and 1 mm laterals, overlapping distributions of two subpopulations may generate what appears to be one log-normally distributed population.

1.1.5 Old primary meristems

In the old primary root meristems there is a 9 fold range in nuclear volumes, 202 to 1873 μm^3. The mean nuclear volume $\pm$ S.D. is 691 $\pm$ 255 μm^3. The range of

nuclear volumes in the old primary meristem is very similar to that in the small primordium (Table 3), but their mean nuclear volume is slightly smaller. Range and mean nuclear volume is slightly smaller in old primaries than in the other meristems studied (Table 3) and there are differences in the frequencies of different classes of nuclei (Table 4). In the old primary, 4.5% of the cells have spherical nuclei and 31.4% have nearly spherical nuclei; 54% of the cells have oval nuclei and 10.2% have elongate nuclei. Thus, old meristems, i.e., mature laterals or old primaries have high frequencies of oval and elongate nuclei and low frequencies of spherical or nearly spherical nuclei, while in young meristems, i.e., small primordium and young primaries, spherical and nearly spherical nuclei are much more frequent than oval or elongate nuclei (Table 4; Figure 14).

The histogram of frequencies of nuclear volumes (Figure 11 b) appears to be symmetrical in form and the probit plot of nuclear volumes yields a straight line for at least 90% of the values, i.e., from 7^{th} to 97^{th} percentiles (Figure 15). A probit plot of the cumulative percentage frequency of nuclear volumes on a normal equivalent deviate scale (Figure 16) also revealed that the volumes of the nuclei lying between -1.5 and +1.45 S.D. units form a straight line. There is also a close agreement between the mean, 691 $\pm$ 255 μm^3,

Figure 14

Frequencies of nuclei of different shapes in the meristem cells of roots of V. faba at different developmental stages.

spherical and nearly spherical nuclei

oval nuclei

elongated nuclei

SP	Small primordium: 650 nuclei were scored.
LP	Large primordium: 1500 nuclei were scored.
VLP-JNO	Very large primordium = Just not out: 1500 nuclei were scored.
JO	Just out lateral root meristem: 1500 nuclei were scored.
1 mm L	1 mm lateral root meristem: 650 nuclei were scored.
LL	Long lateral root meristem: 650 nuclei were scored.
YP	Young primary root meristem: 650 nuclei were scored.
OP	Old primary root meristem: 650 nuclei were scored.

The data from LP, VLP-JNO and JO have not been included in this report.

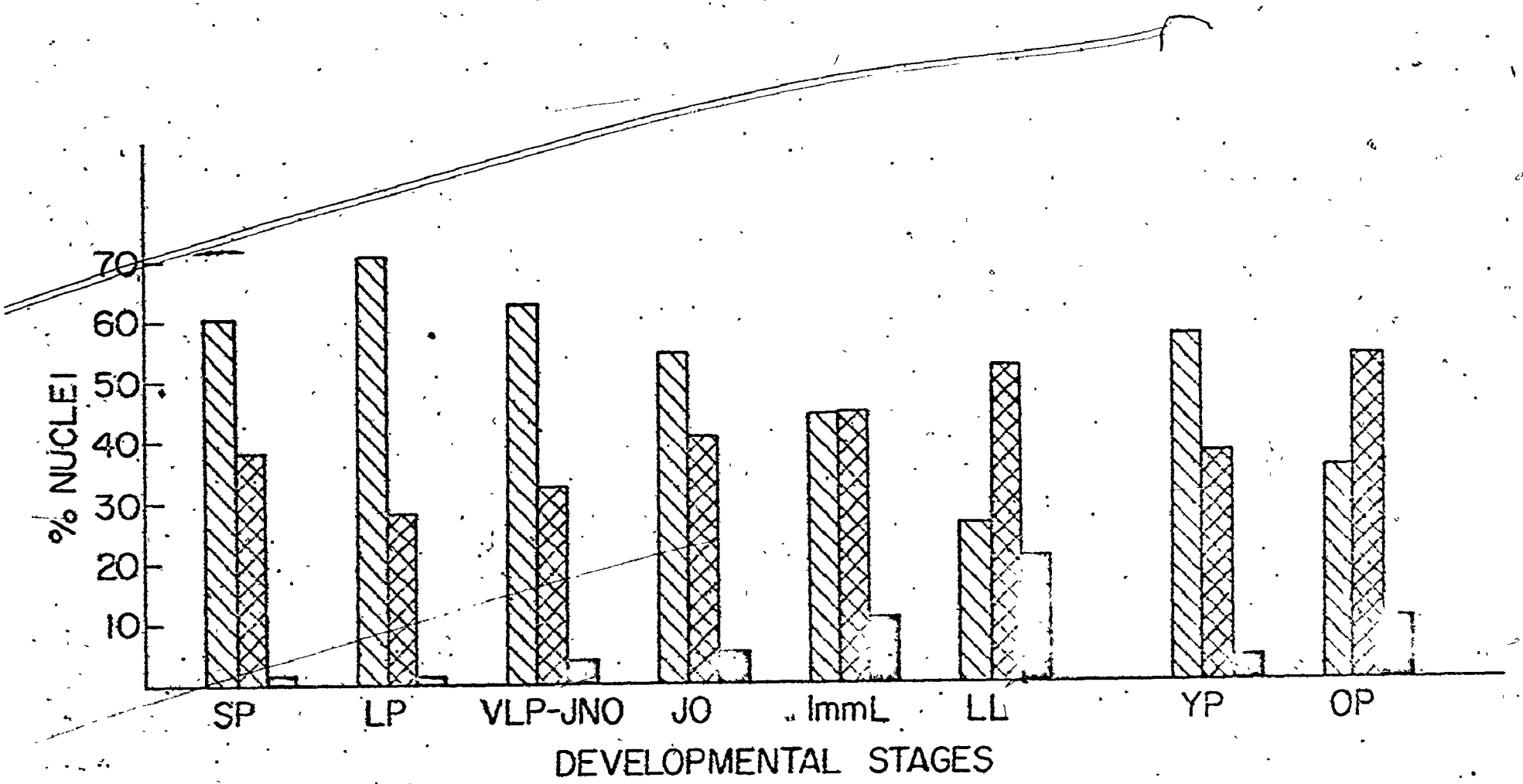

70
60
50
40
30
20
10
% NUCLEI
SP
LP
VLP-JNO
JO
ImmL
LL
YP
OP
DEVELOPMENTAL STAGES

Figure 15

V. faba old primary root meristems.

Plot of cumulative percentage frequencies of nuclear volumes on probability scale. Based on 650 nuclei.

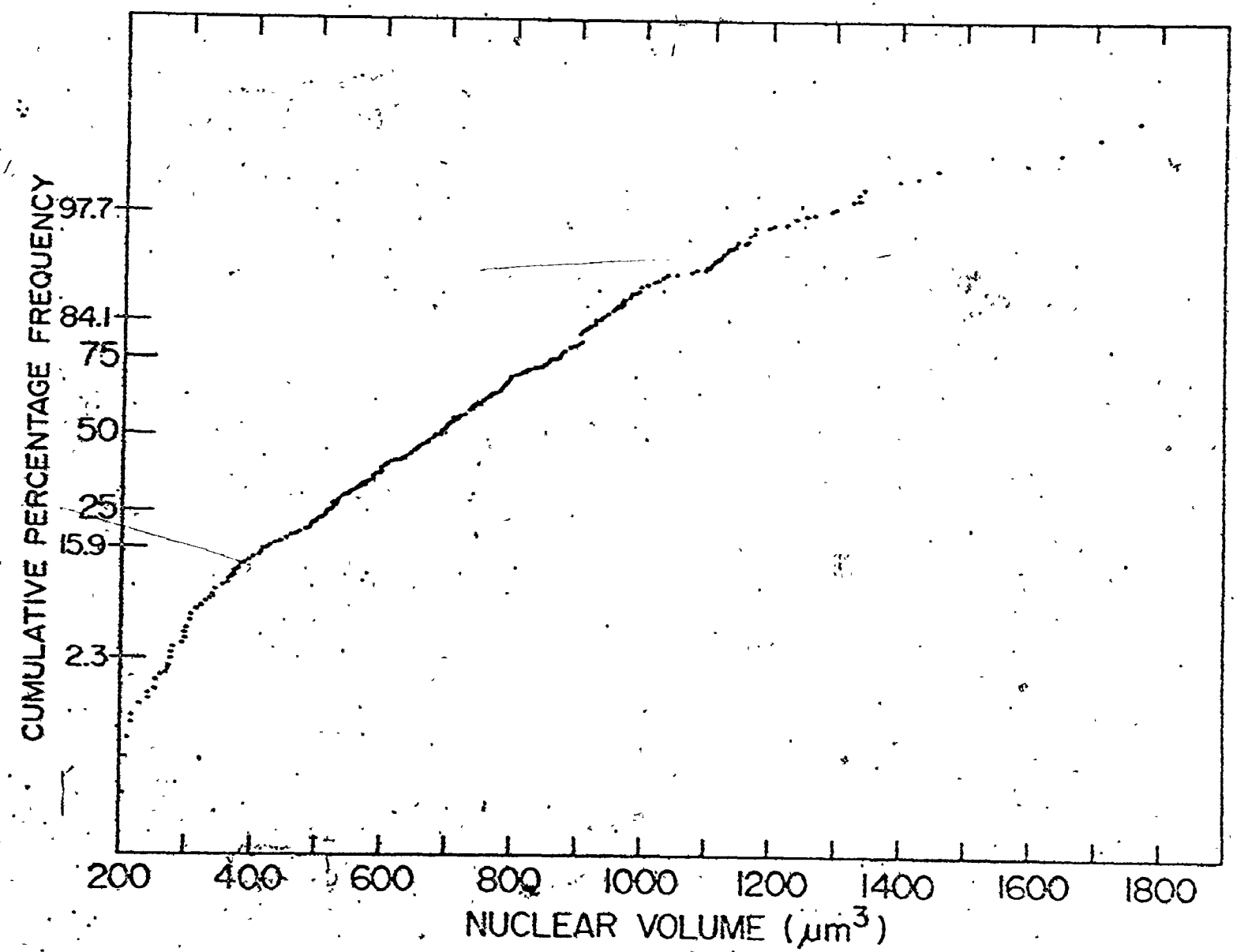
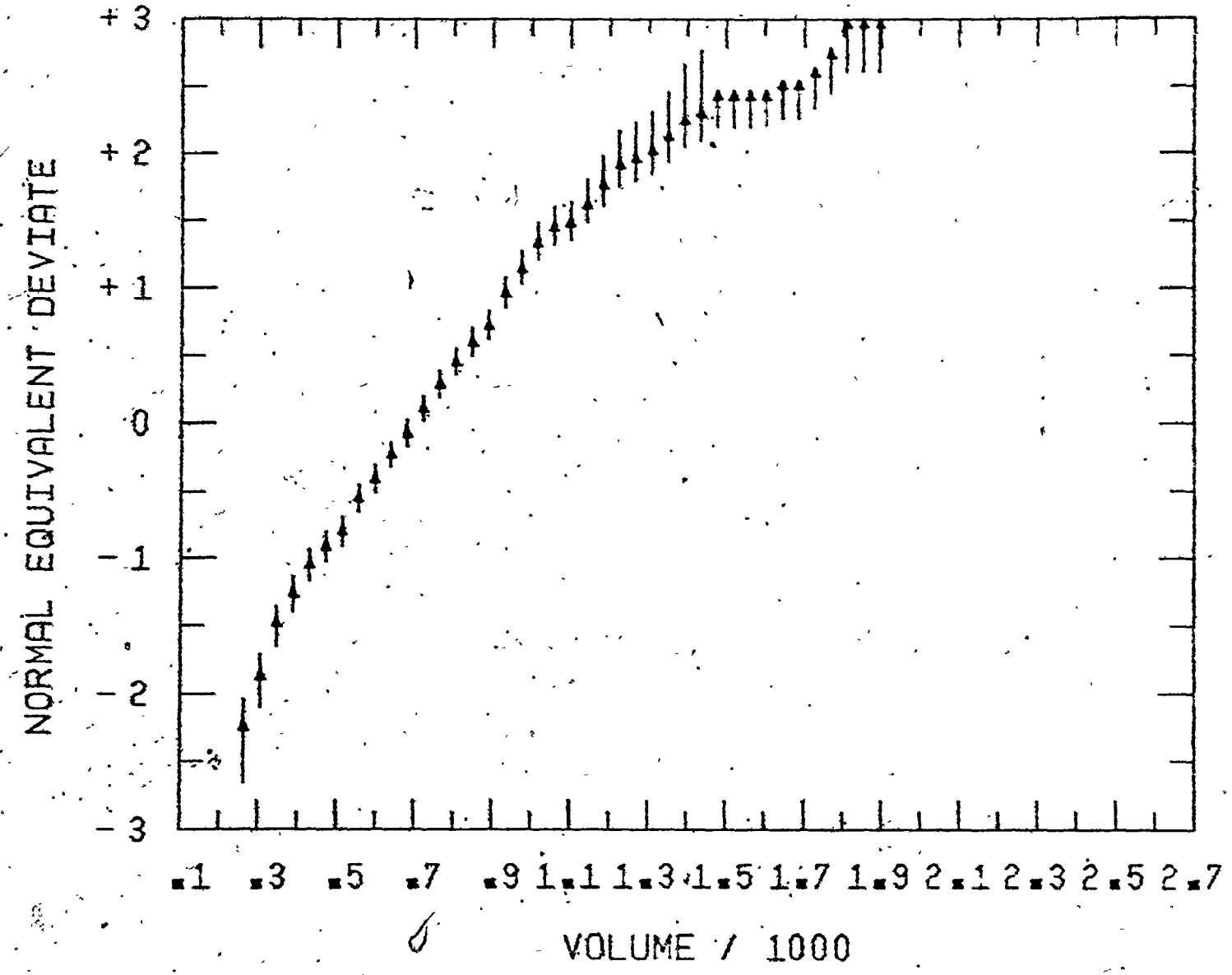

CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
200
400
600
800
1000
1200
1400
1600
1800
NUCLEAR VOLUME (μm³)

Figure 16

V. faba old primary root meristems.

Plot of cumulative percentage frequencies of nuclear volumes on normal equivalent deviate scale. Ordinate - volume x 1000 (μm^3). Based on 650 nuclei.


