NUCLEIC ACID AND PROTEIN METABOLISM DURING THE MITOTIC CYCLE IN *VICIA FABA*

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ABSTRACT

In order to investigate some of the cytochemical processes involved in interphase growth and culminating in cell division, a combined autoradiographic and microphotometric study of nucleic acids and proteins was undertaken on statistically seriated cells of *Vicia faba* root meristems. Adenine-8-C^{14} and uridine-H^3 were used as ribonucleic acid (RNA) precursors, thymidine-H^3 as a deoxyribonucleic acid (DNA) precursor, and phenylalanine-3-C^{14} as a protein precursor. Stains used in microphotometry were Feulgen (DNA), azure B (RNA), pH 2.0 fast green (total protein), and pH 8.1 fast green (histone). The autoradiographic data (representing rate of incorporation per organelle) and the microphotometric data (representing changes in amounts of the various components) indicate that the mitotic cycle may be divided into several metabolic phases, three predominantly anabolic (net increase), and a fourth phase predominantly catabolic (net decrease). The anabolic periods are: 1. Telophase to post-telophase during which there are high rates of accumulation of cytoplasmic and nucleolar RNA and nucleolar and chromosomal total protein. 2. Post-telophase to preprophase characterized by histone synthesis and a diphasic synthesis of DNA with the peak of synthesis at mid-interphase and a minor peak just preceding prophase. The minor peak is coincident with a relatively localized DNA synthesis in several chromosomal regions. This period is also characterized by minimal accumulations of cytoplasmic RNA and chromosomal and nucleolar total protein and RNA. 3. Preprophase to prophase in which there are again high rates of accumulation of cytoplasmic RNA, and nucleolar and chromosomal total protein and RNA. The catabolic phase is: 4. The mitotic division during which there are marked losses of cytoplasmic RNA and chromosomal and nucleolar total protein and RNA.

A primary area of cytology concerns the complex and periodic physiological changes that accompany the mitotic cycle. It has become increasingly evident that much of the mitotic process involves a utilization and segregation of materials synthesized during the preceding interphase. It would seem, therefore, that a study of the time and rate of synthesis in the mitotic cycle of the major nucleoprotein fractions of the cell, would form one approach to an understanding of mitosis and interphase growth. In the present study, the nucleoproteins of the nucleolus, chromosomes, and cytoplasm have been studied using both microphotometric and autoradiographic methods. A study of this kind may be made on a homogeneous tissue, the cells of which are undergoing a division cycle which is synchronous, or if non-synchronous, cells may be related to time in one of a number of ways: seriated by selection of cells at the same stage of mitosis (Prescott, 1955) synchronized by tempera-

ture manipulation (Scherbaum and Zeuthen, 1954), or statistically seriated. In the following study a statistical method for estimating relative interphase time was used on root meristems of *Vicia faba*.

METHODS AND MATERIALS

The statistical method of seriation is based on the assumption that the frequency of an interphase and mitotic event is proportional to the duration of time taken by that event. Such a method was used by Laughlin (1919) to estimate the relative time of the mitotic stages, and more recently by Grundman and Marquardt (1953) to plot nuclear volume changes against interphase time in root meristems. Fig. 1 shows several volume frequency curves, all having approximately the same curve shape. These graphs can be interpreted in two ways: either they represent a single population of cells with wide variance in volume at any given time in interphase, or they represent a single population of cells in which each nucleus is changing in volume as a function of time. It should be possible to determine which of these is correct by investigating the variability of nuclear volumes at several recognizable stages of the mitotic cycle. Accordingly, volume determinations were made on several mitotic stages and very early interphase. As Table I indicates, the standard errors of these nuclear volumes were low, in spite of the fact that they will tend to be overestimated by inevitable small inaccuracies in staging. It can be seen not only that all nuclei at a given stage fall in a certain volume class, but that only nuclei at this stage have that volume. Assuming a similar low coefficient of variation at all other stages of interphase growth, we can conclude that changes in nuclear volume are a function of interphase time, and that variability of nuclear volume for a specific time class has little if any influence on the volume frequency curve. It follows then that we may consider nuclear volume frequency proportional to percentage interphase time. All cytochemical changes analyzed can then be related to

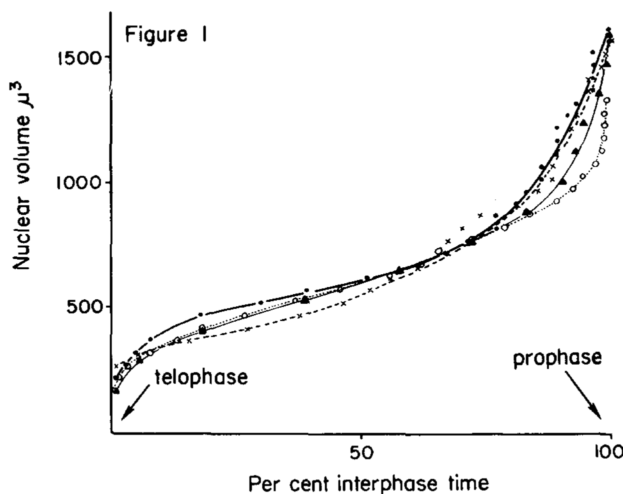


FIGURE 1

Changes of nuclear volume in four *V. faba* root tips plotted as a function of relative interphase time calculated from nuclear volume frequencies (see Methods and Materials).

TABLE I

Nuclear Volume Estimates of Morphologically Recognizable Stages of the Mitotic Cycle

Volumes were computed from planimeter measurements of areas of projected nuclear images

	Root A			Root B		
	Nuclear volume in μ^3 and standard error	N	S. E. as per cent of mean	Nuclear volume in μ^3 and standard error	N	S. E. as per cent of mean
Earliest visible prophase	804 ± 17	15	2.1	839 ± 16	20	1.9
Early prophase	1005 ± 19	20	1.9			
Very late prophase and prometaphase				1553 ± 37	10	2.4
Telophase				145 ± 6	12	4.3
Very early interphase	356 ± 6	20	1.7	334 ± 7	20	2.4

percentage interphase time by placing individual cytochemical values on the time curve according to the volume of the nucleus of the cell from which the value is taken.

Roots were obtained by germinating seeds of *V. faba* in shallow dishes of tap water for 24 hours before removing the seed-coat and placing in damp vermiculite. After 6 to 7 days of growth at 25°C., primary roots of nearly the same thickness and approximately 8 cm. long were selected for study. All cytochemical measurements, both autoradiographic and microphotometric, were made on the cortical cells of roots in the region 700 to 850 microns from the root tip. Nuclease extractions were routinely checked by Feulgen staining in the case of deoxyribonuclease (DNase), and azure B staining in the case of ribonuclease (RNase).

Roots for autoradiography were immersed for varying treatment times in either 5 μc./ml. of adenine-8-C^{14} (1.15 mc./mM) or 5 μc./ml. of DL phenylalanine-3-C^{14} (1.1 mc./mM), or 5 μc./ml. uridine-H^3 (680 mc./mM), and either 5 μc./ml. or 2 μc./ml. thymidine-H^3 (1.9 mc./mM), dissolved in double distilled or deionized water. Several of the roots treated in uridine-H^3 for 1½ hours were transferred to unlabeled glass-distilled water and fixed at intervals up to 50 hours. Four-micron sections of 3:1 ethanol-acetic fixed, paraffin-embedded sections were extracted in various ways:

A. Roots grown in adenine-8-C^{14} (California Foundation for Biochemical Research, Los Angeles) and roots grown in uridine-H^3 (New England Nuclear, Boston).
   1. Tissue sections were placed in 0.02 per cent DNase (Worthington, Freehold, New Jersey) in 0.003M $MgSO^4$ adjusted to pH 6.0 at 25°C. followed by treatment in 5 per cent trichloroacetic acid (TCA) at 2–4°C. for removal of acid soluble nucleotides.
   2. Tissue sections on several slides were digested in DNase as in (1) followed by 0.02 per cent RNase (Armour, Kankakee, Illinois) at pH 6.5. Slides were then treated for 5 minutes in 5 per cent TCA at 2–4°C.
   3. One slide was hydrolyzed in 5 per cent TCA at 90°C. for 30 minutes for removal of both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Pollister and Ris, 1947).

B. Roots grown in phenylalanine-3-C^{14} (California Foundation for Biochemical Research).
   1. Slides were placed in DNase as above followed by 5 per cent TCA at 2–4°C.
   2. Another group of slides was treated in RNase as above followed by 5 per cent TCA at 2–4°C.
   3. Removal of nucleic acids in 5 per cent TCA at 90°C. for 30 minutes was carried out on one slide.

