AN ABSTRACT OF THE THESIS OF

John Morris Johnson (Name) for the Ph. D. (Degree) in Botany (Major)

Date thesis is presented February 28, 1964

Title CONTRACTING NUCLEOLAR VACUOLES IN SOMATIC TOBACCO CELLS GROWING IN MICROCULTURES

Abstract approved [redacted]
(Major professor)

Contracting nucleolar vacuoles are a general and consistent feature of somatic tobacco cells growing in microcultures. Nucleolar vacuoles were studied in cells from callus tissues of two single-cell clones, H-196 and H-239, of hybrid tobacco (Nicotiana tabacum X N. glutinosa). Vacuoles periodically contracted either completely or incompletely, apparently releasing a fluid material into the nucleus, with a corresponding decrease in diameter of the nucleolus. The lack of a stainable material within the nucleolar vacuoles after recommended killing and fixing procedures, collapsed vacuoles in killed and fixed cells, and the fact that no solid material could be seen to move from the vacuole into the nucleus indicated the soluble, fluid nature of the vacuolar contents. In actively growing cells, nucleolar vacuoles were observed to occur in about 70 percent of the cells examined, whereas nucleolar vacuoles were present in only about 33 percent of the senescent or weak cells indicating a relationship between status of the cell and occurrence of nucleolar vacuoles.

Contracting nucleolar vacuoles were also observed in callus cells of hollyhock (Althaea rosea), tomato (Lycopersicon esculentum), horse bean (Vicia faba), and in single-cell clone G-252 of Nicotiana glutinosa. Nucleolar vacuoles were also observed within living cells of incense cedar (Libocedrus decurrens), carrot (Daucus carota var. sativa), potato (Solanum tuberosum), wheat (Triticum aestivum), annual bristleweed (Haplopappus gracilis), corn (Zea mays), lettuce (Lactuca sativa), and Douglas fir (Pseudotsuga menziesii). Many reports of the occurrence of nucleolar vacuoles within nucleoli of other plants and animals are reported in the literature.

Several other points were established in this study concerning the nucleoli of living tobacco cells. In many instances, there was a decrease in nucleolar volume as the tobacco cells aged, became senescent and approached death. Occasionally protuberances of solid nucleolar material formed at the periphery of the nucleolus which were released into the nucleus. The number of nucleoli within the nuclei of the living tobacco cells studied varied from 1 to 30, but usually there were from 1 to 6. In recently divided tobacco cells, nucleoli were seen to fuse following telophase to form either one large nucleolus or a few smaller nucleoli. In some instances, the number of nucleoli increased as the cells became senescent. Apparent fragmentation of nucleoli prior to death was observed.

Evidence from the literature points to the fact that the nucleolus is particularly active in RNA (ribonucleic acid) metabolism and specifically in the metabolism of soluble, transfer RNA. It is

therefore suggested that the contracting nucleolar vacuoles within living tobacco cells may be a mechanism for the controlled release of a soluble metabolic intermediate, perhaps transfer RNA.

CONTRACTING NUCLEOLAR VACUOLES IN SOMATIC TOBACCO CELLS GROWING IN MICROCULTURES

by

JOHN MORRIS JOHNSON

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1964

APPROVED:

[redacted]

Associate Professor of Botany

In Charge of Major

[redacted]

Chairman of Department of Botany

[redacted]

Dean of Graduate School

Date thesis is presented Feb. 28, 1964

Typed by Margaret May Johnson

ACKNOWLEDGMENTS

The writer wishes to express his appreciation to the following persons for their help in making the completion of this thesis possible:

The members of his committee, Dr. Henry P. Hansen, Dr. Roy A. Young, Dr. Frank H. Smith, and Dr. Robert M. Storm,

Dr. Leo E. Jones, major professor under whose direction this thesis was prepared, for his generous assistance and many valuable suggestions,

Dr. Harold Evans for use of his spectophotometer,

Mrs. Kathryn Young for lettering the graphs,

Mrs. Penny Self for typing the tables,

Miss La Rea J. Dennis for help in proof reading, assistance in drawing the graphs, encouragement, and many helpful suggestions,

Mr. and Mrs. Carl T. Johnson, his parents, for their encouragement,

Mrs. Margaret M. Johnson, his wife, for her assistance, understanding, and encouragement, and for typing the final manuscript.