+3
+2
+1
0
-1
-2
-3
NORMAL EQUIVALENT DEVIATE
.1
.3
.5
.7
.9
1.1
1.3
1.5
1.7
1.9
2.1
2.3
2.5
2.7
VOLUME / 1000

and the median, 681 μm^3 (obtained from the 50th percentile in Figure 17), nuclear volumes. From these lines of evidence, it appears that the values of the majority of the nuclei approximate a linear normal distribution. The X^2-test, however, reveals that these values do not follow one linear normal distribution ($p = 0.001$; Table 1). A probit plot of the cumulative percentage frequencies of log values of nuclear volumes (Figure 17) indicates that 60% of the values of nuclear volumes, i.e., from 20th to 80th percentiles fall onto one straight line and they may represent the bulk of one log-normally distributed population. But the 20% of the values at the bottom end of the distribution and the 20% of the values at the top end of the distribution appear to form discrete subpopulations (see also Figure 18).

Though the nuclear volumes from the old primary roots appear, from graphical analysis (Figures 15 and 16), to be linear-normally distributed, the population is clearly heterogeneous. A X^2-test showed that it is not made up of a single population that is linear or log-normally distributed ($p = 0.001$ for both distributions; Table 1). Furthermore, use of EDF statistics showed that all statistics are significantly different at 1% level (Table 2) and hence, the distribution of nuclear volumes deviates significantly from one log-normal population.

Figure 17.

V. faba old primary root meristems.
Plot of cumulative percentage frequencies of log values of nuclear volumes on probability scale.
Based on 650 nuclei.

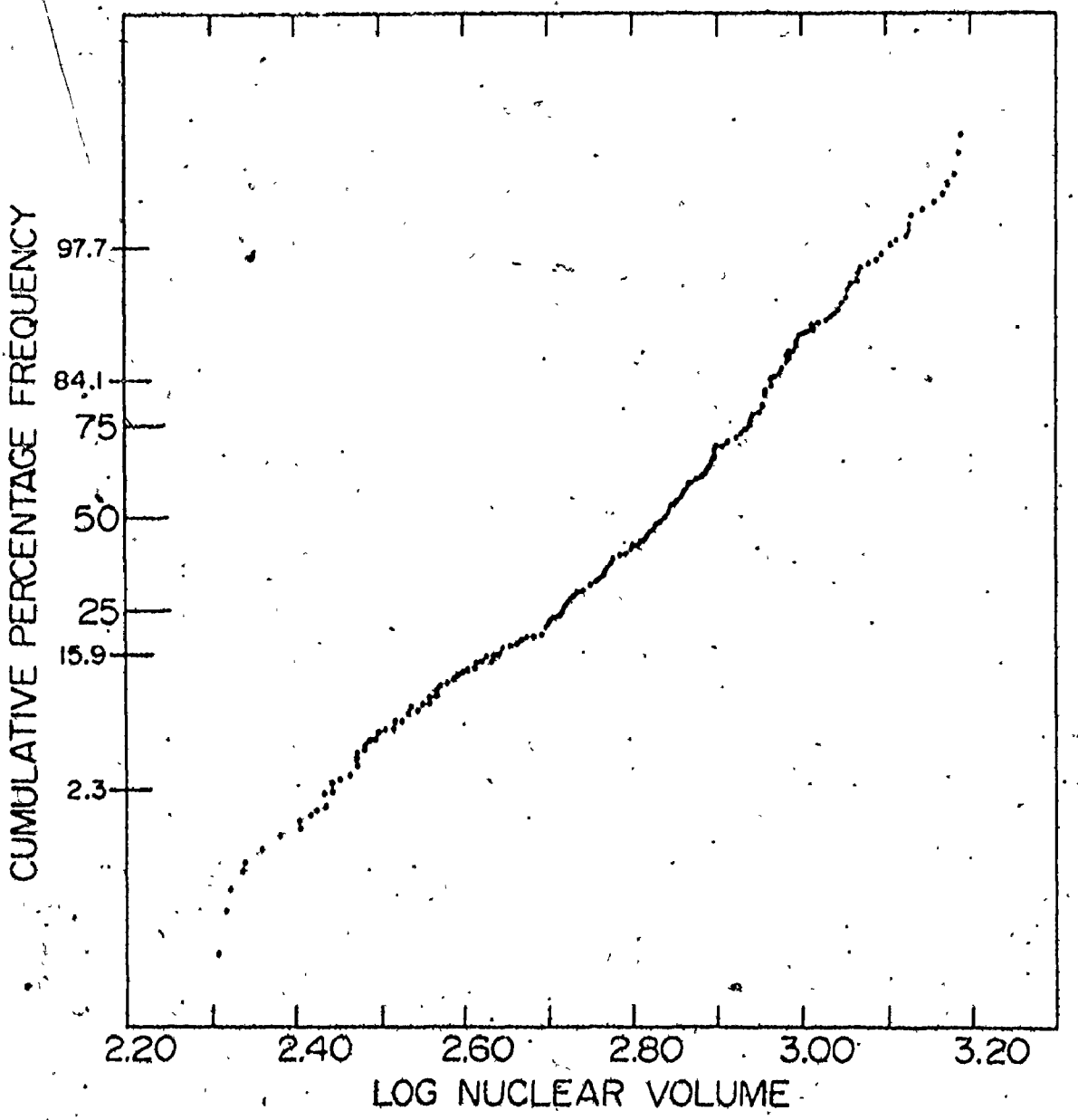
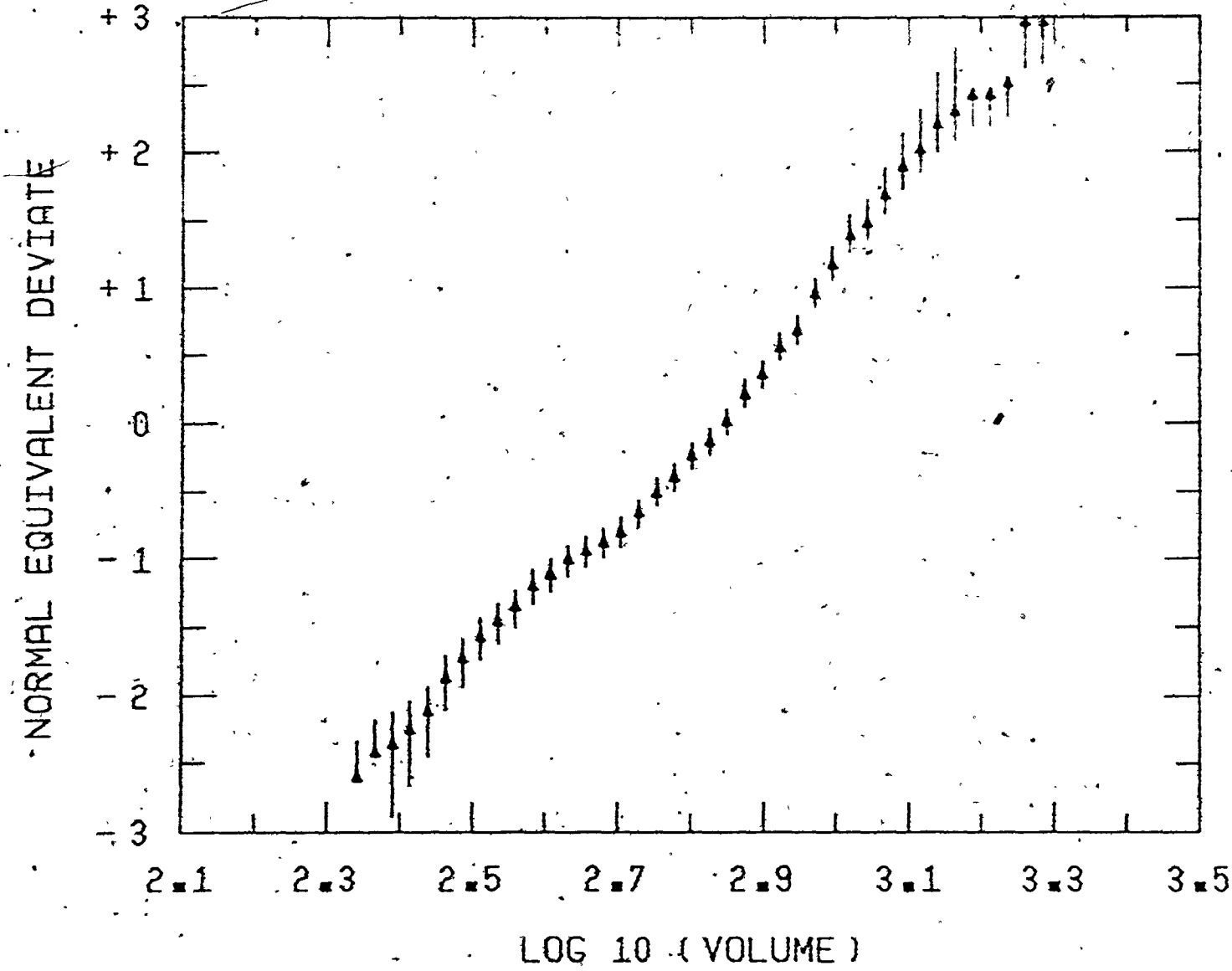

CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
2.20
2.40
2.60
2.80
3.00
3.20
LOG NUCLEAR VOLUME

Figure 18

V. faba old primary root meristems. Plot of cumulative percentage frequencies of log values of nuclear volumes on normal equivalent deviate scale. Based on 650 nuclei.


+ 3
+ 2
+ 1
0
- 1
- 2
- 3
NORMAL EQUIVALENT DEVIATE
2.1
2.3
2.5
2.7
2.9
3.1
3.3
3.5
LOG 10 (VOLUME)

1.2 Prophase

Meristems of V. faba roots, as we have seen, are asynchronous and heterogeneous. Since cells in one subpopulation, e.g., fast, slow or non-cycling; or in G_0, G_1, S or G_2, cannot be identified, the degree of variation in nuclear volumes for a particular phase of the cell cycle cannot be determined. One point in the cell cycle, however, that can be identified morphologically is prophase; prophase nuclear volumes:

(i) are values for one particular phase of the cell cycle,

(ii) represent the cumulation of all nuclear growth that has taken place from the beginning of interphase,

(iii) provide some indication about the form of distribution of volumes in late G_2 in proliferating cells.

Volumes of prophase nuclei were determined in the cells of the small primordium and in the meristem cells of 1 mm laterals, long laterals, young and old primaries. These results are summarized in Table 5. They resemble the results from the interphase nuclei, i.e.:

(1) the range of prophase nuclear volumes is different in the different meristems, but is always large, 4-7 fold (Table 5),

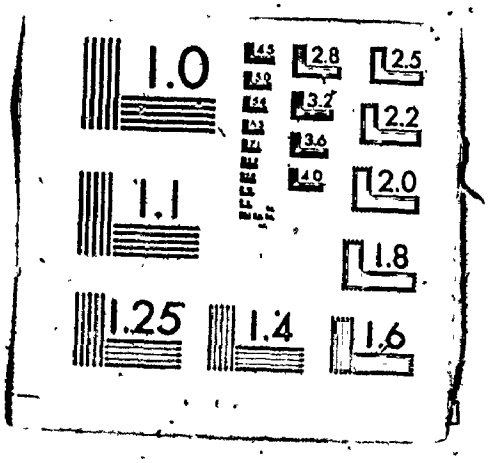
TABLE 5

Mean, median and range of volumes of prophase nuclei in root meristems of V. faba. Probit analyses were used to determine the number of populations of nuclei present and to show whether or not the population values were normally distributed. 250 prophases were scored for each meristem.

Developmental Stage	Nuclear Volumes (μm^3)			Results of Probit Analyses	
	Range	Mean±S.D.	Median (50 percent point on a probit plot)	Linear Volumes	Log Volumes
Long Lateral	517-3427	1413±437	1334	not one linear-normally distributed population	one log-normally distributed sub-population between 10th and 95th percent points (~85% values only)
Small Primordium	797-2847	1504±349	1365	not one linear-normally distributed population	not one log-normally distributed population
1 cm Lateral	956-4878	1991±711	1786	not one linear-normally distributed population	not one log-normally distributed population
Young Primary	449-2860	1335±404	1279	not one linear-normally distributed population	one log-normally distributed sub-population between 15th and 95th percent points (~80% values only)
Old Primary	572-3165	1240±463	1143	not one linear-normally distributed population	one log-normally distributed sub-population between 15th and 70th percent points (~55% values only)

2 2

OF/DE



(ii) the mean nuclear volume is different in the different meristems, e.g., 1418 ± 437 μm^3 in long laterals and 1991 ± 711 μm^3 in the 1 mm laterals,

(iii) probit plots of log values of prophase nuclear volumes never yield a single straight line that includes all the values plotted. The closest approximation to a single straight line occurs in long laterals and in young primaries; in these 85% and 80% of the log values fall on one straight line in the probit plot. This indicates that the complex form of the distribution of interphase nuclear volumes is carried over into prophase.