C. Roots grown in thymidine-H^3 (Schwarz, Mt. Vernon, New York) were hydrolyzed in N HCl, Feulgen-stained, and either sectioned or squashed. In initial experiments, some slides were digested in DNase as above.

After washing in cold distilled water, all slides were covered with stripping film (Kodak AH 10) and stored at 4°C. Following varying exposure times, stripped slides were developed in D-19 (18°C. for 5 minutes) and stained where appropriate with 0.025 per cent azure B in pH 4 McIlvaine's buffer for periods up to 30 minutes, rinsed in cold distilled water for several minutes, and air dried. Autoradiographs of the various fractions are shown in Figs. 2 to 7.

Areas for grain counting were delimited by using a net reticule whose component squares had an area of 45 μ^2 at the magnification used. Background grain counts were made adjacent to tissue sections, and all grain counts over tissues were corrected accordingly. To avoid scatter (silver grains resulting from oblique emissions) from the highly radioactive nucleoli of adenine-8-C^{14}-treated cells, grain counts for chromosomal RNA[1] were made only over nuclei from which the nucleoli had fallen out of the nucleus (Fig. 5) during slide processing and which were at least 5 to 6 microns away from the nearest nucleolus. The radioactivity of whole spherical nucleoli and nuclei (chromatin) may be estimated by assuming that the ratio of radioactivity of an organelle to a 4-micron median section of this organelle is equal to the ratio of their volumes. Thus for nucleoli:

$$N = \frac{nD^3}{6D^2 - 32},$$

and for nuclei (excluding the nucleolus):

$$N = n_1 \frac{D_1^3 - D^3}{6(D_1^2 - D^2)},$$

where N is the total estimated number of silver grains, D is nucleolar diameter in μ, D_1 is nuclear diameter in μ, and n and n_1 are the number of silver grains in the emulsion above a 4 μ section of the nucleolus and nucleus (chromosomes) respectively.

[1] The terms "chromosomal RNA" and "chromosomal protein" are here considered for convenience to be the total RNA and protein of the nucleus exclusive of the nucleolus. These fractions thus actually include components of both chromatin and nucleoplasm. The actual amount of RNA and protein in the nucleoplasm is not known. Furthermore, we are obviously dealing here only with those nucleoprotein fractions which are resistant to the solvents used in the preparation of tissues.

To characterize and define the various radioactive fractions dealt with here, it was necessary to perform a number of extractions with nucleases and TCA. Extractions made on tissues that had incorporated adenine-C^{14} indicated that DNase and RNase treatment, in that order, followed by immersion in 5 per cent TCA at 2–3°C., removed all of the nucleolar label and only 60 per cent of the chromosomal label. This nuclease-resistant fraction was removed by treatment in 5 per cent TCA at 90°C. for 30 minutes, and is thus presumably a nucleic acid (Pollister and Ris, 1947). Several lines of evidence indicate that this fraction is not a DNase-resistant DNA for such slides are Feulgen-negative, and furthermore, if this were DNA, one might expect a mid-interphase increase in the grain counts after DNase corresponding to the time of peak DNA synthesis. That this does not occur can be seen in the plot of chromosomal RNA grain counts (Fig. 16). It is likely then that this is an RNase-resistant RNA present in the chromosomes, but absent in the nucleolus. Taylor and McMaster (1954) also described a fraction in lily anthers, presumably RNA, removable in hot TCA, but resistant to RNase.

In experiments with phenylalanine-3-C^{14}, incorporation was probably by a protein fraction, for grain counts over cells that were treated for 1 hour showed no loss of radioactivity following DNase and RNase extraction, when compared with unextracted controls.

As to the incorporation of uridine-H^3, grain counts made on roots grown in uridine-H^3 for 3 hours, showed that RNase digestion removed all of the nucleolar and cytoplasmic label, but only $\frac{2}{3}$ of the chromosomal label. The remaining $\frac{1}{3}$ was evidently in the DNA molecule for it was removable with DNase. Further extraction studies indicated that roots grown in uridine-H^3 for $1\frac{1}{2}$ hours and transferred to glass-distilled water for 6 hours retained a hot TCA-resistant fraction at all sites, presumably a protein which made up approximately 11 per cent of the total radioactivity.

Finally, it was found that tissues grown in thymidine-H^3 (Schwarz, lots 5813 and 5805) showed specific utilization of this precursor in the synthesis of DNA, for DNased slides were free of radioactivity. All grain counts over DNA were made on roots treated with these 2 lots of thymidine-H^3. Another batch of tritiated thymidine (Schwarz, lot 2910), however, showed some cytoplasmic labeling (Figs. 2 and 3). Since this fraction was resistant to hot TCA, it was presumed to be a protein.

Roots to be used for microphotometric measurements of RNA and total protein were also fixed in

FIGURE 2

DNA autoradiograph of metaphase chromosomes showing the heavy labeling of certain segments. The root was grown for 6 hours in thymidine-H^3, Feulgen-stained, and squashed. Stripped slides were exposed for 30 days. × 2000.

FIGURE 3

Same as Fig. 1 except the chromosomes are in anaphase. High concentrations of silver grains are located in the vicinity of the centromeres. × 3000.

FIGURE 4

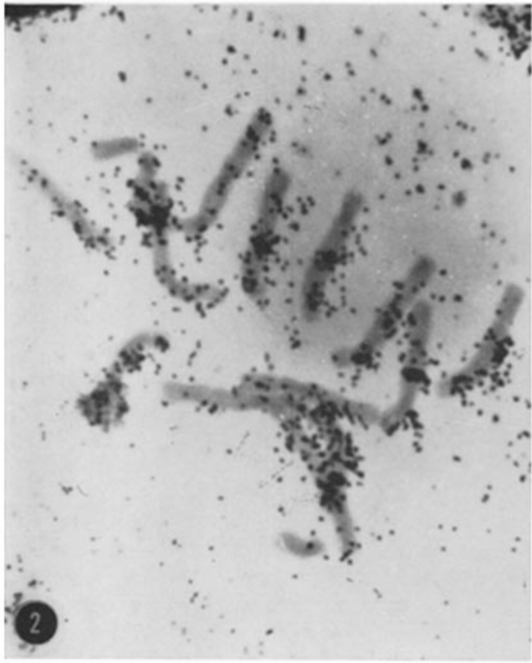
Autoradiograph of chromosomal RNA over nuclei from which the nucleolus had dropped out during slide preparation. Root was grown for 3 hours in uridine-H^3. Four-micron sections were stained in azure B and stripped slides were exposed for 30 days. × 2000.

FIGURES 5 AND 6

RNA autoradiographs from a root grown 1 hour in adenine-8-C^{14}. Fig. 4 shows a chromosomal RNA autoradiograph of nuclei from which the nucleolus had dropped out during slide preparation. Film was exposed for 30 days. Four-micron sections were stained with azure B. × 2000.