The writer also wishes to acknowledge the National Science Foundation who provided the funds, under N. S. F. Grant G-13355, for his research assistantship and for support of the research.

TABLE OF CONTENTS

CONTRACTING NUCLEOLAR VACUOLES IN SOMATIC TOBACCO CELLS GROWING IN MICROCULTURES

INTRODUCTION

A nucleolus is generally described as being a highly refractive, spherical body which occurs within the nucleus of a cell. Since the discovery of the nucleolus by Fontana in 1871 (77, p. 270 and 66, p. 146) it has been an object of study by numerous cytologists. Many extensive reviews and studies have been written concerning the nucleolus; some examples are: Montgomery (56) in 1898, Ludford (49) in 1922, Dermen (23) in 1933, Gates (26) in 1942, Stockinger (72) in 1953, Vincent (77) in 1955, Stich (71) in 1956, Brachet (7) in 1957 Swift (74) in 1959, Sirlin (66 and 67) in 1961 and 1962, and Busch, et al. (11) in 1963. As stated in these reviews, the nucleolus contains much protein as well as ribonucleic acids (RNA), lipids, minerals and some enzymes. Studies of electron micrographs fail to demonstrate a definite membrane between the nucleus and nucleolus. The nucleolus generally disappears and reforms during mitosis. The exact physiological role of the nucleolus, however, is still not completely known.

A search of the literature has revealed no extensive studies concerning the behavior of the nucleoli or nucleolar vacuoles in living somatic cells of seed plants growing under shock-free conditions. The present study was undertaken to gain knowledge of the behavior of the nucleolus and in particular the contracting nucleolar vacuoles in living somatic tobacco cells growing in microcultures.

LITERATURE REVIEW

Nucleolus: Physiological Roles

Many functions have been proposed for the nucleolus. Montgomery in 1898 (77, p. 293 - 294) suggested three hypotheses concerning nucleolar composition and physiology. They were:

(a) The nucleolus represents cytoplasmic material taken into the nucleus to be used in nutrition and growth of the nucleus. (No mention was made as to what type of cytoplasmic material is taken into the nucleus.)

(b) The amount of nucleolar substance is proportional to the functional exchanges which take place between the cytoplasm and the nucleus. In other words, the more exchange that takes place between the cytoplasm and nucleus, the larger or more prominent will be the nucleolus.

(c) The nucleolus represents reserve supplies of nutritive substances retained in the nucleus. (No explanation of what the reserve nutritive substances might be was given.)

Dermen (23, p. 311), in 1933, proposed that the nucleolus is active in the general metabolic processes of the organism rather than being a reserve food.

In 1939 Mensinkai (55, p. 770) summarized functions that had been proposed for the nucleolus. These were: the nucleolus is an

accumulation of waste products of the nucleus, which periodically is fragmented and passed into the cytoplasm; the nucleolus carries the excretory and secretory products out from the nucleus to the cytoplasm; the nucleolus is a storehouse of chromatin within the resting nucleus; the nucleolus is a storehouse of nucleic acid to be drawn upon as chromosomes resume their basophilic character; fragments of the nucleolus pass into the cytoplasm of animal egg and give rise to yolk granules; nucleolar globules are bearers of stimulating or finishing materials of the genes; the nucleolus is a reserve product of metabolic activity and is used in the synthesis of certain parts of the cell; and, in some insects, the nucleolus contributes to the activity of the silk glands. Mensinkai (55, p. 770) suggested hypothetically that the nucleolus acts partly as a fuel substance in the cell economy and partly in reviving chromaticity of the emerging chromosomes by being absorbed and incorporated into the nucleoproteins during mitosis.