The wide range of values in prophase nuclear volumes shows that though there is a minimum value a nucleus must achieve before it can enter prophase, there does not appear to be a critical volume in very late G_2 that will automatically be followed by entry into prophase. The progress of a cell to the point at which it will trigger a G_2 nucleus to enter prophase is, to a considerable extent, independent of the growth of its nucleus. The extent of the lack of this coordination between nuclear volume and entry into prophase is revealed by the observation that a fraction of nuclei enter prophase when their volume is less than the median interphase nuclear volume for the meristem in which they

are growing. In the long laterals this occurs with 1% prophases, in young primaries with 3% and in old primaries with 4.2% prophases. This implies that the factors controlling the entry of nuclei into division are, to some extent, independent of those controlling the growth of the nucleus.

Prophase lasts about 1 hour. It comprises 1/14th of the cycle of fast cycling cells and at least 1/30th of the total cycle of slow cycling cells. Prophase, therefore, is the shortest phase of the cell cycle with an intact nuclear membrane and it is the only phase that can be identified morphologically. Though it lasts only 7% of the total cycle of a fast cycling cell, nuclear volumes are almost as variable in prophase as they are in interphase.

The range of volumes seen in interphase nuclei include values for cycling and non-cycling cells. In prophase, however, the 3 to 7 fold range of volumes is derived only from proliferating cells (a non-cycling cell will never enter prophase). The prophase volumes should, on this basis, be a less heterogeneous group of values than interphase nuclear volumes. That they cover a wide range of values is further evidence for variability in rates of growth of nuclei.

The ratio of fast to slow cycling cells is about 3:1. If prophase volumes in fast cycling cells

differed from those in slow cycling cells, a probit plot of the volumes should have revealed the presence of two distinct subpopulations. That is, about 75% of the values should have formed one group of values that was distinguishable from the second group, of 25% of the values. Probit plots did not reveal the presence of two distinct subpopulations of prophase volumes. It is concluded that the distribution of prophase volumes in fast cycling cells overlap the distribution in slow cycling cells. Since prophase volumes in these two populations are not distinguishable from one another, the rate of increase in the volume in slow cycling cells, which take 30 hours or more to complete a cell cycle, must be considerably lower than it is in fast cycling cells, which complete a cell cycle in 14 hours. Roughly, it is estimated that a nucleus of a slow cycling cell increases in volume at about 0.47 of the rate shown by a fast cycling cell nucleus.

1.3 Interphase and prophase: conclusions

The main conclusions that can be drawn from the data on interphase and prophase populations are:

1. Root meristems of V. faba are heterogeneous for a number of characteristics: they were known to be heterogeneous for cell cycle duration. Results presented here have shown that they are also heterogeneous for nuclear shape and nuclear volume. It has also been shown

that the form of the distribution of nuclear volumes differs at different developmental stages. The occurrence of such heterogeneity could be related to the organization of the meristem in two ways:

(a) heterogeneity for these nuclear characteristics may be essential for the orderly growth of the meristem, i.e., they may be important characteristics of meristematic cells.

(b) the heterogeneity is a constant feature of meristems, which suggests that it is regulated or controlled in some way.

2. The large variations of nuclear volume in the interphase are maintained even in prophase; at this stage there is a 3 to 7 fold range in volume.

3. At any point in the cell cycle, nuclear volumes are not uniform, e.g., prophase and therefore cannot be used as a criterion to estimate the position of the nucleus or cell in the cell cycle.

4. In none of the meristems could we identify, by graphical analysis, three distinct subpopulations that might correspond to fast, slow and non cycling cells. This is a consequence, in part, of the large variations in rates of increase in nuclear volume in different cells. Since non cycling cells cannot be identified as a

distinct subpopulation from their nuclear volumes, their nuclei must grow to some extent. Thus, the range of nuclear volumes in fast, slow and non-cycling cells must show considerable overlap.

5. Since nuclei of non-cycling cells increase in volume in interphase they must be active in some way. They may even be functioning in some way that enables them to contribute to overall growth of the meristem.

6. There are variations in nuclear shapes as well as volumes along the same developmental pathway, i.e., during the transition of small primordium to a mature lateral and from young primary to old primary. During the transition of a small primordium to a mature lateral, these variations occur simultaneously with a number of physiological changes. These nuclear changes may be correlated to the physiological changes.

2. Total nuclear proteins

Variation in nuclear size must result, in part, from quantitative changes in its major constituents, i.e., DNA, RNA, proteins; it may also be related to changes in the degree of hydration. The doubling of DNA content (from 2C to 4C in diploid cells) that takes place in interphase is unlikely, on its own, to account for the enormous variations in nuclear volumes. It has been shown for Pisum root meristem cells (Lyndon, 1967) that variation in nuclear volume can take place independently of DNA content and this is correlated with a 2-3.5 fold increase in the total dry mass as well as an increase in the degree of hydration. This leaves two kinds of macromolecular constituents of nuclei, RNA and protein. RNA is only a small fraction of the constituents of a nucleus and it seems unlikely that variations in the amount of nuclear RNA could be responsible for variation in nuclear sizes. Thus increases in protein content may account for some of the increase in nuclear volume that occurs during interphase.

In order to estimate the extent of variation in the total amount of nuclear protein, nuclei were isolated from the meristems of long lateral roots and stained with 2:4-Dinitro-1-fluorobenzene. Total protein content of the isolated nuclei was measured photometrically. The

sample of 95 nuclei included 35 spherical and nearly spherical nuclei, 30 oval and 30 elongate nuclei. Two methods were used for the estimation of the amount of total nuclear protein: (i) using relative area and (ii) using nuclear volume. When the relative area is used, there is a 5 fold range in total nuclear protein from 26 to 140 arbitrary units (Table 6 ; Figure 19). The mean total nuclear protein ± S.D. is 51 ± 15 arbitrary units. Both oval and spherical and nearly spherical groups of nuclei showed about a 3 fold range, while the elongated nuclei showed 5 fold range in the amount of total nuclear protein (Table 6). However, all these three groups of nuclei are very similar in the mean total nuclear protein amount (Table 6). On the other hand, when volumes of nuclei were used, there was an 11 fold range in the amount of total nuclear protein from 132 to 1479 arbitrary units (Table 6; Figure 19); the mean total nuclear protein ± S.D. is 369 ± 169 arbitrary units. The range in the amounts of total nuclear protein was 4 fold among the spherical and nearly spherical nuclei, 5 fold among the oval and 8 fold among the elongate nuclei (Table 6). Despite such differences in the range of protein contents, the mean amount of total protein was very close (Table 6), though mean total protein content of the elongate nuclei is slightly higher than the others. Thus, there is a large variation in the amount of total

TABLE 6

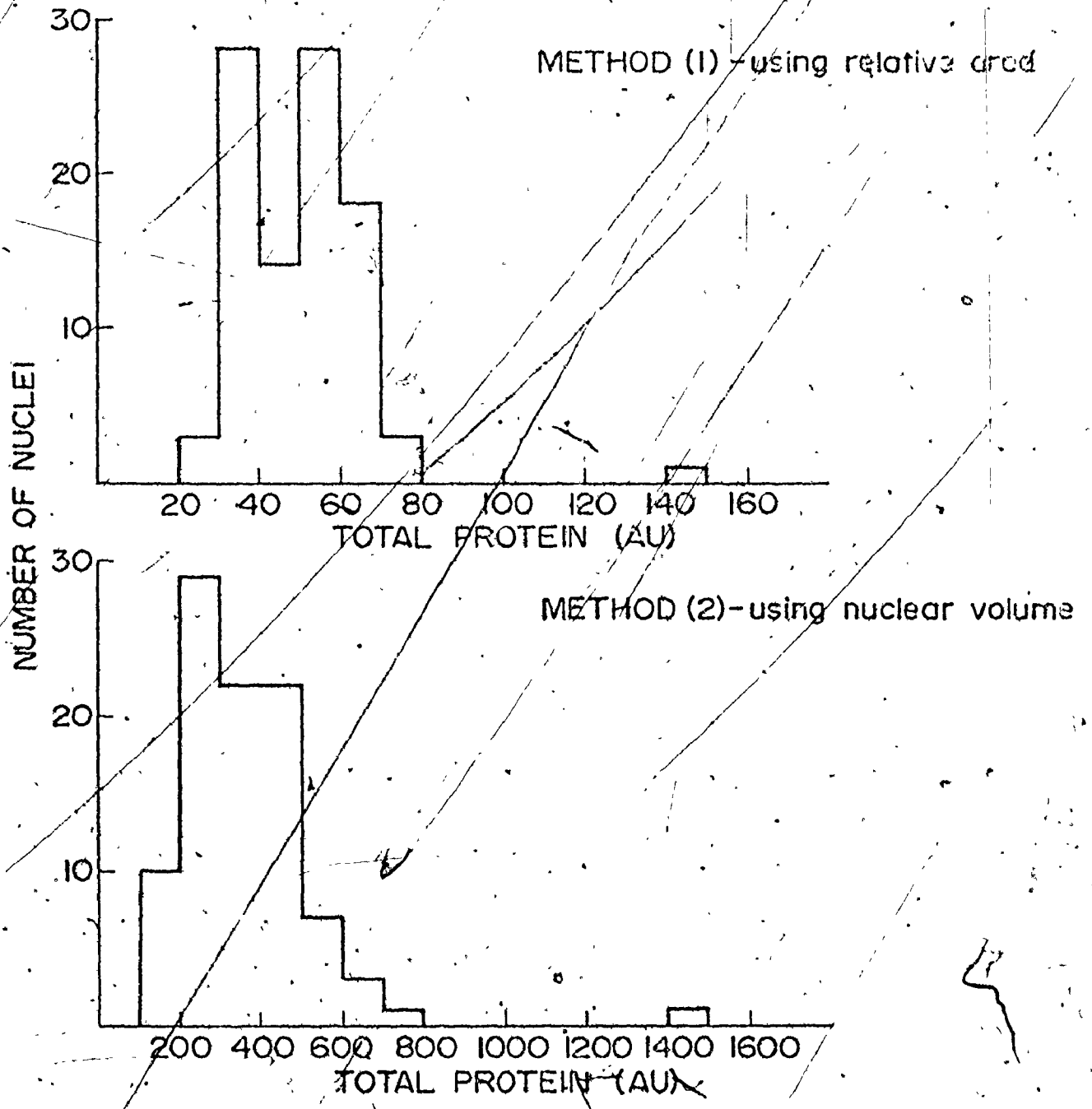
Range and Mean ± S.D. of total nuclear protein contents (in arbitrary units) of nuclei isolated from meristems of long lateral roots of *V. faba*. Nuclei were stained with dinitrofluorobenzene and measured photometrically (sample size = 95).

	using relative area		using volumes of nuclei	
	Range	Mean ± S.D.	Range	Mean ± S.D.
Total sample	26-140	51±15	132-1479	369±169
Spherical and nearly spherical nuclei (n = 35)	26-69	52±12	132-583	342±105
Oval nuclei (n = 30)	28-77	51±13	132-731	369±123
Elongate nuclei (n = 30)	27-140	49±21	177-1479	400±253

n = sample size

Figure 19

Total protein content of nuclei isolated from long lateral root meristems of V. faba. Results from 95 nuclei are shown in each histogram. Total protein content per nucleus is expressed in arbitrary units (AU).


METHOD (1) - using relative area
METHOD (2) - using nuclear volume
NUMBER OF NUCLEI
30
20
10
20 40 60 80 100 120 140 160
TOTAL PROTEIN (AU)
200 400 600 800 1000 1200 1400 1600
TOTAL PROTEIN (AU)

nuclear protein among the meristem cells. Except for some elongate nuclei which have fairly high amounts of total nuclear proteins, all other nuclei have similar ranges of total protein amounts.

A plot of the values of total nuclear proteins (estimated by using nuclear volumes) against nuclear volumes (Figure 20) revealed that, in general, the protein content of the nuclei increased as their volume increased. The correlation coefficient of these two variables is 0.71. This suggests that there is a fairly good positive correlation between the values of nuclear volumes and protein contents. However, even such a statistically significant correlation does not necessarily mean that all variation in nuclear volume results from variations in protein content. Also, it is clear that some large nuclei have low protein content, while some small nuclei have a high protein content (Figure 20).