FIGURE 7

Total protein autoradiograph from a root grown 1 hour in phenyalanine-3-C^{14} and stained with azure B after digestion in RNase. × 2000.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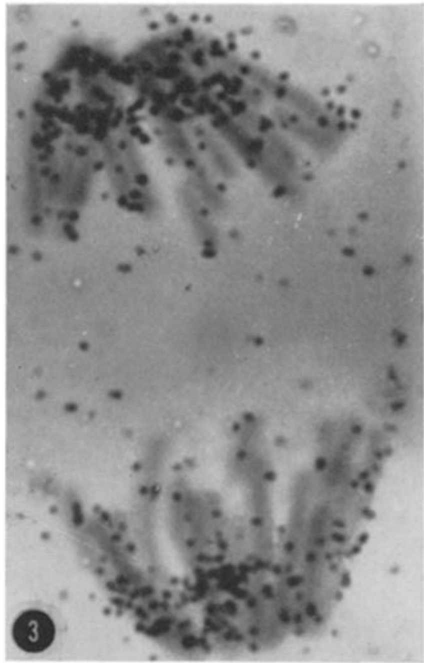
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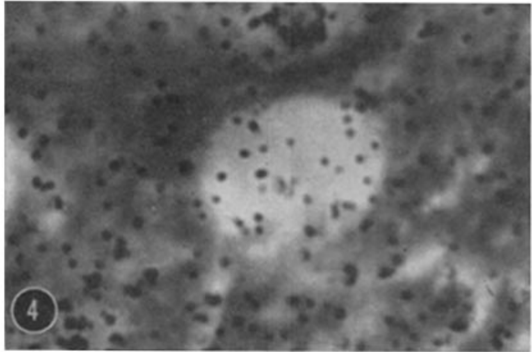
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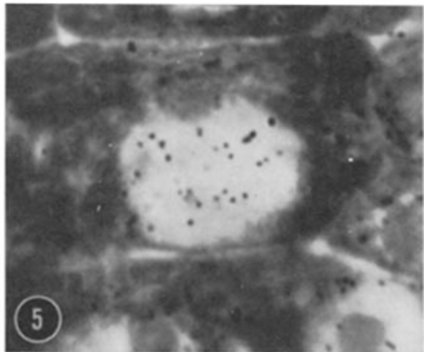
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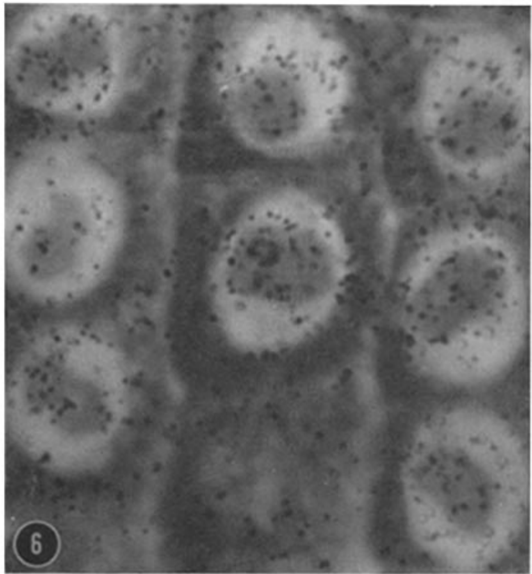
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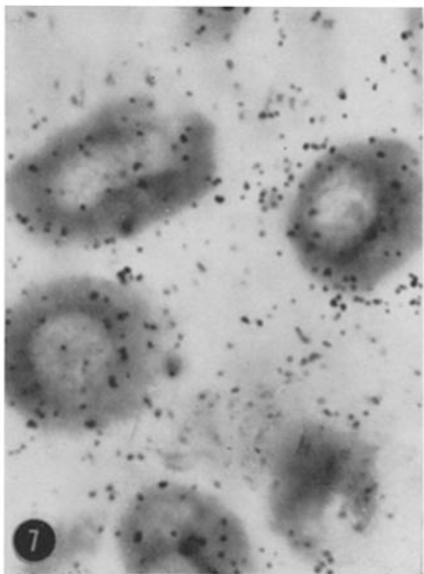
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3:1 ethanol-acetic, embedded in paraffin, and sectioned at 4 microns. Tissue sections for RNA studies were treated in DNase and stained by the azure B method of Flax and Himes (1952), (Fig. 8), while those for total protein studies were digested in both DNase and RNase before staining in 1 per cent aqueous fast green at pH 2 for 1 hour (Schrader and Leuchtenberger, 1950) (Fig. 10). Differentiation for 30 minutes at pH 6 in McIlvaine's buffer was followed by a brief rinse in distilled water, dehydration in absolute ethanol for 10 minutes, and clearing in xylene.

Macerates of single cells (Fig. 9) were prepared using the pectinase method of Setterfield *et al.* (1954). A sliding microtome was used to cut 150-micron transverse sections from roots fixed in 3:1 ethanol-acetic, and paraffin-embedded. These 150-micron thick discs made up that portion of the root 700 to 850 microns from the root tip. The discs were deparaffinized in xylene, hydrated, and shaken in 20 per cent pectinase (Nutritional Biochemicals, Cleveland) at pH 4, for 25 minutes. A drop of the resulting macerate was placed on an albuminized slide and covered with a coverglass. The material was then frozen over dry ice, after which the coverglass was pried off and the slide air-dried. After several distilled water rinses, the slide was placed in 0.02 per cent DNase at pH 6 for 1 hour, again rinsed in distilled water, and then stained with azure B using the procedure described above.

Both DNA and histone measurements were taken from 20-micron sections of a root fixed in 10 per cent formalin for 3 hours, and washed overnight in running tap water. Sections for DNA determinations were stained by the Feulgen technique, and histones were stained in alkaline fast green using the method of Alfert and Geschwind (1953).

Material to be used for microphotometric measurement was mounted in a refractive index oil which matched the refractive index of the tissue. The microphotometric instrument and the methods used with it have been previously described (Swift and Rasch, 1956). In calculating total dye amounts for sections of nucleoli and chromatin the general formula

$$M = \frac{VE}{d}$$

was used, where M is the total amount of dye bound, V is the computed volume of a spherical nucleus or nucleolus, E is the average extinction of core samples through the sectioned organelle, and d is the section thickness. In measuring whole nuclei, the "plug" method (Swift and Rasch, 1956) was used. Total RNA of single cells of the root macerate was measured by the two-wavelength method of Ornstein (1952) and Patau (1952). These values will be referred to as cytoplasmic RNA since there was little or no chromosomal staining in this preparation, and the RNA of the nucleolus made up only about 3.5 per cent of the total amount in the cell.

It would be well to point out that, although the stoichiometry between RNA and bound basic dye is influenced by a variety of factors such as pH, fixation, method of differentiation, etc., under controlled conditions, highly reproducible results may be obtained and good agreement between photometric measurements of basic dye binding and ultraviolet absorption has been reported (Swift, 1955, for review). However the precise proportionality of azure B to RNA between organelles is not known, for azure B will stain the chromosomal RNA of some root meristems of *V. faba* or onion heavily, some lightly, and others not at all, following DNase. RNA from other parts of the cell is not being adsorbed to sites previously occupied by DNA, for the curves of increase of chromosomal RNA during the mitotic cycle might then be expected to coincide with that of DNA. Reference to Figs. 16 and 19 will indicate that these curves show no resemblance.

FIGURE 8

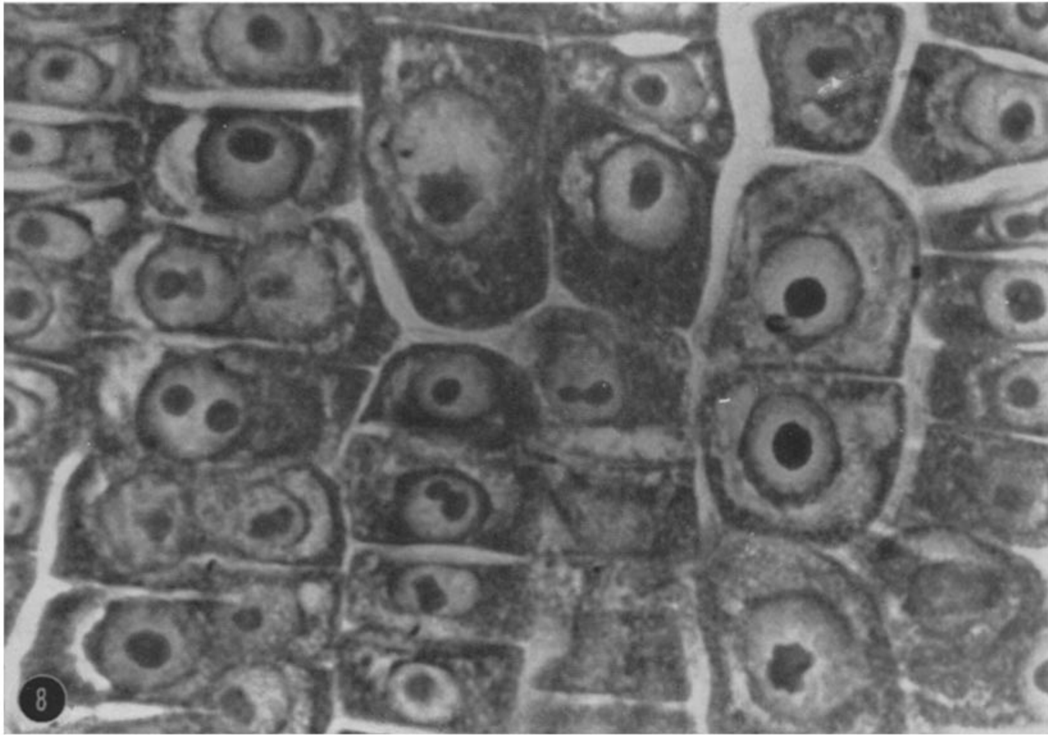
Four-micron section of root tip stained for RNA with azure B. × 1000.

FIGURE 9

Individual meristematic cells separated by pectinase and stained for RNA with azure B. × 1000.