In 1939 and 1940 one of the major advances leading to our present understanding of the structure and function of the nucleolus was made. Independently, Brachet (2, p. 167-202) in 1940 and Caspersson and Schultz (12, p. 602-603) in 1939 established that the nucleolus contains RNA (ribonucleic acid), and each began to relate this nucleolar RNA to protein synthesis in the cytoplasm. In 1940 Caspersson and Schultz (13, p. 514) stated that the nucleolus is closely associated with the appearance of cytoplasmic ribonucleic acids, and they hinted that nucleocytoplasmic relationships might provide some insight into the mode of action of the genes.

In 1947 Caspersson (14, p. 136) proposed three fundamental principles in the relationship between nucleic acids of the nucleus and nucleolus and protein synthesis in the cytoplasm. He stated that all protein synthesis in the cytoplasm requires the presence of nucleic acids, that quantitatively the most important nucleic acids in the chromosomes are of the deoxyribose type, and that the nucleus specifically functions as the main center for the formation of proteins.

Lesher (46, p. 587) in 1951, while working with cells from the salivary glands of Drosophilia, suggested a relationship between the nucleolus, RNA, and the secretory function of the gland. He said that increases and decreases of nucleolar volume and changes in cytoplasmic basophilia of the salivary gland cells paralleled the secretory activity of the gland. From this he concluded that there could be a close relationship between the ribonucleic acid system, the nucleolus, and the secretory function of the cell. Vincent (77, p. 295), in his 1955 review of the nucleolus, discussed most of the preceding hypotheses concerning probable functions of the nucleolus. He concluded that most hypotheses explaining the function of the nucleolus indicate an interchange of material between the nucleolus and the cytoplasm. This interchange, Vincent stated, had not been satisfactorily established. In 1957, however, Brachet (7, p. 135) stated that the very active turnover of nucleotides from the nucleus, which are related to RNA metabolism, is a phenomenon directly attributable to the nucleolus within the nucleus.

Further, he stated that the main characteristics of the nucleolus are the presence of a metabolically labile RNA, powerful mechanisms for the incorporation of amino acids into proteins, and the accumulation of enzymes connected with nucleotide metabolism. Moreover, Brachet said that "although the function of this organelle in the cell still remains enigmatic, there is no doubt that it is very important in RNA and protein anabolism; old ideas considering the nucleolus as reserve material or as a byproduct of nuclear metabolism can now confidently be discarded."

Vincent and Baltus (79, p. 18-23) in 1960 presented evidence that, in starfish oocytes, labeled cytidine is added to RNA already present within the nucleolus. The addition of the cytidine, it was suggested, takes place in a manner similar to that known for mammals during formation of soluble RNA that is concerned with activation of amino acids. Vincent and Baltus, in this same paper, also presented evidence indicating the presence of two pools of polynucleotides within the nucleolus.

In another paper in 1960, Vincent and Baltus (78, p. 300) formulated the following hypothesis: "A major function of the nucleolus is the provision of 'soluble' or 'activation' RNA for use in the synthesis of cellular proteins. The 'activation' RNA provided by the nucleolus is bound to a carrier protein, likewise nucleolar in origin; this protein is considered to be essential for the integrity of RNA."

In 1962 Caspersson (16, p. 130) reviewed results from some of his previous experiments. He had shown, in rapidly growing tissues from higher organisms, that there is a correlation between a marked increase in size of the nucleoli and the appearance of large RNA masses in the cytoplasm near the nuclear membrane. The large nucleoli and RNA masses appear concurrently with an increase in cytoplasmic proteins. This phenomenon appears to be universal in cells which are actively synthesizing proteins in the cytoplasm.

Swift (75, p. 118) in 1962 commented that, although they were inconclusive, radioautographic data suggest a rapid RNA metabolism within the nucleolus which is not entirely dependent upon the accumulation of chromosomal RNA. Evidence for this statement was that fragmented nucleoli not in contact with chromosomes, such as those found in the dipterian Sciara, and amphibian oocytes, rapidly take up labeled RNA precursors. Swift suggested either these fragments accumulate a very active RNA fraction from the chromosomes, or they have an active synthetic process of their own. The latter possibility, he felt, was the most likely.