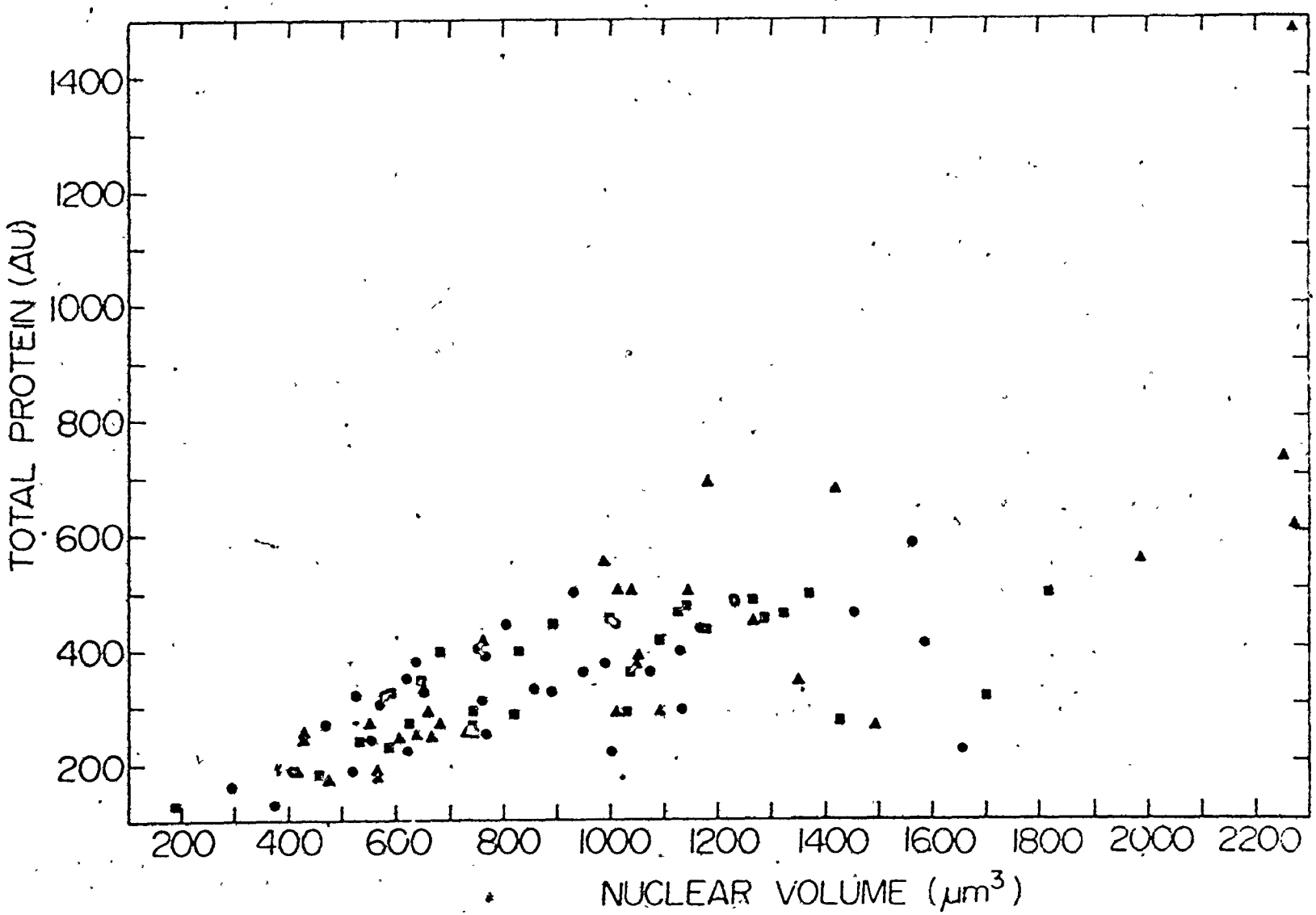
Since the absorbance measurements were of whole nuclei we do not know whether the proteins were intra-chromosomal or extrachromosomal or both. Nevertheless, the variation in nuclear protein contents of meristem cells suggest that they must be of some functional significance to the meristem. Hence, the meristem cells that are already known to be heterogeneous with regard to several characteristics (see p. 3) have additional heterogeneity with regard to one of the major macromolecular constituents i.e., proteins of the nuclei.

Figure 20

Total protein content plotted against volumes of nuclei isolated from long lateral root meristems of V. faba. Total protein content per nucleus is expressed in arbitrary units (AU). Based on 95 nuclei.

- o spherical and nearly spherical nuclei (n = 35)
- □ oval nuclei (n = 30)
- Δ elongated nuclei (n = 30).

n = number of nuclei


TOTAL PROTEIN (AU)
1400
1200
1000
800
600
400
200
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
NUCLEAR VOLUME (μm³)

3. Marked Cells: nuclear volumes in tetraploid cells

Nuclear volumes of interphase cells, as we have seen, show a wide range in values and this variation is maintained when the nuclei enter prophase. The range of nuclear volumes in fast, slow and non-cycling cells cannot be determined because the cells cannot be identified. Similarly it is not possible to estimate the form of the distribution of nuclear volumes in the various stages of interphase, i.e., G_1, S, and G_2, because we cannot identify these phases morphologically. We know that there is considerable variation in nuclear volumes but we do not know if this variability is established at one point in interphase or is cumulative over the whole of interphase. To study variation in nuclear volumes during specific subphases of the interphase, we must be able to identify a group of cells which can be distinguished from the rest of meristematic cells. A marked population of cells was produced in long lateral root meristems by a 1 hour treatment with a dilute solution of colchicine (0,002%); this induces the formation of tetraploid cells. This treatment, a short exposure to a very weak solution of colchicine, has the disadvantage that it produces relatively few tetraploid cells. On the average, only 20-30 cells per meristem could be identified with certainty as having tetraploid nuclei. The advantage of the treatment,

however, is that it is so weak that disruptive effects on cell growth are minimal. Colchicine affects the cells that are in late prophase; it prevents spindle formation and so arrests the cells in metaphase. After a period approximately equal to the total duration of metaphase, anaphase and telophase, the chromosomes of an arrested metaphase revert to an interphase condition. This process is called restitution and it results in the formation of a restitution nucleus. The restitution nucleus formed from a diploid nucleus is tetraploid. In V. faba, diploid nuclei have 2 nucleolar organizers and therefore, have 1 or 2 nucleoli; tetraploid nuclei have 4 nucleolar organizers and up to 4 nucleoli. Hence the tetraploid nuclei can be easily identified; nuclei were scored as tetraploid if they had 3 or 4 nucleoli.

Meristems of long lateral roots fixed 2 or 3 hours from the end of treatment with colchicine were examined in order to study the pattern of recovery of cells from the treatment. At 2 hours, arrested metaphases were present; there were very few anaphases or restitution nuclei. At 3 hours, restitution nuclei were forming and normal metaphases and anaphases were present again. Thus, between 2 and 3 hours from the end of treatment, the cells arrested at metaphase at 2 hours undergo restitution, while the reappearance of normal metaphases and anaphases shows that this treatment with colchicine does not have

a prolonged effect. It appears to be a pulse treatment. The time taken for restitution nucleus to appear is between 2 and 3 hours after the end of colchicine treatment. This, effectively, is the time of formation of tetraploid nuclei.

Tetraploid interphase nuclear volumes were determined at 4, 10 and 14 hours from the end of treatment with colchicine; tetraploid prophase nuclear volumes were determined at 13 and 14 hours from the end of treatment. That is, volumes of tetraploid interphase nuclei were determined when they were about 2, 8 and 12 hours old. Prophase volumes were determined when the tetraploid nuclei were 11 and 12 hours old. A comparison has been made between volumes of tetraploid nuclei 2, 8 and 12 hours after they were formed; this reveals how nuclear volume changes as a cell proceeds through interphase. Mitotic index for the tetraploid population was determined at 15 and 16 hours from the end of treatment; i.e., when the tetraploid cells are 13 and 14 hours old. These mitotic indices give an estimate of the fraction of the tetraploid population that is fast cycling. A high percentage of 4n cells are dividing by 13 and 14 hours; their cell cycle duration must be close to that for diploid fast cycling cells.

3.1 Tetraploid interphase nuclear volume

The mean and median nuclear volumes and the range of nuclear volumes in the tetraploid cells of long lateral root meristems at 4, 10 and 14 hours from the end of treatment with colchicine are summarized in Table 7. The specific features of the populations at these specified times are described in the following sections.

3.1.1 4 hours from the end of treatment with colchicine

Tetraploid nuclei show about a 14 fold range in volume (Table 7). From a probit plot of cumulative percentage frequencies of log values of nuclear volumes on a normal equivalent deviate scale (Figure 21) the log mean volume was obtained; this gives a value of 1193 μm^3. Also from the probit plot values were obtained for the range $\pm$ 1 and $\pm$ 2 standard deviations (Table 7). The range log mean $\pm$ 1 S.D. is 806 - 1767 μm^3, i.e., a 2.2 fold range. In the interval $\pm$ 2 S.D. the range is 551 - 2597 μm^3; this is a 4.7 fold range. This shows that the range of volumes is large even within 2 - 3 hours of nuclei entering G_1: there is a 2.2 fold range in the central 68% of the nuclei scored. They also show that, since a wide range of volumes is present within 2 - 3 hours of the beginning of interphase, there must be variation in the rates of increase in nuclear volume in early interphase. It appears, therefore, that a wide range in nuclear

TABLE 7

Mean, median and range of nuclear volumes of tetraploid nuclei in long lateral root meristems of V. faba. At each fixation time, 650 nuclei were measured. The median value is taken from the log value at the 50 percent point on a probability scale plot of log nuclear volumes. The range, ± 1 S.D. covers the values between the 15.9 and 84.1 percent points; ± 2 S.D. covers the range between 2.3 and 97.7 percent points.

Time from the end of treatment with colchicine (hours)	Nuclear Volume (μm^3)					Difference Between Intervals			
	Range	Mean±S.D.	Median	Range between ±1 S.D.	Range between ±2 S.D.	-2&-1 S.D.	-1S.D. & Median	Median & +1 S.D.	+1&+2 S.D.
4	274-3,875	1285±526	1193	806-1767	551-2597	255	387	574	830
10	426-14,464	2292±1600	1892	1113-3483	640-6779	473	779	1691	3296
14	1173-11,826	3591±1339	3303	2402-4745	1783-6876	619	901	1442	2131

Since there appear to be two subpopulations of values of nuclear volumes at 10 hours, the values between the 15.9 and 84.1 percent points and between the 2.3 and 97.7 percent points do not cover the ranges of ± 1 and ± 2 standard deviation. However these values are given for comparison with the values at 4 and 14 hours.

Figure 21

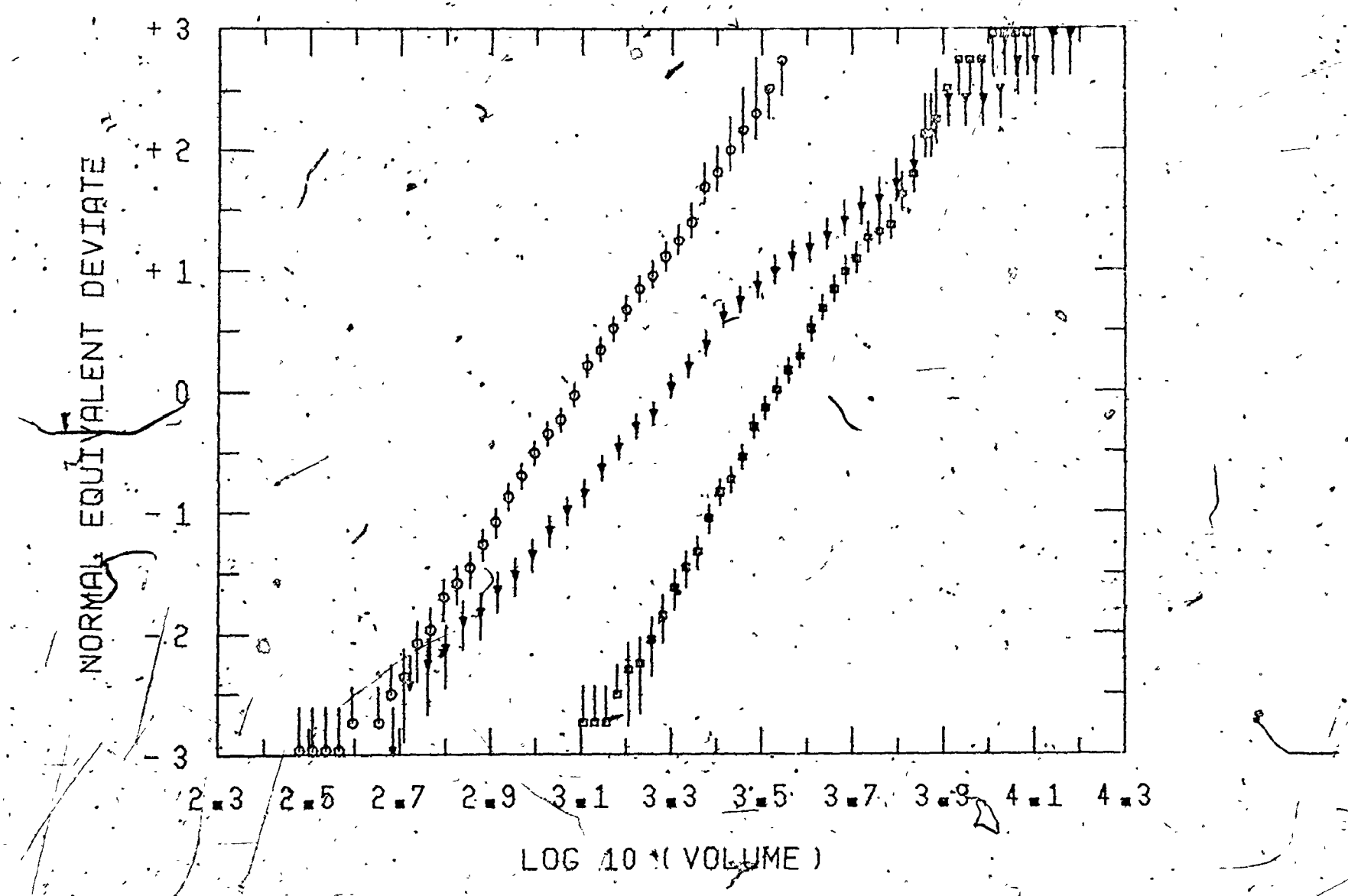
V. faba long lateral root meristems.

Plot of cumulative percentage frequencies of log values of tetraploid interphase nuclear volumes on normal equivalent deviate scale.

21 a. ○ 4 hours from the end of treatment with colchicine; based on 650 nuclei.

21 b. ▽ 10 hours from the end of treatment with colchicine; based on 650 nuclei.

21 c. □ 14 hours from the end of treatment with colchicine; based on 650 nuclei.


NORMAL EQUIVALENT DEVIATE
+ 3
+ 2
+ 1
0
- 1
- 2
- 3
2.3
2.5
2.7
2.9
3.1
3.3
3.5
3.7
3.9
4.1
4.3
LOG 10 (VOLUME)

volumes is already established at the beginning of the interphase, the variation is maintained throughout the interphase (Table 7) but is clearly not a result of a progressive increase in the range of nuclear volumes as cells proceed through interphase. Furthermore, the 2.2 fold range of volumes (Table 7) show that a doubling of nuclear volumes can occur within one phase of the cell cycle. These results, from tetraploid nuclei, show that at least a 4.7 fold range in nuclear volumes exists in G_1 and, therefore, the stage in interphase reached by a cell cannot be determined solely on the basis of nuclear volume (cf. Rasch et al., 1967). The results from tetraploid nuclei, therefore, confirm the conclusion from diploid nuclei (see Chapt. III, sec. I).