FIGURE 10

Four-micron sections through the root meristem stained for amino groups (total protein) with pH 2 fast green. × 1000.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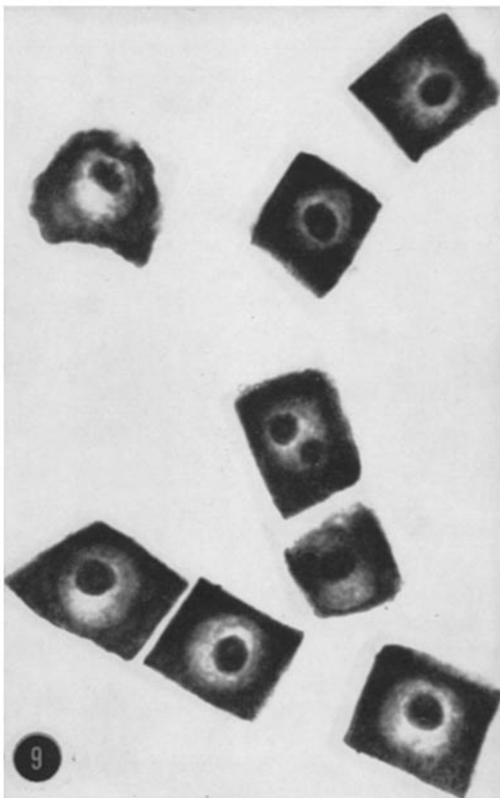
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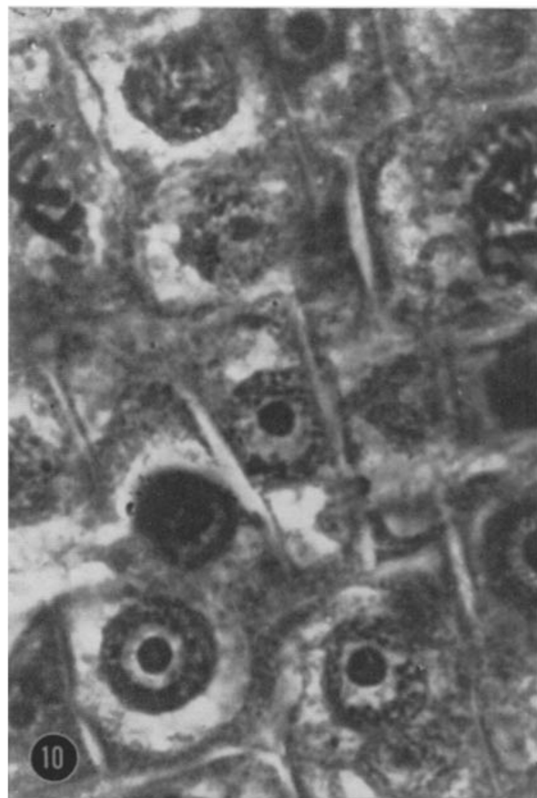
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RESULTS

Autoradiographic Data

Autoradiographic data can be presented in several ways. One method is to express the results as grain concentrations per unit area. Our grain counts for the various fractions have been plotted in this way in Figs. 11 and 12 as silver grains per square micron against interphase time. Since growth of the roots in isotope was restricted to 1 hour, these curves can be considered an expression of rate of incorporation per square micron per hour of interphase time.

On the other hand, activities of the cellular subunits have been expressed as silver grains per unit of bound dye per hour in Fig. 14. This ratio, taken from the curves of grains per square micron and extinction per micron of section thickness (Figs. 11 to 13), might be considered a measure of relative specific activity. These curves showed several significant interphase changes: although the relative specific activity of cytoplasmic RNA remained fairly constant during those portions of interphase studied and the activity of nucleolar RNA gradually declined, RNA of the chromosomes showed a rapid increase in early interphase and maximal activities during the latter half of interphase; activity of chromosomal total protein rose sharply during early interphase, showed maximal activities during the first quarter of the interphase period followed by a gradual decline and a final rise at the end of interphase. The low specific activity of chromosomal RNA and total protein during early interphase may be due to the adventitious inclusion of cytoplasm within the nucleus during telophase.

Another way to express the autoradiographic data is to plot the relative radioactivity of the whole organelle (as derived from grain counts over sections of the organelle) as a function of interphase time. Such a graph expresses rate of incorporation per organelle per hour. The curves of incorporation rate per organelle during the life history of the cell showed several noteworthy relationships. Nuclear (chromatin) uptake of RNA precursor (adenine-8-C^{14}) and total protein (phenylalanine-3-C^{14}) were strikingly similar in that both substances had an increased rate of incorporation during the latter part of interphase (Figs. 16 and 17). As to nucleolar changes, grain counts per nucleolus following 1 hour of growth in adenine-8-C^{14} are shown in Fig. 15. The curve illustrates a high rate of incorporation during the post-telophase period, a leveling off period during the first half of interphase, an increased rate during the latter part of interphase followed by rapid decline.

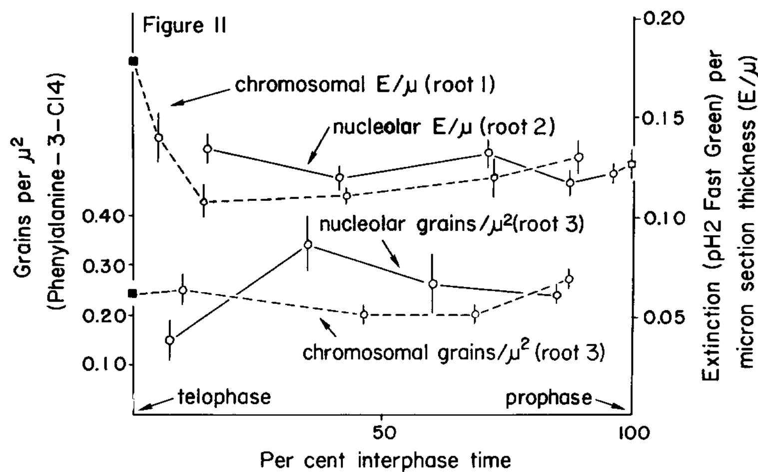


FIGURE 11

Incorporation of phenylalanine-3-C^{14} into nucleolar and chromosomal total protein is expressed as grains per square micron. Concentration of nucleolar and chromosomal total protein (pH 2 fast green) is expressed as extinction per micron of section thickness. Open symbols are means with standard errors. Squares represent telophases on the left and prophases on the right.

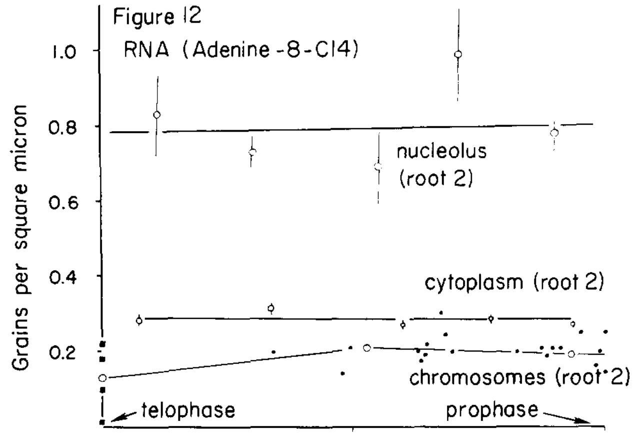


FIGURE 12

Grains per square micron over RNA of the nucleoli, chromosomes, and cytoplasm after growth in adenine-8-C^{14}. Open symbols are means with standard errors; squares are telophases.

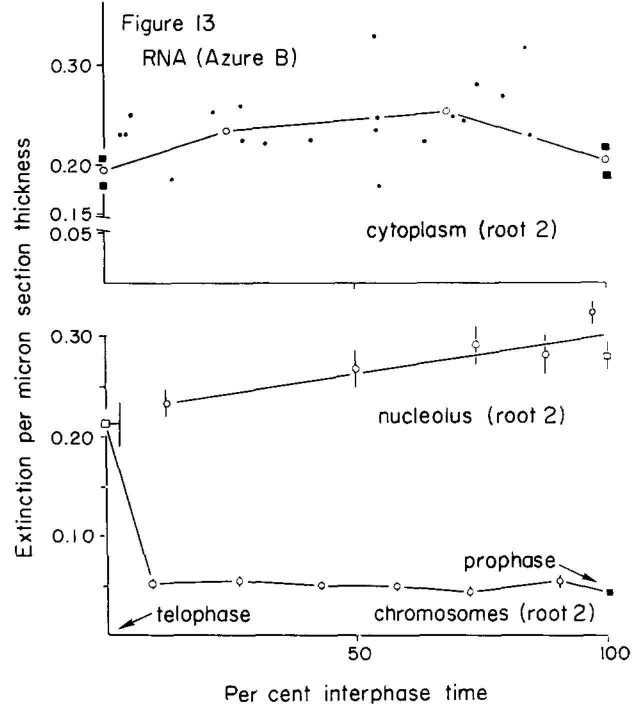


FIGURE 13

RNA (azure B) concentrations are plotted as extinction per micron of section thickness. Open symbols are means with standard errors. Squares are telophases on the left and prophases on the right.