Sirlin (67, p. 39), in his 1962 review article, summarized his discussion of the turnover of RNA in the nucleolus by saying that autoradiographic and chemical studies show that nuclear RNA turnover is mainly due to a continuous turnover of nucleolar RNA. He also stated that it has been shown chemically that part of the nucleolar RNA turnover represents terminal turnover of transfer RNA.

Further, Sirlin said autoradiographic studies show nucleoli have slow and fast turnover components. The fast turnover component has a half life of less than two hours. Also, these two nucleolar RNA's with different turnover rates have been chemically demonstrated. In the same article, Sirlin (67, p. 50) maintained that "The participation of the nucleolus in the RNA metabolism of the cell is apparently the most important nucleolar function, either in the making of RNA itself or of some final protein."

Nucleolar Vacuoles

Vacuoles within nucleoli have been reported in numerous types of organisms. Montgomery (56, p. 272) in 1898 reported that Balbiani first described the movement and discharge of nucleolar vacuoles in the eggs of spiders, as well as in cells from several other invertebrates. In the same article Montgomery (56, p. 508) stated,

> In opposition to Meunier ('86) and in agreement with most investigators, I must conclude the vacuoles are normal structures in nucleoli, since they may be seen after the most diverse methods of fixation, and their size and number are not only to some extent limited for the particular cell, but are also different at different periods of metamorphoses of the nucleus. It is the rule that the youngest nucleoli are homogeneous and that vacuoles first arise when they have increased in size. Their size and number vary at different phases of development of the nucleolus. Very frequently a number of smaller ones appear, and then these subsequently fuse together and produce a larger one.

He also mentioned that nucleoli of egg cells generally have more and larger vacuoles than do somatic cells and that some somatic

cells may not have nucleolar vacuoles.

Jörgensen (41, p. 126) in 1913 noted and described the development of vacuoles within oocyte nucleoli of several invertebrates.

Cleland (19, p. 392), in his 1922 description of the pollen mother cells of Oenothera franciscana, mentioned that hyaline circular areas resembling vacuoles are seen within the nucleolus. Furthermore, he reported that these vacuoles may contain crystal-like bodies.

In 1926 Latter (44, p. 279) stated that, in the pollen mother cells of Lathyrus odoratus, the peripheral region of the nucleolus stains more intensely than the central area. This distribution of stain gives the nucleolus the appearance of having a large, central vacuole. Within this vacuole she found small, crystal-like structures which she thought were protein in nature.

Zirkle (84, p. 93-94) in 1931, while examing living cambial cells of Pinus, found that the nucleoli contain small droplets of less refractive substance and, consequently, appear vacuolate.

Dermen (23, p. 305-311) in 1933 observed vacuolated nucleoli in the living stamen hairs of Callisia. He mentioned that at times he found all the nucleoli vacuolated and at other times some nucleoli were homogeneous. Dermen placed considerable significance upon the presence of vacuoles within the nucleoli and considered them to be normal structures which form and disappear during the normal course of cellular activity.

In 1939 Mensinkai (55, p. 770), after studying several species of Allium, stated that the nucleoli usually are vacuolated. He suggested that the vacuoles may represent droplets of colorless liquid.

Lewis (47, p. 531-536) in 1943 found nucleolar vacuoles in living cultures of normal and malignant fibroblasts from rats and mice. He noted that the vacuoles range in size from 0.5 to 5 microns, that there are some indications that small vacuoles fuse to form larger ones, and that the vacuoles either increase in number, or decrease and disappear in the course of a few days. He found fewer nucleolar vacuoles in cells from "poor" cultures (those that lived only a few days) than in active, growing cultures. He postulated that the nucleolar vacuoles might be caused by the temporary trapping of materials which normally diffuse out of the nucleolus.

Vogt and Vogt (80, p. 66) in 1946 described nucleolar vacuoles in human nerve cells and mentioned that the vacuoles are discharged from the nucleolus.