The distribution of nuclear volumes in this sample taken at 4 hours from the end of treatment with colchicine is not linear normal. It differs significantly from one linear normal distribution; the X^2-test gives a P value of less than 0.001 (Table 8). However, it is not significantly different from a log normal distribution; the X^2-test gives a value of $>0.05<0.02$ (Table 8). The probit plot of the log values of nuclear volumes, on a normal equivalent deviate scale (Figure 21), indicate that the volumes of the majority of the nuclei follow one log normal distribution. Use of the EDF statistics (Table 9) indicated that the distribution of the values

TABLE 8

X^2-test of the fit of volumes of tetraploid interphase nuclei in long lateral root meristems of *V. faba*. Goodness of fit to linear and log normal distribution were tested. 650 nuclei were measured at each of the times specified. X^2-test was done on grouped data.

Time from the end of treatment with colchicine (hours)	Interphase Nuclear Volume: Linear: Observed X^2-sum	Linear: X^2-sum expected at p=0.001	Linear: Degrees of freedom	Linear: Do values fit a normal distribution	Log: Observed X^2-sum	Log: X^2-sum expected at p=0.001	Log: Degrees of freedom	Log: Do values fit a normal distribution
4	334.45	42.31	18	no: significantly different at p=0.001	34.36	45.32	20	yes: values not significantly different at p=<0.025
10	669.01	32.91	12	no: significantly different at p=0.001	51.08	48.27	22	no: significantly different at p=0.001
14	329.62	39.25	16	no: significantly different at p=0.001	54.33	43.82	19	no: significantly different at p=0.001

TABLE 9

Results of the empirical distribution function (EDF) test of goodness of fit to one log-normal distribution for the tetraploid interphase nuclear volumes on the long lateral root meristems of V. faba. The calculated statistics were suitably modified according to the size of the various samples.

Hours from the end of treatment with colchicine	Modified statistics				
	D	V	W^2	U^2	A^2
4	0.794	1.426	0.076	.0731	0.455
10	1.533	2.183	0.353	0.298	2.232
14	0.989	1.835	0.221	0.187	1.467

of nuclear volumes is not significantly different from one log normal population. These results establish that within 2 hours of entering interphase, nuclei show a wide range of volumes and also that they are distributed log normally.

3.1.2 / 10 hours from the end of treatment with colchicine

Probit analysis of log values of nuclear volumes reveals that in the interval between 4 and 10 hours, the amount of increase in the volumes of all the nuclei in the population is not similar (cf. Figure 22). Some nuclei at the bottom end of the distribution at 4 hours, do not grow very much during this interval; about 20% of the nuclei at the top end of the distribution appear to have increased enormously during the same period of time and hence, have presumably grown at a faster rate. Moreover, the probit plot of the log values of nuclear volumes, both on probability scale (Figure 22) and normal equivalent deviate scale (Figure 21) reveals that the population is not uniform; it appears to be made up of two log normally distributed subpopulations. The probit plot (Figure 22) reveals that about 78% of the nuclei (from 1st to 79th percentiles) with a volume range from 546 to 2852 μm^3 (about 5 fold difference) comprise one subpopulation; the second subpopulation consists of the top 20% of the nuclei (volume range: 2856 to 14,464 μm^3; about 5 fold difference).

Figure 22

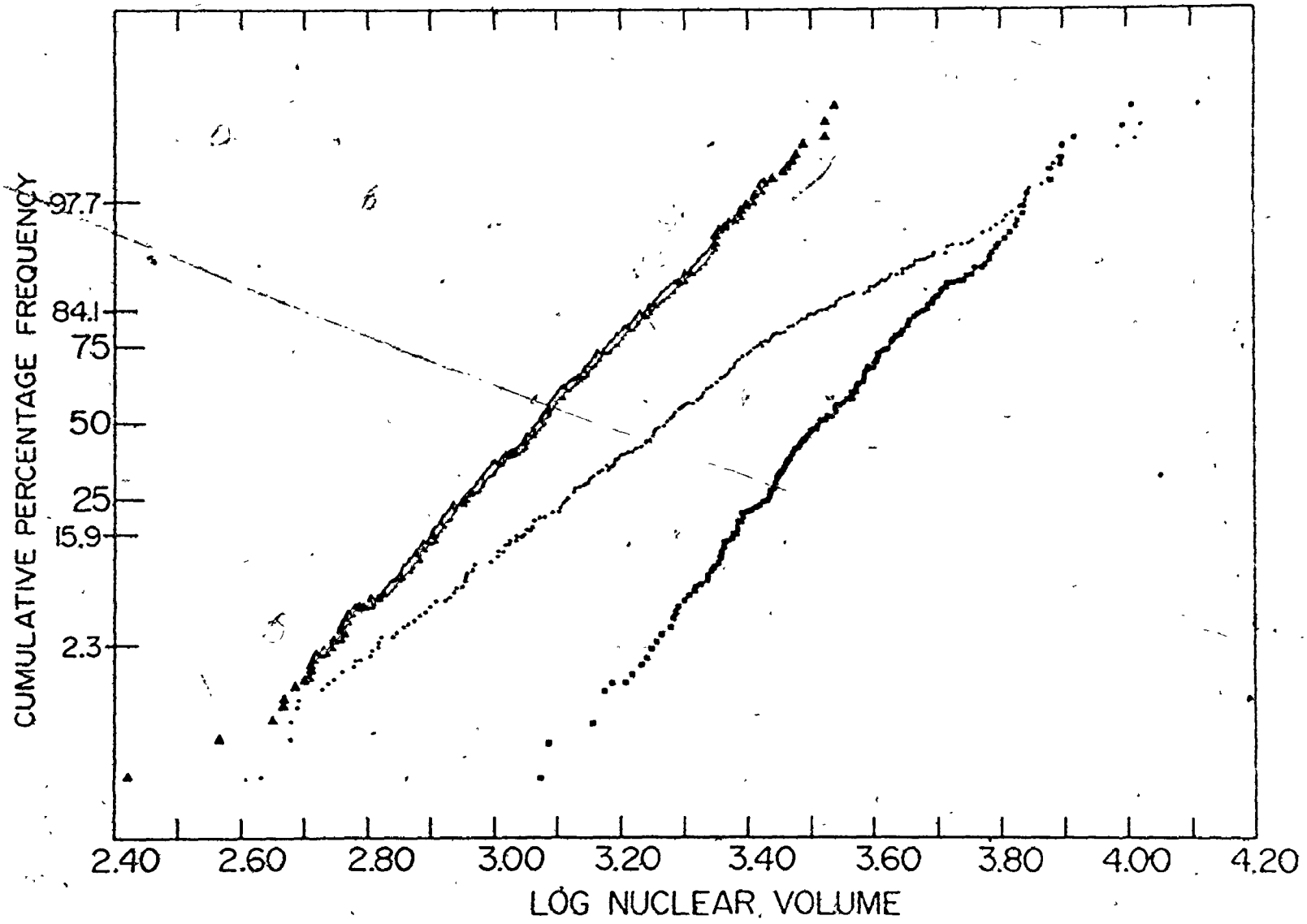
V. faba long lateral root meristems.

Plot of cumulative percentage frequencies of log values of tetraploid interphase nuclear volumes on a probability scale.

22 a. ▲ 4 hours from the end of treatment with colchicine; based on 650 nuclei.

22 b. ○ 10 hours from the end of treatment with colchicine; based on 650 nuclei.

22 c. □ 14 hours from the end of treatment with colchicine; based on 650 nuclei.


CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
2.40
2.60
2.80
3.00
3.20
3.40
3.60
3.80
4.00
4.20
LOG NUCLEAR VOLUME

This suggests that a relatively small fraction of the population undergoes an enormous increase in nuclear volume compared to the majority of the nuclei. Furthermore, the probit plot on a normal equivalent scale (Figure 21) also reveals a similar heterogeneity within the population; values lying in the central part of the distribution, i.e., in the range of -0.5 to +1.0 standard deviation units form one log normally distributed subpopulation; the values lying outside these limits are distinct from the majority of the nuclei. The X^2-test showed that the distribution of the values of nuclear volume was significantly different from that expected for one log normal population ($p = 0.001$; Table 8). This was further confirmed by performing the EDF test; all EDF statistics confirmed that the sample was significantly different (at 1% level; Table 9) from one log normally distributed population. A X^2-test also showed that the distribution of the values of nuclear volume is significantly different from a linear normal distribution.

There is a 30 fold range in nuclear volumes at 10 hours from the end of treatment with colchicine; the mean nuclear volume $\pm$ S.D. is 2292 $\pm$ 1600 μm^3.

The population at 10 hours differs from that at 4 hours in two ways:

(1) range of nuclear volumes is much larger at 10 hours (30 fold difference) than at 4 hours (14 fold difference).

(ii) there appear to be two log normally distributed subpopulations at 10 hours whereas there is only one log normally distributed population at 4 hours.

Therefore, within a period of 6 hours, there is a large increase in nuclear volumes and there is a change in the form of the distribution of nuclear volumes. It is evident that during these 6 hours not all nuclei have undergone similar increases in volume. Hence, within this population there are differences in the rates of increase in nuclear volume; this is reflected in the heterogeneous nature of the distribution of nuclear volumes.

By 10 hours from the end of treatment with colchicine, these nuclei have undergone approximately 8 hours of growth since the beginning of interphase. We know that for the majority of the diploid fast cycling cells in long lateral root meristems, the average durations of G_1 and S subphases of the interphase are 2.5 hours and 6.2 hours respectively; taken together, the duration of these two subphases is 8.7 hours. Colchicine, when given in low concentration, does not alter the duration of the cell cycle and its subphases. Therefore, on this basis, and also because some tetraploid cells are in division at 13 and 14 hours (i.e., have completed the cell cycle in 11 and 12 hours respectively) some of the fast cycling cells either are going through the S phase or

have just completed S phase and entered G_2 by this time. During the S phase the nuclei are metabolically very active: they undergo DNA synthesis. Moreover, it is known that there is migration of proteins both into and out of the nucleus during the S phase, e.g. mouse fibroblast cells in culture (Zetterberg, 1966, 1971). The variability of nuclear volumes could be a reflection to some extent of the metabolic activity of the nucleus; it may also be related to the relaxed state of the chromatin in some nuclei when they are going through the S phase.

3.1.3 14 hours from the end of treatment with colchicine

By 14 hours from the end of treatment with colchicine, the tetraploid nuclei have completed about 12 hours of growth since the beginning of interphase. This time period corresponds to the combined average durations of G_1 (2.5 hours), S (6.2 hours) and G_2 (3.3 hours) for diploid fast cycling cells in the long lateral root meristems. Hence, by this time, most of the fast cycling tetraploid cells should be in G_2 phase or some of them might even be in very late G_2. This is substantiated by the observation that tetraploid cells were in division by 13 and 14 hours from the end of treatment with colchicine. On the basis of the assumption generally held that nuclear volume is directly proportional to the age of the cell in the cell cycle, we should have been able to observe a

cluster of values of nuclear volumes which were approximately identical. No such clustering of values of nuclear volumes was observed at the upper end of the distribution in the probit plot of the log values of nuclear volumes (Figure 22). Probit plot of log values of nuclear volumes on a normal equivalent deviate scale (Figure 21) generates a straight line for the values lying between $\pm$ 1 standard deviation units. This suggests that the volumes of the majority of the nuclei form one log normally distributed population. However, the X^2-test shows that the distribution of values is significantly different from that expected for one log normally distributed population ($p = 0.001$; Table 8); all EDF statistics, except D, show that the population is significantly different at 1% level (Table 9) from one log normally distributed population. The values do not follow a linear normal distribution (from a X^2-test, $P = 0.001$; Table 8).

A comparison of the probit plots of log values of nuclear volumes at 10 and 14 hours (cf. Figure 22b and Figure 22c) reveals that in the interval between 10 and 14 hours, the nuclei with smaller volumes at 10 hours have undergone a greater increase in volume than those with larger volumes at 10 hours. There is about a 2.75 fold difference between the minimum values of nuclear volumes at 10 and 14 hours. This shows that all tetraploid nuclei

in the population, whether they are fast, slow or non-cycling cells, have grown since they were formed.

There is a 10 fold range of nuclear volumes at 14 hours, from 1173 to 11,826 μm^3; the mean nuclear volume $\pm$ S.D. is 3591 $\pm$ 1339 μm^3. Within a range of $\pm$ 1 standard deviation units (Table 7) the nuclei show an approximately 2 fold difference of volumes, i.e., from 2402 - 4745 μm^3; within the range $\pm$ 2 standard deviations, there is a 4 fold difference of volumes. Nevertheless, there are few nuclei in the population that appear to have grown very much larger than the rest of the population.

The nuclear volumes in the population at 14 hours appear to be less variable than those of the population at 4 and 10 hours. This means that although there is variability in nuclear volumes when some of the nuclei are in G_2, the extent of variation is less than when most nuclei are in G_1 or some of the nuclei are in the S phase.

Within a time period of 4 hours, that is, between 10 and 14 hours from the end of treatment, there is a change in the form of the distribution of the values of nuclear volumes; the heterogeneous population (at 10 hours) appears to have resolved into one log normal population (at 14 hours). The population at 14 hours resembles the one at 4 hours in the sense that both appear to have one log normally distributed population of values of nuclear

volumes. A striking point of similarity between the populations at 4 and 14 hours is that in both of them the range of nuclear volumes within ± 1 S.D. is 2 fold and within ± 2 S.D. is 4 to 5 fold.