Grain counts made on roots which were treated for 1½ hours in uridine-H^3 and then transferred to non-radioactive water are represented in Fig. 18. The activity curves of nucleolar and chromosomal RNA are roughly similar in their rapid initial increase, rapid decline beginning at 6 hours of recovery, and final slow decline. Cytoplasmic activity, on the other hand, shows a rapid initial rise followed by a period of slight increase during which time the chromosomes and especially the nucleolus are rapidly losing their RNA label, either through the process of degradation or transfer or both.

From the data on nuclear incorporation of thymidine-H^3 (Fig. 19) it is apparent that DNA synthesis here was a rather slow process taking up about 50 per cent of the interphase for its completion and that the maximum rate of DNA synthesis occurred at approximately mid-interphase. In addition to the principal period of DNA synthesis, it may be seen that there was a secondary synthetic period at the end of interphase. The significance of this diphasic pattern of DNA synthesis, and the extent to which it may be of universal occurrence, is not known.

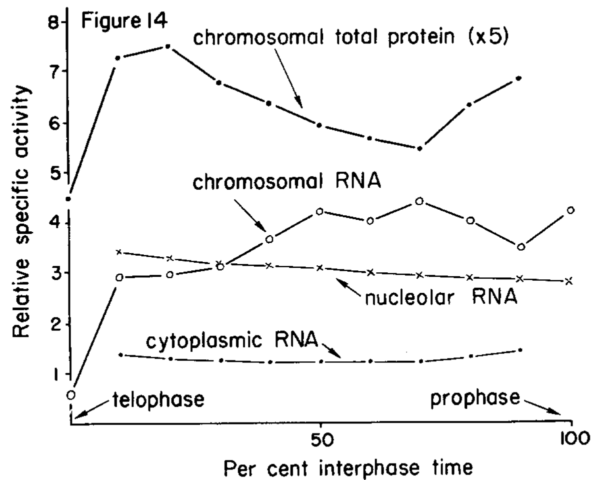
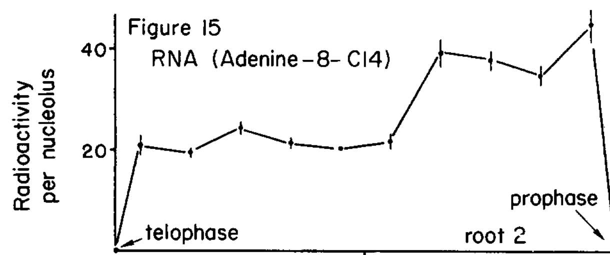
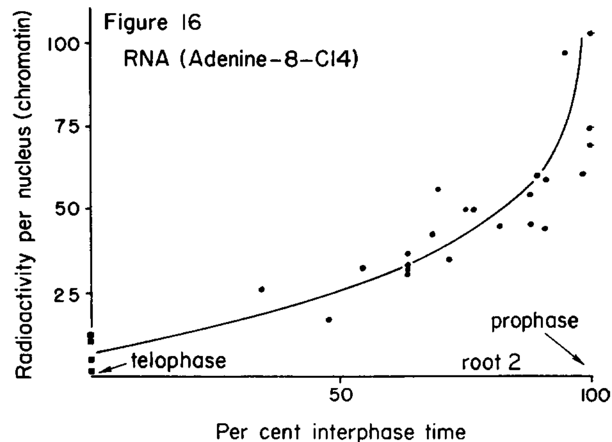


FIGURE 14

Relative specific activities of several fractions expressed as:

$$\frac{\text{Silver grains per square micron}}{\text{Extinction per micron section thickness.}}$$





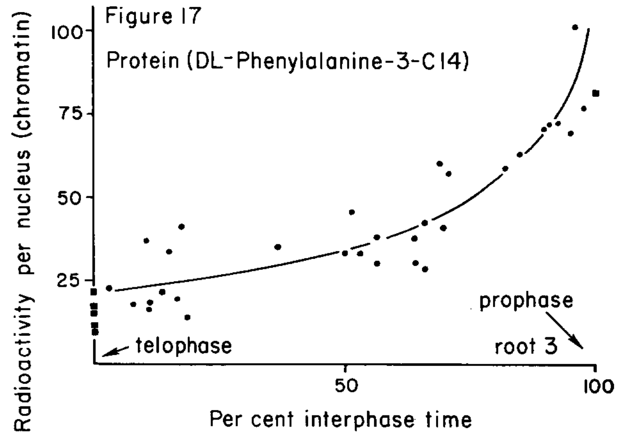
FIGURES 15 AND 16

Radioactivity of chromosomal and nucleolar RNA with standard errors calculated from grain counts over 4 μ sections of a root treated with 5 μc./ml. of adenine-8-C^{14} for 1 hour. Sections were DNased for 1 hour, then placed in 5 per cent TCA at 2–4°C. for removal of low molecular weight nucleotides before covering with stripping film. Exposure time was 30 days and average background count was 0.014 grains/μ^2. Squares represent telophase values.

In other experiments directed toward a study of patterns of DNA synthesis, it was noted that unlabeled mitotic chromosomes occurred among labeled ones in squashed whole Feulgen-stained nuclei that had been treated in thymidine-H^3 for 15 minutes and then placed on cold medium for 11 hours. Since these cells were presented thymidine-H^3 during the latter part of their period of DNA synthesis, the cold chromosomes had presumably synthesized DNA before growth in isotope. It can be concluded then that all chromosomes of the cell do not synthesize DNA at the same time (see also LaCour and Pelc, 1958). Moreover, other preparations which had been presented thymidine-H^3 for 6 hours and then immediately fixed, showed a definite localization of autoradio-

FIGURE 17

Radioactivity per nucleus of chromosomal total protein calculated from grain counts made on 4 μ sections of roots treated in 5 μc./ml. phenylalanine-3-C^{14} for 1 hour, RNased, and placed in 5 per cent TCA at 2–4°C. for 5 minutes. Exposure time was 33 days and the background count was 0.010 grains/μ^2. Squares on the left represent telophase values; squares on the right represent prophase values.



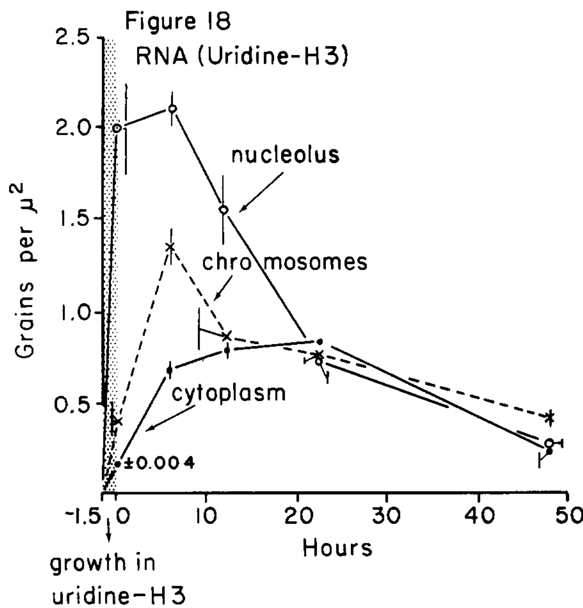


FIGURE 18

Grain counts over chromosomal, cytoplasmic, and nucleolar RNA treated in 5 μc./ml. uridine-H^3 for 1½ hours (stippled area), then transferred to non-radioactive water. Four-micron sections were DNased, then placed in 5 per cent TCA at 2–4°C. for 5 minutes before covering with stripping film. Exposure time was 24 days.

graph. Anaphase chromosomes (Fig. 3) were heavily labeled in the centromere region, while most chromosome arms were relatively free of activity. In addition, daughter nuclei in late interphase were seen to have a localization of autograph in those portions of the nuclei which were mitotic poles and presumably near the sites of the centromeres. Finally, metaphase chromosomes also showed certain heavily labeled bands at various locations, some centromeric, others median, and perhaps one terminal (Fig. 2). Since these chromosomes were treated in thymidine-H^3 during the last part of their period of DNA synthesis, it is concluded that certain segments of DNA are either synthesizing at a greater rate than others during this period, or that these segments are the last parts of the chromosome to synthesize DNA. The heavy localized labeling was simultaneous with the secondary period of DNA synthesis at the end of interphase. Taylor (1960) recently reported asynchronous synthesis of DNA in chromosomes of the chinese hamster. Many of these chromosomes were found to contain segments which, like those of *Vicia*, also duplicate late in interphase.