In 1951 Lesher (45, p. 579) found that the nucleoli of cells from salivary glands of Drosophila larvae characteristically became "vacuolated". He stated that the most striking feature of these nucleoli is the well-defined, lightly-staining areas which, from their general morphology, suggest liquid-filled "vacuoles". He also stated that the "vacuoles" are prevalent in the nucleoli of actively secreting cells in which the cytoplasm is markedly

basophilic. In another 1951 paper, Lesher (46, p. 586-588) showed that the volume of the nucleolus decreases when the gland ceases to function and at this time the so-called "vacuoles" disappear from the nucleoli.

Chayen (17, p. 294) in 1952 reported what he called a nucleolar core in killed and fixed cells from Vicia faba roots. This core did not stain with basic or acidic dyes. He also demonstrated this core in the living stamen hairs of Tradescantia and concluded that the core is not an artifact as it could be found in these living cells.

In 1953 Chayen, et al. (18, p. 190-195), using ultraviolet absorption techniques, reported that the nucleolus consists of an outer peripheral zone and an inner core. These two zones were also demonstrated in fixed root tips of Vicia faba, Allium cepa, and Zea mays, as well as in the living stamen hairs of Tradescantia bracteata. They reported further that the outer peripheral zone of the nucleolus stained densely by the tetrazotized dianisidine coupling reaction of Danielli, but that the inner zone or nucleolar core stained only lightly. These results suggested that both zones contain protein but in varying amounts.

Höpker (30, p. 220-229) in 1953 described nucleolar vacuoles in rat nerve cells. He regarded the vacuoles as physiological products of the nucleolus.

Vincent and Huxley (76, p. 290) in 1954 stated that vacuoles occupy up to 50 percent of the total volume of starfish oocyte

nucleoli, but the vacuoles contain less than five percent of the total dry matter content of the nucleoli.

Vincent (77, p. 272) in 1955 stated that although many persons have noted vacuoles in the nucleolus no basic physiological role could, as yet, be assigned to them.

In 1956 Lowman (48, p. 34), using electron micrographs, showed vacuole-like structures with dark internal bodies in the nucleoli of cells from Drosophila salivary glands.

Mulnard (59, p. 488) in 1956 found, in nucleoli within cells from Drosophila salivary glands, Feulgen-positive granules arranged around large central vacuoles. He reported that the vacuoles within these nucleoli stain negatively for proteins and/or nucleic acids.

Brachet (7, p. 127), in a short review of the nucleolus in 1957, stated that nucleoli are often filled with vacuoles but that the nature and role of vacuoles are not known. He also stated that cytochemical tests for nucleic acids and proteins in the vacuoles are negative, but that it is possible that the vacuoles contain soluble products of metabolic activity.

Sirlin (64, p. 448-450) in 1958 reported sizeable vacuoles in the nucleoli in somites of the frog Xenopus. His photomicrographs reveal neither Methyl Green and Pyronin stain nor L-arginine C^{14} nor adenine 8 C^{14} grains in the nucleolar vacuoles.

In 1958 Lafontaine (42, p. 777-784), in an electromicrographic study of the root tips of Vicia faba and Allium cepa, showed what he terms a central core in the nucleoli of cells from these two

species. Further, he places the nucleoli from these two species into two categories: (a) nucleoli of homogeneous texture (except for small, light spaces of various sizes) and (b) those which have a sharp boundry between a dense outer cortex and a much lighter central core. Moreover, he stated that many nucleoli are intermediate between the two distinct types.

Lafontaine and Chouinard (43, p. 167-174 and 187), in a 1963 paper, presented a correlated light and electron microscope study of the nucleolar material in Vicia faba during mitosis. In this paper they illustrated and discussed nucleolar vacuoles. They stated that in preprophase the dense nucleolar material is formed of two distinct structural components. One of these components is represented by 150 angstrom granules, the other component consists of fibrils 60 to 100 angstroms in diameter. The vacuole-like structures contain loosely and uniformly distributed granules and fibrils similar to those found in the dense nucleolar material. They proposed that the vacuoles probably contain nuclear material in a dispersed form rather than nucleolar sap.