3.2 Tetraploid prophase nuclear volumes

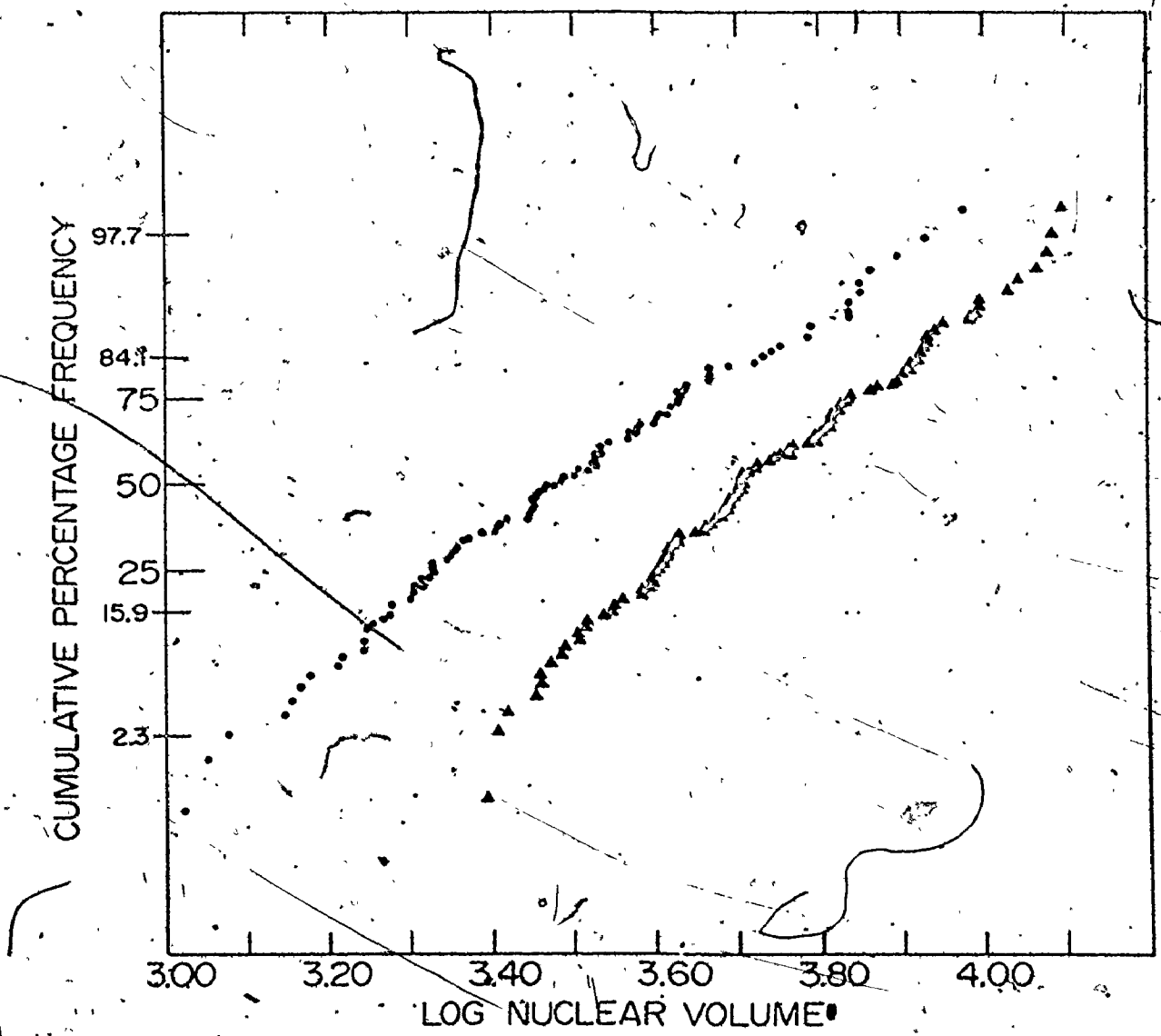
Tetraploid cells were seen in prophase at 13 and 14 hours. They have taken 11 and 12 hours to complete interphase, i.e., about the same time as fast cycling diploid cells. Prophase nuclear volumes are as variable as interphase volumes. The volumes of the tetraploid prophase nuclei showed an 11 fold range (from 1052 to 11,950 μm^3) at 13 hours and a 7 fold range (from 2189 to 15,576 μm^3) at 14 hours from the end of treatment with colchicine. The mean nuclear volume ± S.D. at 13 and 14 hours are 3552 ± 1997 μm^3 and 5824 ± 2567 μm^3 respectively. The values of prophase nuclear volumes do not form a uniform population. Probit plots of log values of nuclear volumes (Figure 23) reveal that only volumes of the nuclei in the central part of the distribution form one straight line and these values may represent one log normally distributed population. The rest of the values at the bottom as well as the top end of the distribution are distinct from the central group of values. About 62% of the nuclei lying between 20^{th} and 82^{nd} percentiles form the central log normally distributed population at 13 hours (Figure 23a). At 14 hours

Figure 23

V. faba long lateral root meristems.
Plot of cumulative percentage frequencies of log values of tetraploid prophase nuclear volumes on a probability scale.

23 a. o 13 hours from the end of treatment with colchicine; based on 100 nuclei.

23 b. Δ 14 hours from the end of treatment with colchicine; based on 100 nuclei.


CUMULATIVE PERCENTAGE FREQUENCY
97.7
84.1
75
50
25
15.9
2.3
3.00
3.20
3.40
3.60
3.80
4.00
LOG NUCLEAR VOLUME

only 55% of the nuclei, that is, those between 5th and 60th percentiles form one log normally distributed population (Figure 23b).

The median prophase nuclear volumes at 13 and 14 hours respectively are 3046 μm^3 and 5071 μm^3 (Table 10). At both these times the range of volumes for the upper 50% of the nuclei is about 4 or 5 times larger (cf. 13 hours: 8904 μm^3; 14 hours: 13,387 μm^3) than the lower 50% of the values (13 hours: 1994 μm^3; 14 hours: 2282 μm^3). This suggests that the distribution of nuclear volumes is asymmetrical about the median volume and also that the asymmetrical nature of the distribution is carried over from interphase to prophase.

The volumes of at least 15.5% prophase nuclei at 13 hours and 13.5% prophase nuclei at 14 hours are less than the median interphase nuclear volume at 10 hours, which is 1892 μm^3. Since an appreciable number of nuclei that have entered prophase are smaller than the majority of the nuclei that are still in interphase, there appears to be no correlation between nuclear volume and entry into division; these results confirm the conclusion from diploid prophase cells of the different meristems.

At 10 hours, the maximum value of nuclear volume is 14,464 μm^3. At 14 hour the maximum value is 11,826 μm^3 (Table 7); this drop is not due to contraction

TABLE 10

Tetraploid prophase nuclear volumes in the long lateral root meristems of *V. faba*. 100 nuclei were scored at each fixation time.

Time from the end of treatment with colchicine (hours)	Nuclear Volume (μm^3)		
	Range	Mean±S.D.	Median (50% point on probit plot)
13	1052-11,950	3552±1997	3046
14	2189-15,576	5824±2567	5071

of the nucleus for by 14 hours some nuclei have entered prophase. The maximum value of prophase nuclear volume (at 14 hour) is 15,576 μm^3. Thus, it appears that the largest prophase nuclei are from the largest interphase nuclei; and also that the first nuclei to enter prophase include many of the largest nuclei in G_2 population. When the nuclei with large nuclear volumes seen at 10 hours move into prophase by 14 hours, the result is a loss of nuclei from the top end of the distribution of nuclear volumes. Furthermore this suggests that some of the cells that are fastest dividing have very large volumes.

The prophase nuclear volumes of the fast cycling tetraploid cells are very variable, probably as a result of differences in the rate of growth of nuclei among the cells of the same subpopulation. This suggests that the rate of growth of nuclei is to some extent independent of the cell cycle duration that affect the nuclear growth rates.

The variability of prophase nuclear volumes as well as the entry of an appreciable number of nuclei into prophase while they are smaller than the majority of interphase nuclei, suggests that there is no correlation between the size of a nucleus and its age in the cell cycle. Furthermore, it also appears that although a minimum critical amount of growth may be essential for

entry into division, the total amount of nuclear growth does not regulate the triggering of the transition from interphase to mitosis.

3.3 Mitotic index of tetraploid cells

Tetraploid cells have reached prophase 13 hours from the end of colchicine treatment (Table 11). At 13 and 14 hours, metaphases and anaphases are also present but in low frequency; there were only five metaphases and anaphases in the sample of tetraploid cells that contained 100 prophases at 13 hours (Table 11). Telophases were not present until 15 hours and at this time 10 tetraploid cells had divided. Their daughters must have been in early G_1. The number of prophases scored was kept at 100, since these nuclei were required for nuclear volume determinations. The sample of tetraploid cells that included 100 prophases also included metaphase-telophase stages at 15 and 16 hours and so at these times M.I. was determined; the values were 47.6 and 62.8 at 15 and 16 hours. At 16 hours, 25 tetraploid cells had divided, i.e., 6.6% of the total sample of 4n cells.

Tetraploid cells have entered prophase by 13 hours; i.e. when they are 11 hours old, and prophases are still present at 16 hours, in 14 hour old cells. At 13 hours, there are few metaphases and anaphases, 5 per 100 prophases; at 14 hours there are 31 per 100 prophases. Thus, in 1 hour, about 25% of the prophases move into

TABLE 11

Frequencies of tetraploid cells in the various stages of mitosis and in early G_1 in the long lateral root meristems of V. faba. (Time is indicated from the end of treatment with colchicine.)

Time (Hours)	Prophase	Metaphase	Anaphase	Telophase	Interphase	Mitotic Index	Early G_1
13	100	2	3	-	*	*	-
14	100	14	17	-	*	*	-
15	100	24	20	36	198	47.6%	20
16	100	56	37	45	141	62.8%	50

- not present

* not scored

metaphase-anaphases. The increase in numbers of metaphases and anaphases continues from 14 to 15 and 15 to 16 hours.

The data on mitotic indices are incomplete because their collection was incidental to the study of prophase volumes. However, they provide some estimates of cell cycle kinetics of the tetraploid cells.

(a) Minimum cell cycle duration. By 15 hours, 2.6% of the tetraploid cells have divided; therefore, minimum cell cycle duration is 13 hours.

(b) Interphase duration. Prophases are present at 13 hours. They may have been present somewhat earlier than 13 hours and, therefore, interphase duration is probably somewhat less than 11 hours. This will be the minimum interphase duration for tetraploid cells.

For most tetraploid cells, interphase is longer than 11 hours: tetraploid cells are still entering mitosis between 15 and 16 hours, i.e., when 4n cells are 13 to 14 hours old. Mitotic index increases from 47.6 to 62.8 between 15 and 16 hours, i.e., a 15.2% increase; in the same period, other 4n cells divide. Their frequency increases from 2.6 to 6.6% between 15 and 16 hours. Thus, overall, 15.2 + 4.0 i.e. 19.2% of tetraploid cells have either divided or entered mitosis, between 15 and 16 hours. This value, 19.2%, has been used to estimate the total period over which tetraploid cells divide (see Discussion).

Another conclusion from the MI of 4n cells concerns the proportion that are fast cycling. Since 67.8 + 6.6% of tetraploid cells have divided or are in mitosis by 16 hours, it is concluded that 69.4% of tetraploid cells have a cell cycle duration of ~ 14 hours; this is in close agreement with the estimate for fast cycling diploid cells. From this we can say that ~ 70% of the nuclear volumes and all of the prophase volumes determined are from fast cycling cells.

3.4 Tetraploid cells: Conclusions

The tetraploid nuclei show a large range in volume in interphase and the range differs in different stages of interphase. Within the first two hours of G_1 there is a large range in nuclear volume: even in the 68% of the nuclei in the centre of the distribution, i.e., mean ± 1 S.D., there is a 2 fold range in volume. Thus nuclei can at least double in volume within the first two hours in interphase. The largest range in interphase nuclear volumes is observed at ten hours from the end of treatment with colchicine, when some nuclei are probably in S phase or have just completed S phase. At 14 hours, when some tetraploid nuclei are already in prophase and others are in G_2, the degree of variation is less than that in the earlier parts of interphase. One reason for smaller variation in nuclear

volume at 14 hour is perhaps that some of the very large nuclei that were in interphase at 10 hours have entered mitosis by this time.

The changes in the degree of variation of nuclear volumes at different times within the interphase are accompanied by changes in the form of distribution of these values. One log normal population at G_1 diverges into what appears to be two log-normally distributed subpopulations when some nuclei are in S or have completed S phase. This heterogeneous population thereafter resolves to one log normal population by the time when some nuclei are in division.

The changes in the degree of variation of nuclear volumes as well as in their form of distribution are occurring within one interphase of a large number of tetraploid cells. Taken together these changes suggest that:

(i) there is variation in the rates of growth of nuclei within any one subphase of the interphase, i.e., G_1, S, or G_2, and

(ii) the rates of growth of nuclei are not uniform throughout the interphase.

These suggestions are supported by the data from graphical analyses of nuclear volumes. The nuclei at the top and bottom end of distributions differ in the amounts of increase in their volumes. Between four and ten

hours, the nuclei at the top end appeared to have increased greatly in volume relative to those at the bottom end. Between 10 and 14 hours the nuclei at the bottom end of the distribution appear to have increased relatively more than those at the top end.

The largest increase in nuclear volume appears to occur in S. The variation in nuclear volume at ten hours may, therefore, reflect the distribution of cells between parts of the S-phase.

At least 70% of the tetraploid cells are fast cyclers; if the others are slow or non-cycling cells, their nuclei must also be growing.