Microphotometric Data

The microphotometric measurements of nucleoprotein changes during the life history of the cell,

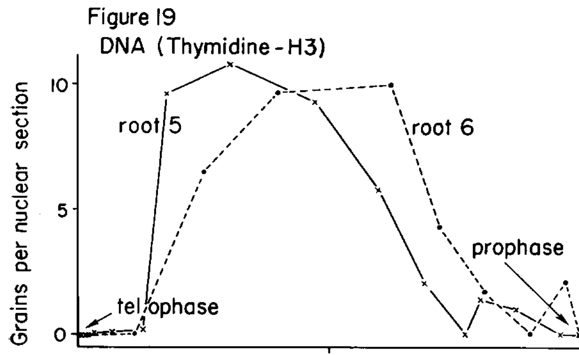


FIGURE 19

Means of silver grain counts over 4 μ sections of nuclei are plotted against percentage interphase time. Roots were grown for 30 minutes in 5 μc./ml. thymidine-H^3 (solid line), and 2 μc./ml. (broken line). Sections were hydrolyzed in 1 N HCl for 10 minutes at 60°C. and stained with the Feulgen reagent before stripping. Slides were exposed for 8 days (solid line) and 15 days (broken line).

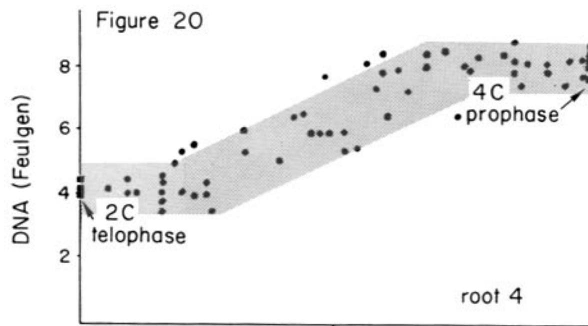


FIGURE 20

DNA amounts measured microphotometrically at 6100 A and plotted against percentage interphase time. Squares on the left represent telophase values and squares on the right represent prophase values.

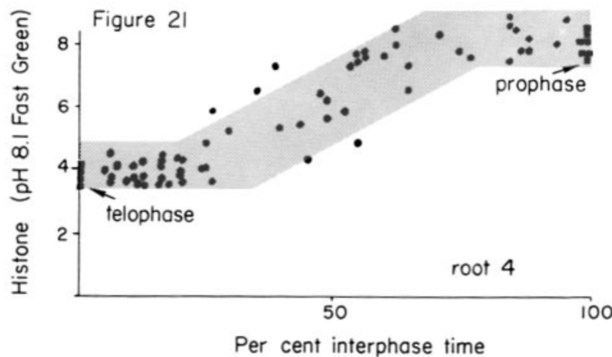


FIGURE 21

Amounts of nuclear histone measured at 5800 A with the microphotometer and plotted against percentage interphase time. Squares on the left represent telophase values; squares on the right represent prophase values.

presented in Figs. 24 and 26, indicate that both RNA and total protein of the chromosomes show an approximately parallel interphase history in that amounts of both substances had maximal increases at the end of interphase. This high rate of accumulation may resume in early interphase as suggested by Fig. 26. In general, the microphotometric data on interphase changes of chromosomal total protein and RNA showed a good correspondence to autoradiographic data insofar as both suggest maximal increases in late interphase.

In reference to nucleolar changes, it is well known that in most cells nucleoli disappear rapidly in prophase and reappear rapidly in telophase. There was consequently a sharp increase in nucleolar RNA during the telophase-post telpohase period and a sudden drop during prophase (Fig. 23). It may be seen from microphotometric measurements presented in Fig. 25 that changes in nucleolar protein levels closely paralleled changes in nucleolar RNA levels throughout interphase.

Measurements of cytoplasmic RNA of whole single cells of the root meristem are graphed in Fig. 22. Once again the curve shows an expo-

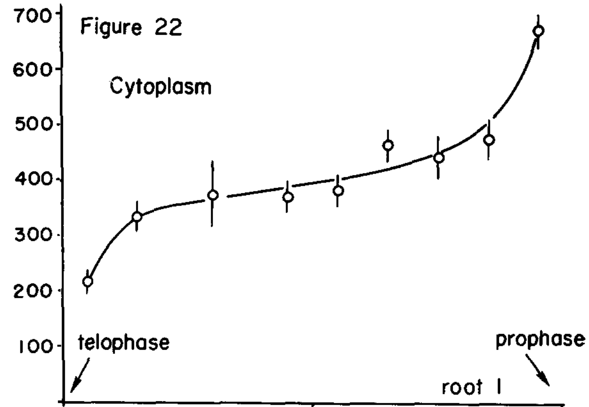


FIGURE 22

Means of cytoplasmic RNA amounts with standard errors of cells of the root macerate measured by the two wavelength method ($\lambda 1$ = 4650 A, $\lambda 2$ = 5000 A) and plotted against percentage interphase time. Two wavelength values were adjusted to a wavelength of 5900 A, using the table of correction factors given by Swift and Rasch (1956).

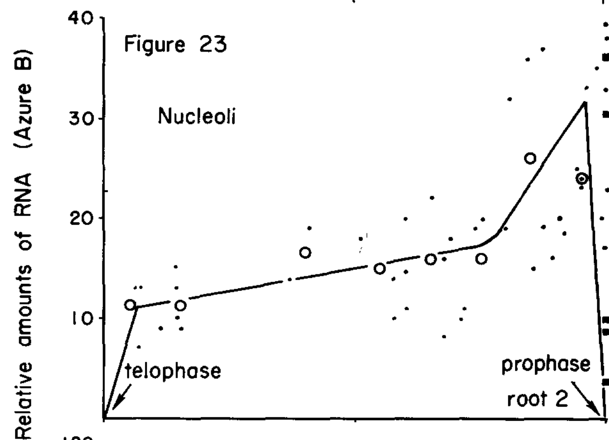


FIGURE 23

Nucleolar RNA amounts during interphase measured microphotometrically at 4900 A and adjusted to a wavelength of 5900 A by multiplying by the factor $\frac{\text{E at 5900 A}}{\text{E at 4900 A}}$ taken from the azure B absorption curve of a nucleolus. Open circles represent mean values; squares represent prophases.

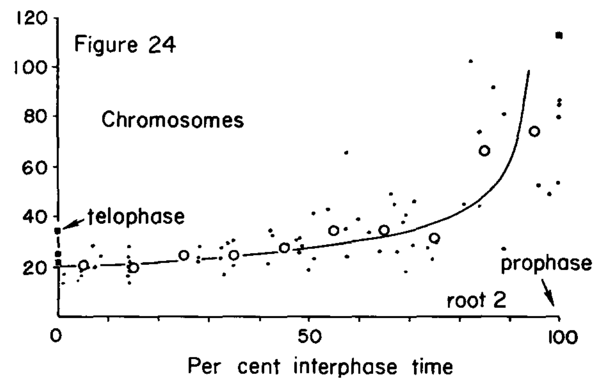
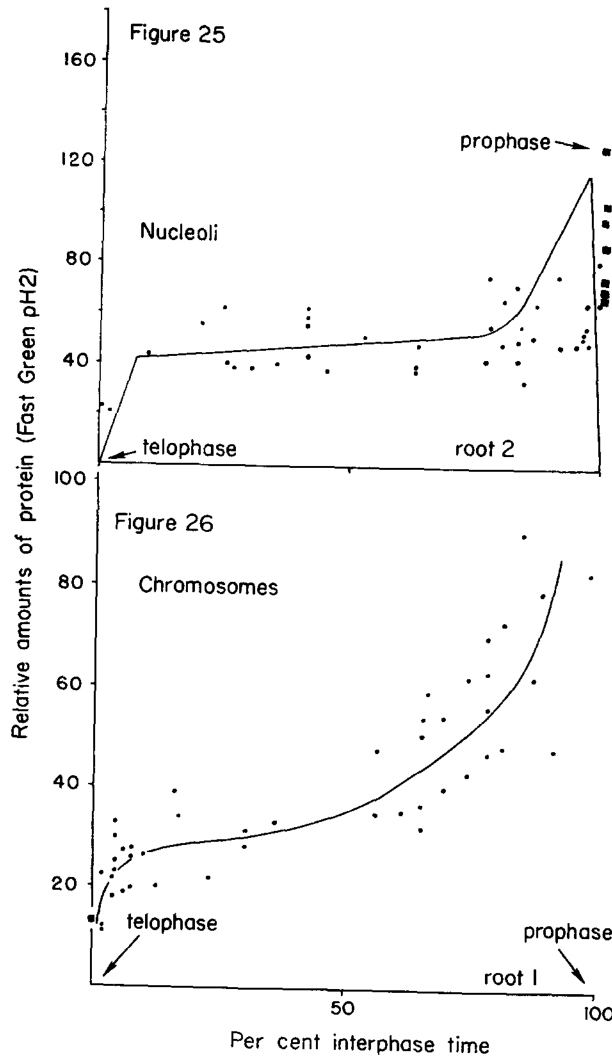