Soudek and Stránská (68, p. 241-243) in 1958 reported that vacuoles are formed in the nucleoli of the fungus Basidiobolus ranarum, and that these vacuoles periodically are emptied into the nucleus. Further, they observed that formation of the vacuoles takes place only in actively growing cells. They stated that in cells that were no longer growing the nucleolus decreases in size and no longer forms vacuoles. They suggested that the vacuoles

are a sign of secretory activity of the nucleolus, which is evidently closely related to growth of the cell and hence to protein synthesis.

In 1960 Soudek (69, p. 447-452) presented quantitative data showing a relationship between the occurrence of nucleolar vacuoles in Basidiobolus ranarum and growth of the fungus. He found that nucleolar vacuoles are more prevalent in actively growing cells than in non-growing cells, and that the ratio between nucleolar volume and vacuolar volume is 10:1 in growing cells and 20:1 in non-growing cells.

Coons (21, p. 55 and 60) suggested and presented some evidence in 1958 to indicate that the nucleolus and nucleolar vacuoles of lymph cells may be associated with antibody production.

Raven (62, p. 7-8), in a discussion of molluscan oogenesis in 1958, stated that "the metabolic activity of the nucleolus leads to the formation of nucleolar products, which takes place in two ways: (1) by the intranucleolar formation of vacuoles in the inner part of the nucleolus, which break through at its surface, and (2) by the appearance of epinucleolar buds in the cortical part of the nucleolus, which are then constricted off." He also stated that the above nucleolar products first pass into the nucleus and then may be transfered to the cytoplasm through the nuclear membrane.

Dalcq (22, p. 892-894) in 1959 reported the cytochemical localization of adenosinetriphosphatase in the nucleolar vacuoles of rat and mouse eggs.

In 1959 González-Ramírez, et al. (27, p. 52-56) showed, in time lapse motion pictures of living HeLa cells, the passage of nucleolar vacuoles into the nucleus from the nucleolus and also a passage of solid material from the nucleolus into the cytoplasm. They concluded that the vacuoles might represent proteins which are formed by the nucleoli and later released into the nuclear sap.

González-Ramírez, et al. (28, p. 153-160) in 1960 showed vacuolar areas in electron micrographs of the nucleolus of HeLa cells. These appear similar to the vacuolar areas seen in living HeLa cells with phase-contrast optics.

Swift (74, p. 270) in 1959 reported that the nucleoli of killed and fixed interphase onion nuclei contain small vacuoles of non-staining materials. These vacuoles, he claimed, were occasionally connected with the nucleoplasm through a small opening.

Sirlin (66, p. 147), in a 1961 review of the nucleolus, stated that the formation of large nucleolar vacuoles is characteristic of cells from actively secreting tissues and, sometimes, of cells from degenerating tissues.

Dutta, et al. (25, p. 542-550) in 1963 described an intranucleolar body or "nucleololus" in nerve cells of monkey and in Euglena gracilis. They considered the "nucleololus" to be the same as the vacuole or opaque body seen with the light microscope and the same as the light central core described from the electron micrographs of plant nucleoli taken by Lafontaine (42). Dutta et al.'s

electron micrographs were interpreted by them as showing that the "nucleololus" contains a delicate meshwork of fine filaments and granules and has no limiting membrane. Their cytochemical tests showed that the "nucleololus" contains neither DNA (deoxyribonucleic acid) nor RNA (ribonucleic acid), but that it stains readily for proteins with Millon's Reagent or by the Danielli Process, and is positive for bound lipids with Berenbaums's Acetone-Sudan Black B Technique. Dutta et al. suggested that the "nucleololus" or intranucleolar body may be concerned with the synthesis of nucleolar proteins.

Using cells from the liver of guinea pig, Maggio, et al. (50, p. 295) in 1963 showed electron micrographs of the nucleoli which appear to be full of vacuoles or lacunae as the authors termed them. The authors presumed that the vacuolar contents were extracted during preparation.