The variability of sizes of interphase nuclei seem to be maintained in prophase, although the degree of variation is smaller in prophase. This confirms the conclusions from the diploid cells. The cells in prophase by 13 and 14 hours do not have identical nuclear volumes. The variability of prophase nuclear volumes, along with overlapping of volumes of nuclei when they are in G_1 (4 hours) and at least some nuclei are in G_2 (14 hours), suggests that there is no absolute correlation between the volume of a nucleus and its age in the cell cycle.

From the time of the appearance of tetraploid cells in mitosis the duration of their interphase is estimated to be between 11 and 13 hours. This is very close to the average duration of interphase for the fast-cycling cells,

i.e., 12 hours. The duration of the complete cell cycle is 13 hours for the tetraploid cells with the shortest cell cycle (2.6%; Table 11) and is 14 hours for the 4% of the tetraploid cells. Thus, cell cycle duration of at least some of the tetraploid cells is similar to that for fast cycling diploid cells, that is, 14 hours.

4. Chromosome volumes

In the marked population of tetraploid cells, the nuclear volumes are very variable throughout the interphase. The variability in nuclear volumes is present from very early interphase and is present even in the prophase which is the end point of the nuclear growth cycle. What we do not know about this population of cells are the initial volumes of the nuclei at the very beginning of interphase. The minimum possible volume of a tetraploid nucleus would be equal to the volume of the total chromosome complement at metaphase. It should be realized that this estimate provides a minimum value. Clearly, some cytoplasm and parts of the spindle will probably be included as the nucleus reforms and they would increase its volume. To estimate the minimum possible volume of a tetraploid interphase nucleus, volumes of total chromosome complements at metaphase were determined.

Two methods were used to calculate the volume of the metaphase chromosome complement (see Material and Methods, p. 13). Method one (Bennett and Rees, 1969) treats the chromosome as a single cylinder; method two treats the chromosome as made up of two cylinders, the two chromatids. In each method two different average measurements of chromatid width were used: (i) mean of 5 chromatid widths/per cell for each of the 50 cells, (ii) mean of the width of all the chromatids from 50

cells, i.e. 250 chromatid widths. When the mean width of 5 chromatids per cell was used, for both methods, there is approximately 5 fold range of volumes, i.e., from 94 to 460 μm^3 and 47 to 230 μm^3; the mean $\pm$ S.D. is 276 $\pm$ 109 μm^3 and 138 $\pm$ 54 μm^3 respectively (Table 12). The range in chromosome volumes is as large as the range of volumes of prophase nuclei in the long lateral root meristems, i.e., 6 fold (cf. Table 5). However, when the mean of 250 chromatid widths is used for both methods of determination of chromosome complement volumes, there is approximately a 2 fold range, i.e. 175 to 327 μm^3 and 88 to 164 μm^3 respectively. The mean $\pm$ S.D. is 264 $\pm$ 31 μm^3 and 138 $\pm$ 15 μm^3 respectively. It appears, therefore, that large variations with respect to total chromosome complement volumes occur among the meristem cells, i.e., a group of undifferentiated, proliferating cells with identical genotypes. These variations must be inherent within the chromosomal constituents. Although the range of volumes of the total chromosome complement is much smaller than in the early interphase, the occurrence of variation in total complement volumes indicates that there is perhaps a similar, if not identical, range in nuclear volumes at the beginning of interphase within the cells of the same meristem.

In the first method of calculation of volumes of total complement of chromosomes (Bennett and Rees, 1969)

TABLE 12

Mean ± S.D. and range of total chromosome complement volumes in the long lateral root meristems of V. faba. 50 metaphase complements were scored.

Method-1		Method-2	
using mean width of 5 chromatids/cell	using mean width of 250 chromatids (5/cell;50 cells)	using mean width of 5 chromatids/cell	using mean width of 250 chromatids (5/cell;50 cells)
volume (μm^3)			
Range Mean±S.D.	Range Mean±S.D.	Range Mean±S.D.	Range Mean±S.D.
94-460 276±109	175-327 264±31	47-230 138±54	88-164 132±15

it is assumed that the chromosomes are cylindrical in shape. Each chromosome, however, is not cylindrical; it is made up of two individual chromatids, which are cylindrical. Therefore, the use of the first method results in an over-estimation of the volume of the total chromosome complement. This is reflected in the differences in the total chromosome complement volumes calculated by using two different methods (cf. method 1 and 2 in Table 12). The values of the total complement volume obtained by the second method were approximately half those obtained by using the first method. Therefore, the second method gives a more accurate estimate of the volume of the total chromosome complement.

At 4 hours from the end of treatment with colchicine, volumes of only 0.5% cells (range of volumes: 274 - 460 μm^3) fall within the range of the volumes of the total chromosome complements. About 24.8% nuclei have volumes in the range of 567 to 920 μm^3 which is approximately double that of the total complement volumes; the rest, 74.4% of the nuclei, have volumes that are much larger than those of the total chromosome complement. Since the volumes of the total chromosome complements are taken as the estimates of the minimum possible volumes of the tetraploid nuclei at the very beginning of the interphase, the volumes of the tetraploid nuclei at 4 hours suggests that all but very few tetraploid nuclei increase enormously in volume within the first two hours in G_1.

IV. DISCUSSION

The root meristems of Vicia faba have a complex organization. They are known to be heterogeneous for a number of structural and physiological characteristics:

(i) At the structural level, there are variations in cell shapes and axes of cell division (Davidson, 1975).

(ii) At the physiological level there are variations in cell cycle duration (Howard and Dewey, 1960; Murin, 1966; Webster and Davidson, 1968), nuclear volumes (Rasch, Rasch and Woodard, 1967; Davidson, 1972, 1975) and in growth rates of nuclei (Davidson, 1975).

d. Nuclear volumes:

The results reported here extend the evidence for heterogeneity within the root meristems of V. faba. The meristems of roots at different developmental stages have large variations in interphase as well as in prophase nuclear volumes. The form of the distribution of interphase nuclear volumes differs in the different meristems; in long laterals there is one log-normally distributed population but in all other stages there appear to be either two or more subpopulations.

Nuclear volumes differ in different developmental stages of roots of V. faba (Bennett, 1970; Bennett, Smith and Smith, 1972; Macleod and Mclachlan, 1974). Mean nuclear volume is lower, 704 μm^3, in 21 day old primary

roots than in seven-day old primaries, where it is 805 μm^3 (Bennett, 1970); each value is an average of the values from two replicates, each of 50 nuclei. Bennett gives no standard deviation or indication of the range of values but his results agree with those reported here (Table 3). Bennett, Smith and Smith (1972), however, for the same variety of V. faba quote a mean interphase nuclear volume of 252 μm^3, based on 300 nuclei. They also report a mean value of 528 μm^3 for the volume of a diploid metaphase complement. Thus the mean interphase volume they report is about the same as the mean volume of an anaphase complement of chromatids (528/2 μm^3 = 264 μm^3) and, therefore, is the minimum volume expected in very early G_1 nucleus. It appears that the sample they have scored is not representative of nuclei of all stages of interphase. Macleod and Mclaghlan (1974) reported changes in the nuclear volumes in the cap, epidermis, cortex and stele during lateral root elongation in V. faba. Serial transverse sections of roots were used for determinations of nuclear volume; only 45-50 nuclei and only larger spherical or nearly spherical nuclei were measured. Nuclear volumes of all tissues of newly emerged laterals were greater than the corresponding values in the longer laterals and were generally minimal in all tissues of roots 0.5 - 2 cm in length. Nuclear volumes were greatest in the cortex and lowest in the cap; values of nuclear

volumes were similar in epidermis and stele. Because of the different procedures used in their determinations of nuclear volumes, their results cannot be directly compared with those reported here. Even though the values reported by Macleod and Mclachlan are somewhat biased, their results agree with the observations (Chapt. III, sec. 1.1) that mean nuclear volume is greater in 1 mm laterals than in 2.0 - 2.5 cm laterals, and 2) that mean nuclear volume changes as a lateral root develops.

Variations in nuclear volumes also occurs in other systems. In primary root meristems of Pisum sativum (Lyndon, 1967) there is a 5 fold range in mean volume; the mean volume for 20 telophase nuclei was 93 μm^3 and for ten prophase nuclei was 460 μm^3. The range in telophase nuclei was 30 - 300 μm^3, and in prophase nuclei; 260 - 860 μm^3. Thus there is a 10 fold range of volumes in telophase and a 3.5 fold range in prophase nuclei. Measurements were made on sectioned meristems. Lyndon (1967) also showed that there is considerable overlap in sizes of nuclei having 2C or 4C DNA contents; these results are based on a total sample of about 150 nuclei. For roots of P. sativum, therefore, it appears that there is considerable variation in nuclear volume and that G_1 and G_2 values overlap.

Similar variation in nuclear volume and evidence for overlapping of volumes of nuclei with different DNA values

have been reported for cultured fibroblast like cells from human skin (Mittwoch, 1968). Within a range of ± 1 standard deviation of the mean, the nuclei with a 2C DNA value (mean of 77 nuclei) showed a 10 fold range, from 137 to 1349 μm^3; those with 3C value (mean of 91 nuclei) showed a 4 fold range, from 474 to 1910 μm^3, and the nuclei with a 4C DNA value (mean of 127 nuclei) had a 3 fold range, 758 - 2552 μm^3. There is some correlation between nuclear size and DNA content, mean volume of 4C nuclei was 2145 ± 949 μm^3, of 3C nuclei, 1665 ± 897 μm^3 and of 2C nuclei, 793 ± 556 μm^3; but, as Mittwoch suggests at each level of DNA content there is a range of nuclear volumes. Thus it appears that constituents of nuclei, other than DNA play a part in determining nuclear size. Further evidence for this view is provided by human lymphocytes. These cells are derived from proliferating cells, have 2C amounts of DNA and are arrested in the G_0 phase of the cycle. Nuclear volumes in these cells ranged from 45 to 96 μm^3, with a modal value of 66.6 μm^3 (Mũniz, Houston, Cruz-Abad, Ritzman and Levin, 1970). Even in G_0, therefore, there is a 2 fold range in nuclear volume.

The results from these studies provide evidence that nuclei show more than a doubling during interphase. With the exception of Mittwoch's study, however, the sample sizes are small and the values are based on samples that were not taken at random. The main drawback to the use

of small samples from roots of V. faba or P. sativum is that the investigators have ignored the presence of heterogeneity of cell cycle duration and therefore their samples may not include representatives of fast, slow and non-cycling cells. It seems important to bear in mind this heterogeneity. In this investigation, an attempt was made to identify the three types of interphase nuclei. One method that might reveal these nuclei is graphic analysis.

Large samples of nuclei of interphase cells, dissected from root meristems, were measured; the roots were not sectioned and therefore selection of median sections of nuclei was avoided. On the basis of graphic analyses of nuclear volumes, discrete subpopulations corresponding to the fast, slow and non-cycling cells, could not be identified in this study. This means that rates of nuclear growth vary within each subpopulation and hence there is overlapping of volumes. This conclusion from random samples of nuclei is confirmed by the range of volumes in G_1 and G_2 nuclei of a marked population of tetraploid cells (Chapt. III, sec. 3).

In none of the meristems of emerged lateral roots and primary roots could a discrete subpopulation with very small nuclear volumes, corresponding to the subpopulation of non-cycling cells be identified. This implies that the nuclei of non-cycling cells must have

increased in volume since they were formed; nuclei of non-cycling cells grow during interphase and some of the growth must occur so early in G_1 that they overlap in size with newly formed G_1 nuclei of fast and slow cycling cells. Since nuclei of non cycling cells show some growth they must be metabolically active to some extent. What is not known is whether or not they are metabolically active in other ways that enable them to contribute to over-all growth of the meristem.

It is known from at least two other systems that the nuclei of resting cells are larger than those of the dividing cells. The average nuclear diameter is 7% higher in the resting than in dividing primary root meristems of Triticum durum var. cappelli (Innocenti, 1971). The histone content per nucleus was also higher in the resting than in the dividing meristems. This is in agreement with the functional role of the histones in the repression of gene action. Innocenti (1971) also suggested that the resting meristems may also have higher amounts of acidic proteins and this might influence nuclear size. The cells of the quiescent region of shoot meristems of Tradescantia paludosa have larger nuclear volumes than the adjoining actively dividing cells (Naylor, 1958; Yun and Naylor, 1973). Tobin, Yun and Naylor (1974) have shown that the nuclei in the quiescent region have a higher arginine:DNA ratio and a higher proportion

of an arginine-rich protein fraction that occurs in mitotically active cells of the shoot meristems.

2. Relationship of nuclear volume with interphase time:

In contrast with the results presented in this study, Rasch, Rasch and Woodard (1967) reported that they could identify two discrete subpopulations of values of nuclear volumes in the primary root meristems of V. faba. About 10% of the nuclei had smaller volumes and were suggested to be the non-cycling cells. However, this work differs from the present work in three important ways:

(1) the determinations of nuclear volumes were based on measurements of nuclei in sections of roots; moreover, Rasch et al. (1967) made two assumptions in the treatment of their data

(2) nuclear volume is directly proportional to the time spent by the cell in the cycle, and

(3) rate of growth of nuclei is uniform throughout the interphase.

Woodard, Rasch and Swift (1961) also suggested that all nuclei at a given stage have a particular volume, which is unique to that stage. They reported for 10 - 20 nuclei, a mean nuclear volume ± S.E. of 145 ± 6 μm^3 for telophase nuclei and a range of 334 - 356 μm^3 for very early interphase nuclei in V. faba roots. The assumption that nuclear volume is directly proportional to the time spent by a cell in interphase has also been

made by Lyndon (1967) and by Lafontaine and Lord (1974). In the latter study nuclear volume was used to identify the age of a cell in interphase for a study of nuclear ultrastructure.