FIGURE 24

Microphotometric measurements of chromosomal RNA taken at a wavelength of 5900 A. Open circles represent mean values; squares to the left represent telophase values, squares to the right represent prophase values.

nential rise of RNA levels in early interphase, a decreased rate of accretion during mid-interphase, and an abrupt rise in late interphase. It is significant to note that the ratio of cytoplasmic RNA amounts per cell at telophase and at prophase was of the order 1:3. Generally similar ratios were found also for chromosomal RNA (1:3) and for chromosomal total protein (1:5).

Measurements of individual Feulgen-stained nuclei are plotted in Fig. 20. In agreement with data on thymidine-H^3 incorporation (Fig. 19), the time-wise spread of intermediate Feulgen values indicated a relatively slow synthesis of DNA throughout 50 per cent of the interphase. The curve for histone synthesis (Fig. 21) followed exactly that for DNA and indicates simultaneous reduplication of these two nuclear components, as previously described by Alfert (1955) and Bloch and Godman (1955).



FIGURES 25 AND 26

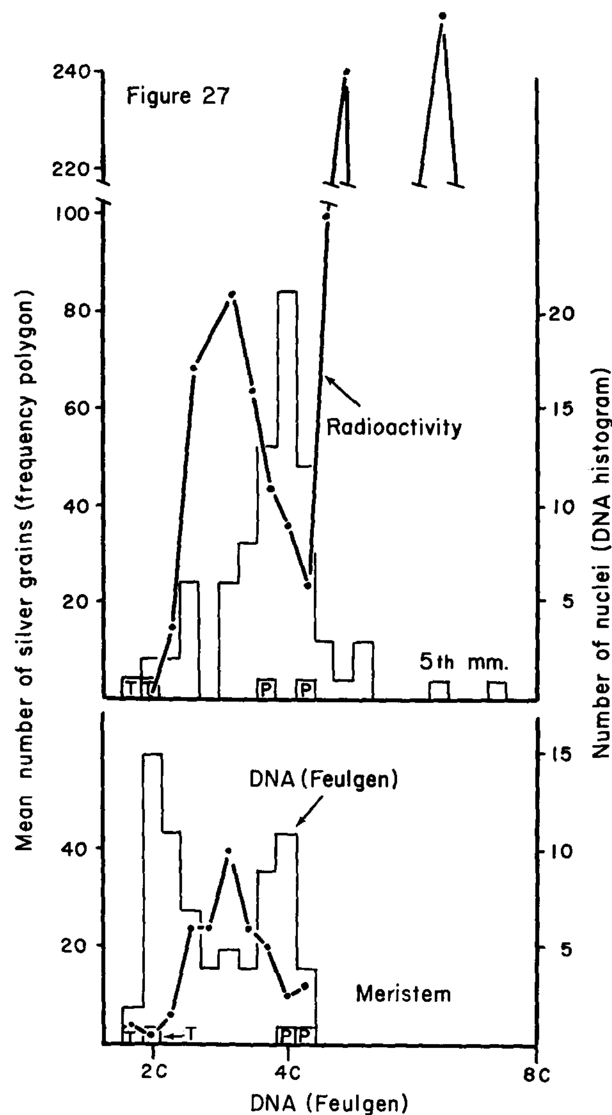
Nucleolar and chromosomal total protein measured microphotometrically at wavelengths of 5800 A and 6100 A respectively, and plotted against percentage interphase time. Chromosomal values were adjusted to a wavelength of 6100 A. Squares to the left represent telophases; squares to the right represent prophases.

DISCUSSION

In a recent study comparing grain densities over nucleoli and cytoplasm in *V. faba* root meristems which had been either treated continuously in cytidine-H^3 or briefly in cytidine-H^3 and then allowed to grow in non-radioactive medium, Woods and Taylor (1959) have concluded that cytoplasmic RNA is nuclear in origin. Their conclusion is based on three major points: 1. Since the cytoplasm shows no label during the 1st hour of continous treatment, there can be only trace amounts, if any, of cytoplasmic RNA synthesis. 2. Incorporation of nucleolar RNA reaches a plateau during continuous treatment and is ostensibly losing labeled material at a time when the radioactivity of the cytoplasm is still increasing. 3. Cytoplasmic RNA labels during recovery following treatment despite dilution of the radioactive precursor pool; the label must, therefore, be coming from outside the cytoplasm. They conclude that the label comes from the nucleolus since during recovery the radioactivity of the nucleolus is decreasing. Our data on *V. faba*, using uridine-H^3 as an RNA precursor, are in essential agreement (for example, see Fig. 18), although we cannot agree with this somewhat narrow interpretation, for actually their observations also fit equally well two other current hypotheses of nuclear-cytoplasmic interaction: (1) that cytoplasmic synthesis is independent of the nucleolus and that the incorpo-

Figure 27

Silver grain counts and microphotometric DNA measurements of the same nuclei in the meristem and elongation zone (5th mm.) of *V. faba* roots grown in thymidine-H^3 (5 μc./ml.) for 1 hour. For histograms of relative amounts of DNA (Feulgen) estimated by the 2 wavelength method, use right hand scale. Points on the frequency polygons are means of silver grain counts over nuclei making up the bars of the DNA histogram (left hand scale). Squares containing *T* represent telophases; squares containing *P* represent prophases.



ration behavior of these organelles can be interpreted as an expression of differential rates of synthesis and degradation of RNA, high in the nucleus, low in the cytoplasm, or (2) that the cytoplasm contains RNA of both cytoplasmic and nuclear origin in an unknown ratio. In the latter case, the observations of Woods and Taylor would fit a system in which there was a movement of RNA from the nucleolus to cytoplasm concurrent with a differential rate of synthesis and breakdown of RNA in nucleolus and cytoplasm.

The origin of cytoplasmic RNA in *V. faba* is as yet unsolved and it is unlikely that studies of differences in incorporation rates of nucleolus, chromosomes, and cytoplasm will solve it unless the availability of the labeled precursor pool can be curtailed or, preferably, eliminated during recovery on non-radioactive medium. In the absence of such a pool, the loss of nucleolar label coincident with a gain in cytoplasmic labeling during recovery on non-radioactive medium would be unequivocal proof of a transfer of nucleolar RNA, or its breakdown products, to the cytoplasm. However, in the presence of a radioactive precursor pool, it is impossible to determine whether cytoplasmic RNA is nucleolar in origin or whether it is synthesized

in situ from precursors of the labeled pool. Unfortunately for such experiments, *V. faba* accumulates an enormous pool during a short period. Our data show that when *V. faba* roots were treated for 1½ hours in 5 μc./ml. uridine-H^3 and then grown on unlabeled glass-distilled water for 22½ hours, there was a 5½-fold increase in the activity of the cytoplasmic RNA despite the fact that the radioactivity of all or most of the cells had been 50 per cent diluted by cell division (Fig. 18).

Turning now to our analysis of nucleoprotein changes during the life history of the cell, the mitotic cycle may be shown to consist of several phases, each characterized not only by its relationship to mitotic time, but by its distinctive metabolic behavior. A discussion of these phases, three anabolic, and one catabolic, will be presented below.