METHODS AND MATERIALS

The primary experimental materials were stem callus from a hybrid tobacco (Nicotiana tabacum X N. glutinosa), established as single-cell clones, H-196 and H-239, by Muir, et al. (57, p. 877-878 and 58, p. 589-597). The tobacco callus for the microculture preparations was grown in liquid cultures. Detailed observations and photographic studies were made in small filaments of tobacco callus cells growing in microculture chambers similar to those described by Jones, et al. (37, p. 468-469).

All observations and photographs were made using a Leitz Ortholux microscope stand fitted with Zeiss dark phase-contrast optics. 35 mm photomicrographs were taken on Kodak High Contrast Copy film (A. S. A. 64) using a Leitz MIKAS (Micro-Ibso) attachment and a Leica 35 mm camera body. Cinemicrographs were taken on Kodak Tri-X reversal 16 mm movie film using a Bolex, H-16 Rex, single lens, reflex movie camera without lens. The movie camera was held by a drill press stand and pivoted into position over the phase-contrast microscope.

All volumes of nucleoli and nucleolar vacuoles were computed by using this basic formula for the volume of a sphere:

$$\text{volume} = 4/3\,\pi R^3 = 1/6\,\pi D^3.$$

The data presented in Table I are concerned with the decrease in diameter of the nucleolus following contraction of a nucleolar vacuole. These data were obtained by photographing nucleoli and nucleolar vacuoles, before and after the contraction of a vacuole. The photographic negatives were enlarged, and the images of the nucleoli and vacuoles were then traced. [The final magnification for all traced photographs taken with the 63x Neofluar objective (N. A. 0.90) was 3000x, and those taken with the 40x Neofluar objective (N.A. 0.75) was 1950x.] These traced images were measured

in millimeters to determine the diameters of the nucleoli and vacuoles. In some instances the nucleoli and vacuoles were oval in outline; in such cases several measurements along different diameters were taken of both the nucleoli and vacuoles, and an average of the measurements was used as the diameter to calculate the volumes. It is realized a slight error could result from using these combined measurements for calculating volume. Finally, the measurements in millimeters were converted to the actual sizes of the nucleoli and vacuoles in microns. This conversion was made by using the appropriate conversion factor obtained from photographs of a calibrated stage micrometer projected at the same magnification as the measured nucleoli and nucleolar vacuoles.

The calculated diameters for the nucleoli following a contraction, as shown in Table I, were derived from the formula,

$$1/6\pi D_1^3 - 1/6\pi D_2^3 = 1/6\pi D_3^3.$$

Since $1/6\pi$ is a constant value on both sides of the equation, the formula can read $D_1^3 - D_2^3 = D_3^3$. D_1 is the diameter in microns of the nucleolus before the contraction of a nucleolar vacuole; D_2 is the diameter of the nucleolar vacuole before contraction; D_3 is the calculated diameter of the nucleolus following a complete contraction of the nucleolar vacuole. D_3 then equals the cube root of the value obtained by subtracting D_2^3 from D_1^3. In instances where an incomplete contraction of a nucleolar vacuole occurred, the calculated diameter of the nucleolus was obtained by using the following formulas:

$1/6\pi D_1^3$ = Volume A (volume of nucleolus before contraction of nucleolar vacuole)

$1/6\pi D_2^3$ = Volume B (volume of nucleolar vacuole before contraction)

$1/6\pi D_3^3$ = Volume C (volume of nucleolar vacuole after incomplete contraction)

Volume B - Volume C = Volume D (decrease in volume due to incomplete contraction of nucleolar vacuole)

Volume A - Volume D = Volume E (calculated volume of nucleolus after incomplete contraction of nucleolar vacuole)

$$\text{Volume E} = 1/6\pi D_4^3$$

$$D_4^3 = \frac{\text{Volume E}}{1/6\ \pi}$$

$$D4 = \sqrt[3]{\frac{\text{Volume E}}{1/6\ \pi}}$$

D_4 equals the calculated diameter of the nucleolus after a partial contraction of a nucleolar vacuole. The same series of formulas were used to calculate data for Table II in which diameters were measured using a calibrated ocular micrometer. These measurements were also converted into microns by using an appropriate conversion factor for the ocular micrometer.