This assumption appears to be completely unfounded. The volumes of prophases of diploid and tetraploid nuclei cover a range of volumes; there is not a consistent value for all prophases. Furthermore, some prophase values are less than the volumes of interphase nuclei. Also tetraploid nuclei in G_1 and G_2 are variable in volume and their values overlap. It is concluded, therefore, that there is no strict correlation between nuclear volume and the age of the nucleus in the cell cycle.

The results reported here also show that nuclear volume can vary independently of the DNA content of the nucleus i.e., tetraploid G_1 nuclei with a 4C DNA content may be larger than G_2 nuclei with an 8C DNA content (Chapt. III, sec. 3).

It has been concluded that there is no absolute correlation between nuclear size and age in interphase. This means that nuclei must vary in their growth rate during interphase. Two lines of evidence support this: (1) variation in volume of prophase nuclei and (2) variation in volume of tetraploid G_1 nuclei.

3. Prophase nuclei:

About 75% of diploid prophase nuclei are from fast cycling cells (Webster and Davidson, 1968). The mean cell cycle duration of these cells is 14 hours. Prophase of fast and slow cycling cells cover a 6.6 fold range of volumes, so whether prophases of fast cycling cells cover the lower or upper end of the range of volumes, they do not form a narrow group of values. Cell cycle duration in fast cycling cells does not vary more than 1.3 fold i.e. about 12 - 16 hours, but prophase volumes in these cells cover at least a 4 fold range of volumes. This suggests that, within the subpopulation formed by fast cycling diploid cells, there is variation in the rate of nuclear growth. Data from tetraploid nuclei support this conclusion. A sample of 100 tetraploid prophase nuclei at, for example, 14 hours are known to be from fast cycling cells; they show a 7 fold range of volumes from 2189 to 15,576 μm^3. They appear to have grown at different rates. In the period 13 - 14 hours after colchicine, 200 tetraploid prophase nuclei were measured. Their volumes range from 1052 to 15,576 μm^3. This is a 15 fold range. Thus, over a one hour period there is a 15 fold range in prophase volumes, which is further evidence for non-uniformity of growth rates of interphase nuclei.

4. Tetraploid G_1 nuclei:

Within 2 - 3 hours of their formation, tetraploid nuclei vary 14 fold in volume. It has been shown that at least 70% of these cells have a cell cycle duration of ∿14 hrs. (Chapt. III, sec. 3). Even within one hour of their formation, therefore, G_1 nuclei vary in rate of growth. Variation in nuclear growth is more pronounced between 4 and 10 hours and 10 and 14 hours from the end of colchicine treatment (Figure 22). Some nuclei increase in volume between 4 and 10 hours, and then appear to grow slowly. Others grow little between 4 and 10 hours and then grow rapidly between 10 and 14 hours. In tetraploid cells we see ample evidence of variation in growth rates of nuclei; it varies in different nuclei in one part of interphase and it varies within one nucleus at different parts of the cycle. These results show that heterogeneity of cell cycle duration is accompanied by heterogeneity of rates of nuclear growth. A rapid increase in nuclear size in the first hour in G_1 has been reported for HeLa S3 cells (Maul, Maul, Scogna, Lieberman, Stein, Hsu and Borun, 1972). Surface area increased to 200 μm^2 by one hour after mitosis followed by a plateau level of 240 μm^2 at 2 - 3 hours. At five hours from the end of mitosis i.e., at the G_1-S transition, a second increase in surface area occurs and precedes DNA synthesis for

most cells. By the end of S, nuclear surface area has risen to 393 μm^2.

5. Growth of nucleus in interphase:

From the range of volumes of prophase nuclei it appears that growth of nuclei to a particular size is not automatically followed by entry into mitosis. Hence, not all growth of interphase nuclei may be necessary for nuclear division. Growth of nuclei may have two components: (a) an increase in the amount of constituents needed for nuclear division and (b) an increase in the constituents that regulate rate of growth through interphase or are needed, in some way, for the regulation of nuclear behaviour in the daughter cells. In any case, it cannot be argued that only nuclear factors regulate entry into prophase; cytoplasmic factors are also involved. Zetterberg (1971) suggested that although a certain amount of cell growth appears to be necessary for cell division, the total growth of a cell during a cell cycle may not be directly linked to the initiation of cell division. A similar situation may occur with nuclei. The growth of a nucleus is probably dependent, partly, upon the volume of the telophase chromosome complement from which it is formed. Differences in growth rates may mean that different nuclei enter interphase with chromosome complements of different volumes; those with small initial nuclear volumes may grow slowly in early G_1 while those with large initial volumes may grow rapidly. Variation occurs, as we have seen, in chromosome volume (Chapt. III, sec. 4).

6. Chromosome volumes:

Metaphase chromosome volumes were calculated in two ways (Chapt. III, sec. 4). 1) A mean of five chromatid widths were determined for each cell and then used to calculate chromosome volume for that cell; 50 complements were measured. 2) The mean of 250 chromatid widths were used to calculate chromosome volume for all 50 cells. With the first method the range in volumes was 94 to 460 μm^3, a 5 fold range, while with the second method the range was 175 to 327 μm^3, a 2 fold range.

The results mean that diploid nuclei enter G_1 with a minimum volume of 47 to 88 μm^3 and a maximum volume of 164 to 230 μm^3. The minimum nuclear volume observed in a diploid cell in a long lateral was 169 μm^3; i.e., it is in the range of values expected from the calculations of chromosome volumes. The minimum volume of a tetraploid nucleus was 274 μm^3. This is also close to the expected value for a nucleus formed from a complete diploid metaphase complement.

Thus for diploid and tetraploid nuclei, the volumes of the complements from which they are formed are not uniform. Nuclei, therefore, are variable in volume from the onset of G_1.

Chromosome volume changes in response to a change in the supply of phosphate (Pierce, 1967; and Bennett and Rees, 1969). High phosphate treatments produced larger

chromosomes; in Allium cepa root tips, mean chromosome volume increased from 243 μm^3 to 334 μm^3 after seven days on a high phosphate treatment (Bennett and Rees, 1969). These authors also showed that the increase in chromosome volume was accompanied by parallel increases in total nuclear proteins; there was no change in DNA content. Bennett (1970), Bennett, Smith and Smith (1972) and Flannagan and Jones (1973) reported the occurrence of a natural variation in the volumes of the total chromosome complement; e.g., there was variation between lateral and primary roots of V. faba (Bennett, 1970). He suggested that such variation may be correlated with different levels of cellular metabolism and Flannagan and Jones (1973) report, in Secale cereale, that the largest chromosome volumes were found in cells undergoing rapid growth. Slower growth was associated with a reduced chromosome volume.

These studies of chromosome volume suggest that nuclei formed from a chromosome complement of large volume begin G_1 with more protein than nuclei formed from a small chromosome complement. This does not mean that nuclear growth in G_1 is regulated by the protein content of the chromosomes; nevertheless, it is an interesting possibility. It would mean that the protein content of a chromosome complement is important for the regulation of nuclear growth in the subsequent interphase. Thus,

it would provide a chromosomal mechanism by which a nucleus influenced the growth of its daughter nuclei.

7. Protein content of nuclei:

Nuclei as well as contracted chromosomes vary in protein content. It has been shown that there is a 5 - 11 fold difference in protein content of nuclei in laterals of V. faba (Chapt. III, sec. 2). Though the variation in protein content is not as great as that of nuclear volume, it is clear that for protein content, nuclei of a meristem are also heterogeneous.

Furthermore, total nuclear protein content varies in nuclei of different shapes; it is not confined to any one type of nucleus. Whether or not the protein content in G_2 nuclei directly determines the protein content of the metaphase chromosomes is not known. Though it is interesting that prophase nuclear volumes vary 6.6 fold while metaphase chromosome volumes vary 5 fold.

From what is already known about V. faba and other systems and from the present study, it appears that there are variations at the level of nuclei, chromosomes and individual nuclear component e.g. proteins. These variations occur in cells of meristematic tissues which have so far been characterized as a group of proliferative, undifferentiated cells with identical genotypes. Perhaps the variations inherent in the system add flexibility to

and made meristematic cells able to withstand fluctuations in environmental conditions.

8. Nuclear morphology:

It is now clear that meristematic cells in V. faba vary in nuclear growth rate, time of active growth in the cell cycle, cell cycle duration and protein content. In addition, it has been found that there is variation in nuclear shapes. Four shapes of nuclei were found: spherical, nearly-spherical, oval and elongate. The frequencies of cells with different nuclear shapes differs in the meristems of roots at different developmental stages. There is similarity between meristems of younger roots, i.e., small primordia and young primaries (Table 4). Similarly, the meristems of older primary roots resembled those of older laterals in the frequencies of cells with different shapes of nuclei. Similar changes in the frequencies of differently shaped nuclei occur in the growth of primary and lateral roots.

Changes in nuclear shape result in changes in nuclear surface area. For example, a spherical nucleus has a lower surface area than an elongate nucleus with the same volume. The changes in frequencies of nuclear types as roots develop may be important in providing an increased surface area for the exchange of materials between the nucleus and the cytoplasm. Perhaps an increased surface area facilitates uptake of cytoplasmic components during

early G_1: elongate nuclei may grow more rapidly in early G_1 than spherical nuclei. However, no data bearing on this are available at present.

Nuclear shape may change during interphase; it would be difficult to show this directly except in cells in culture that could be followed as they proceed through a cell cycle. However, observations on tetraploid cells suggest that no change occurs. The frequencies of spherical and nearly spherical nuclei are 20% at 4 hours, 26% at 10 hours and 20% at 14 hours; for oval nuclei the values are 37% at 4 hours, 39% at 10 hours and 34% at 14 hours and for elongates, the corresponding values at 43%, 35% and 46%. These results suggest that the nuclear shape established in early G_1 is maintained throughout interphase. How nuclear shape is controlled and why there are differences in nuclear shape are not known. Variation in nuclear shape, however, adds another dimension to the heterogeneity among meristematic cells.

9. Mitotic index in tetraploid cells:

About 70% of the tetraploid cells have divided or are in mitosis when the population is 14 hours old. It was concluded that their cell cycle duration, 14 hours, is close to that for fast cycling diploid cells. Over a one hour period, 19.2% of the tetraploid cells enter mitosis or divide (Table 11). If this frequency was repeated every

hour then the MI at 14 hours, i.e., 62.8 would be 62.8 + 38.4 = 101.2 by 16 hours; also it would have been expected to be 47.6 - 19.2 = 28.4 at 13 hours and 28.4 - 19.2 = 7.2 at 11 hours. Therefore, if the tetraploid cells are all fast cycling the range in cell cycle duration would be 11 - 16, i.e. 5 hours. On the other hand, if only 70% are fast cycling then their cycle time will vary only by 3 hours, i.e., 11 - 14 hours.

The proportion of tetraploid cells that are fast cycling, 69.4%, is high. Of the diploid cells induced to become tetraploid 75% are fast cycling (Webster and Davidson, 1968). We see that 70%, at least, of their progeny are also fast cycling. This leads us to speculate about the factors that regulate cell cycle duration and so determine whether a cell will be fast, slow or non-cycling. Are these factors cytoplasmic or nuclear? The answer, unfortunately, is not known; this points out how little critical information is available on:

(1) the extent to which proliferating cells differ in their cytoplasmic components,

(2) the frequency of divisions that are, in some way, differential and

(3) the effect of chromosomal proteins on nuclear growth in daughter cells.

Heterogeneity exists within a meristem and between meristems of *V. faba*. The wide range in the variability

of a number of nuclear and cellular parameters suggests that:

(i) they are of some significance for the functioning of the meristem

(ii) the different characteristics must be under the control of factors that operate at the levels, both of single cells and groups of cells. This means that different parts of a meristem as well as meristem cells of roots at different developmental stages may differ in the intracellular conditions. These diversities do not appear to interfere with the ability of the cells to proliferate. However, some regulating mechanism must be acting to integrate the activities of the various control mechanisms and so produce normal root growth.

V. CONCLUSIONS

1. Root meristem cells of *V. faba* are heterogeneous for growth rates, volumes, protein contents and shapes of nuclei, besides being heterogeneous for cell cycle duration and cell shapes. Moreover, the changes in the growth rates, volumes and shapes of nuclei accompany changes in physiological activities that occur during the maturation of the roots.

It is clearly important to recognize the heterogeneous nature of the meristematic cells whenever roots are used as experimental material.

2. Nuclei undergo large increases in volume during interphase. Even in G_1 nuclear volumes cover a wide range of values. Since this variation occurs without a change in DNA content, changes in other nuclear constituents must contribute to variation in nuclear volumes.

3. It has been established that there is no fixed correlation between nuclear volume and age in cell cycle. It appears that nuclear size is not the only factor that determines entry into mitosis. The growth of nuclei during interphase may have two components: (a) an increase in the nuclear constituents needed for nuclear

and, perhaps cell division, (b) an increase in constituents that may regulate the growth of the two daughter nuclei and cells.

4. Nuclear growth is not restricted to cycling cells. Nuclei of non-cycling cells also grow.

5. There is variation in nuclear growth during interphase. This is suggested by two lines of evidence:
(i) variations in volumes of nuclei in prophase,
(ii) variations in volumes of tetraploid nuclei in G_1. Variations in the amounts of increase in volumes of tetraploid nuclei within as well as between different subphases further confirm the variation in the growth rates of nuclei during interphase.

6. One possible factor that might be affecting the growth rates of nuclei during the interphase, is the volume of the chromosome complement from which the nuclei are formed. However, cytoplasmic components of the cells may also affect growth rates of the nuclei, e.g., nuclei of cells with a smaller cytoplasmic mass may grow differently than those from cells with larger cytoplasmic mass.

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