According to both methods of analysis used here, the microphotometric (amount of substance present) and autoradiographic (rate of incorporation per hour per organelle), total protein and RNA at all sites studied showed a common trend during interphase—all demonstrated a greatly increased rate of incorporation per organelle during the latter stages of interphase. In addition, cytoplasmic RNA, chromosomal total protein, and nucleolar total protein and RNA also showed sharp increases during very early interphase. It is possible that this early interphase increase occurred in the RNA and protein of all organelles but that because of the short duration of the rise, the method of seriation used here was not sufficiently sensitive to detect it. During most of the interphase there is a slow, almost linear increase of RNA and total protein in the organelles studied. It remains to be seen, however, whether the late interphase-preprophase increase of ribonucleoproteins observed in *V. faba* is a general phenomenon associated with mitosis. That this indeed may be the case is suggested by data from other biosynthetic systems. For instance, an exponential rise in levels of RNA and total protein of the cytoplasm and total protein of the macronucleus was found at late interphase in synchronous cultures of *Paramecium aurelia* (Woodard *et al.*, in press). In addition, a similar preprophase accumulation of total RNA has been found in *Amoeba proteus* by Mazia (1954) and Woodard (unpublished). Moreover, nuclear volume changes are similar when plotted against time in root meristems of *V. faba* (Fig. 1) and *Tradescantia* (Rasch and Woodard, 1959), in *Amoeba proteus* (Prescott, 1955), *Acanthamoeba* sp., *Paramecium aurelia* (Woodard, unpublished), crown gall tumors of *V. faba* stems, and root meristems of corn and onion (Rasch, unpublished). If nuclear volume changes are proportional to total protein changes, as reported by Schrader and Leuchtenberger (1950), and Alfert (1955), then there is indeed a marked similarity in the pattern of nuclear protein synthesis in a variety of organisms.

Another point demonstrated in the present study was that, in addition to the rapid RNA and total protein loss from the nucleolus during prophase, there was also a reduction in amounts of RNA and total protein of the chromosomes and RNA of the cytoplasm during mitosis. For instance, although the metaphase-anaphase amount of cytoplasmic RNA was 697 ± 39, the telophase value, rather than being ½ this amount, was 205 ± 28 or 29 per cent of the parent cell value. Other workers have also reported such mitotic losses. A decrease in cytoplasmic RNA during late prophase and metaphase was noted by Brachet (1942) and Montalenti *et al.* (1950), while Pollister (1952) has reported a decrease in nuclear protein (Millon) during mitosis. The instability of RNA and total protein demonstrated here emphasizes the relative stability of DNA and histone, for the telophase amount of both the latter substances was ½ the parent cell amount. The over-all loss of RNA throughout the cell during mitosis might suggest that much, perhaps all, of the loss may be attributed to a continued breakdown of these metabolites in the absence of synthesis.

Since the presence of chromosomal RNA in *V. faba* root meristems has been questioned by Woods and Taylor (1959)[2], who reported that "little radioactive material that could be attributed to RNA appeared in the chromatin portion," it is pertinent to cite our evidence for the existence of such a fraction. In the first place, the azure B basophilia of chromatin following DNase treatment, was demonstrated by microphotometric measurements which indicated that the mean extinction of 4-micron sections was 0.245 at a measuring wavelength of 5900 A. Furthermore, grain counts over nuclei (chromatin) showed that there was significant incorporation of isotope (0.19 grains/micron^2) into a chromosomal RNA fraction after 1 hour of growth in adenine-8-C^{14} (Fig. 5). Finally auto-

[2] Woods (1959) has recently reversed this position and reported a chromosomal RNA fraction in *V. faba* roots.

radiographs over the chromatin of cells treated 3 hours in uridine-H^3 had a grain density of 0.48 grains/micron^2 after DNase digestion (Fig. 4). It has, therefore, been concluded that there exists a sizable chromosomal RNA fraction in *V. faba* root meristems.

Another period of metabolic significance during the life cycle of the cell is the time of DNA and histone synthesis, events which take up a large part of the interphase. Simultaneous reduplication of these components took place at a time when RNA and total protein synthesis at all sites was minimal. However, in this connection, Siskin (1959) has reported that DNA and nuclear (nucleolar and chromosomal) RNA are not synthesized at the same time in root tip meristems of *Tradescantia paludosa*. Siskin further observed that "all nuclei which have incorporated thymidine in an individual experiment belong to a single size class" in *T. paludosa* root tips, although, oddly, his graphs actually show incorporation over a rather large range of nuclear volumes. In order to determine accurately the volume class during which DNA synthesis occurs, presentation of labeled precursor should be of short duration, preferably 1 hour or less. Perhaps the long treatment in isotope (8 and 10 hours) contributed to the wide range of volumes showing a DNA autoradiograph in Siskin's experiments. However, our data for *V. faba* root meristems show DNA synthesis to be a slow, diphasic process with a maximal rate at about mid-interphase. It could be argued that the incorporation data of Fig. 19 might also be explained by asynchrony in the onset and duration of synthesis from cell to cell. The microphotometric data, however (Fig. 20), indicated that such asynchrony was insufficient to account for the autoradiographic data. This behavior is not restricted to *V. faba* meristems, for autoradiographic analysis of both corn and onion root tips showed that DNA synthesis (thymidine-H^3 incorporation) was a long interphase process. Similarly, it has been found that adenine-8-C^{14}, uridine-H^3, and P^{32} are also incorporated into DNA over nearly the entire interphase period. A more extensive treatment of rate and duration of DNA synthesis will be presented elsewhere (Rasch, Rasch, Woodard, and Swift, in preparation).

CONCLUSIONS

With respect to nucleic acids and proteins in the nucleolus, the chromosomes, and the cytoplasm of the root meristem cell of *V. faba*, autoradiographic data (representing rate of synthesis per organelle) and microphotometric data (representing changes in amount) indicate that the mitotic cycle may be divided into several metabolic phases, 3 predominantly anabolic (net increase), and a fourth phase predominantly catabolic (net decrease). The anabolic periods are:

1. Telophase to post-telophase, during which there are very high rates of accumulation of chromosomal and nucleolar total protein and cytoplasmic and nucleolar RNA.
2. Post-telophase to preprophase, during which there is a doubling of DNA and histone amounts accompanied by minimal increases of cytoplasmic RNA and nucleolar and chromosomal total protein and RNA. DNA synthesis is diphasic with the peak of synthesis at mid-interphase and a minor peak just preceding prophase. This minor peak occurs at a time when DNA synthesis is largely confined to certain segments of the chromosome.
3. Preprophase to prophase, throughout which there are once more markedly high rates of accumulation of cytoplasmic RNA, chromosomal and nucleolar total protein, and RNA.

The catabolic period is:

4. The mitotic division, during the course of which there are significant losses of cytoplasmic RNA, and chromosomal and nucleolar total protein and RNA.

NOTE ADDED TO PROOF:

It was noted that the elongation zone of *V. faba* roots grown in thymidine-H^3 for 1 hour showed far heavier labeling of nuclei than the meristem. Pelc and Lacour (1959) attributed this heavy labeling to an exchange of cold thymidine for thymidine-H^3 in 4C nuclei and claimed further that no DNA synthesis occurred in this region. We felt, on the other hand, that the simplest, most likely explanation for a nuclear incorporation of thymidine-H^3 is that it is being used in the synthesis of new DNA. One method of testing this notion is to learn whether radioactive nuclei are undergoing DNA replication; *i.e.*, show interclass values. This can be determined by measuring the radioactivity and the amount of DNA in the same nuclei. Accordingly, after photographing fields selected for analysis, grain counts were made over squashed nuclei of both the meristem (1st mm.) and a segment of the elongation zone (5th mm.) Autoradiographs were then removed with Farmer's reducer, and the same nucleus relocated and measured microphotometrically, using the 2 wavelength method. It is evident from data presented in Fig. 27

that nuclei of the 5th mm. show about twice as much radioactivity as meristematic nuclei containing the same amount of DNA. Furthermore, the 5th mm. contains nuclei with DNA amounts intermediate between the 2C and 4C values, and these nuclei are incorporating thymidine-H^3 as are their counterparts in the meristem. Consequently, we have concluded that the radioactivity of nuclei of the elongation zone in *V. faba* results from the incorporation of thymidine-H^3 during replication of new DNA. No evidence for a "metabolic" thymidine fraction was found. The heavier labeling of nuclei of the elongation zone cannot be accounted for at present. In tnis connection it is interesting to note that nuclei in the elongation zone of roots treated in uridine-H^3 or cytidine-H^3 also show the relatively intensive DNA labeling. It is possible that these nuclei are synthesizing twice as fast as meristematic nuclei so that during short term presentation of labeled precursor they incorporate twice as much label; or perhaps the specific activity of the radioactive precursor pool of the elongation zone is larger than that of the meristem. Thymidine-H^3 autoradiographs of longitudinal sections of unfixed, frozen roots have, indeed, indicated a larger radioactive pool in the 5th mm. than in the meristem.

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