Two procedures were used to stain for nucleic acids. These were the Methyl Green and Pyronin method as outlined by Jensen (32, p. 251) and Conn, et al. (20, p. 166-167), and the Azure-B method as outlined by Jensen (32, p. 251-252) and Conn, et al. (20, p. 220).

RNA (ribonucleic acid) digestion, as a criterion for the specificity of Pyronin and Azure-B stains for RNA, was carried out as outlined by Brachet (3, p. 6). Possible proteolytic activity of the RNase (ribonuclease) enzyme was destroyed by heating the RNase in a boiling water bath for five minutes in a 0.2 saturated ammonium sulfate solution as suggested by Swift (73, p. 80). The RNase used in all digestions and in all experiments was 5X crystalline RNase obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Proteins were stained using the Mercuric Bromphenol Blue method outlined by Mazia, et al. (51, p. 57-67). Lipids were determined by using Sudan IV, as described by Johansen (33, p. 191).

The RNase solutions used in experiments concerned with the effect of RNase on living tobacco cells were prepared by the addition of measured amounts of RNase to the standard liquid medium. This medium was liquid D medium described by Hildebrandt, et al. (29, p. 591-597), and the mixture of RNase and liquid D medium was designated RNase-D. The RNase-D medium was sterilized by using Millipore filters. The pH of the RNase-D medium was 5.5 to 5.8, similar to that of the standard liquid D medium. RNase activity of the RNase-D medium was measured using the absorption of ultraviolet light at 260 mμ as described by McDonald (52, p. 228).

Some special procedures and methods used in certain experiments are presented with the discussion of these specific experiments.

GENERAL OBSERVATIONS CONCERNING THE NUCLEOLUS

When examined with dark phase-contrast optics, nucleoli within the nuclei of living somatic tobacco cells had three general appearances: (a) they appeared as dark gray spheres containing one to three vacuolar areas which were one-third to nine-tenths the diameter of the enclosing nucleolus (Figures 1 and 4); (b) they appeared as homogeneous dark gray or black spheres (Figure 2); and (c) they appeared as dark gray spheres containing 1 to 30 small vacuolar areas (Figure 3); these numerous vacuoles were usually spherical and less than one-fifth the diameter of the nucleolus.

During mitosis and cell division, the nucleoli of living tobacco cells were seen to disappear late in prophase and to reappear during early telophase; this is in agreement with the behavior generally described for nucleoli of animal and plant cells (23, p. 255). In one instance the nucleolus, in a prophase nucleus, was observed to separate into two parts, each of which rounded up and then disappeared later during prophase.

The number of nucleoli within a nucleus varied from one tobacco cell to another. The number also varied within the same cell depending upon the time relationship to mitosis and cell division, and upon the morphogenic status of the cell concerned. Generally the number of nucleoli per nucleus varied from one to six; however, up to 30 nucleoli were found within the nuclei of some cells. In several instances a nucleus was observed to contain only one nucleolus prior to mitosis and cell division; however, after such a cell divided the number of nucleoli was from two to eight in the daughter nuclei. Frequently the numerous nucleoli in new daughter cells fused during the following interphase. In one particular instance, a young daughter nucleus contained eight nucleoli at eight a. m. and only four when observed at four-thirty p. m. of

Figure 1

Typical view of a living young, active tobacco cell showing nucleus (A), nucleolus (B), large nucleolar vacuole (C) and cell wall (D). (1000x)

Figure 2

Nucleus of a living tobacco cell containing three homogeneous appearing nucleoli, labeled (B). (1300x)

Figure 3

Large nucleolus of a living tobacco cell showing small nucleolar vacuoles (C). (2000x)

Figure 4

Large nucleolus in a living tobacco cell. Nucleolus contains a very large nucleolar vacuole (C) and shows the beginning of a peripheral budding of solid nucleolar material, note protuberance labeled (M). (1300x)

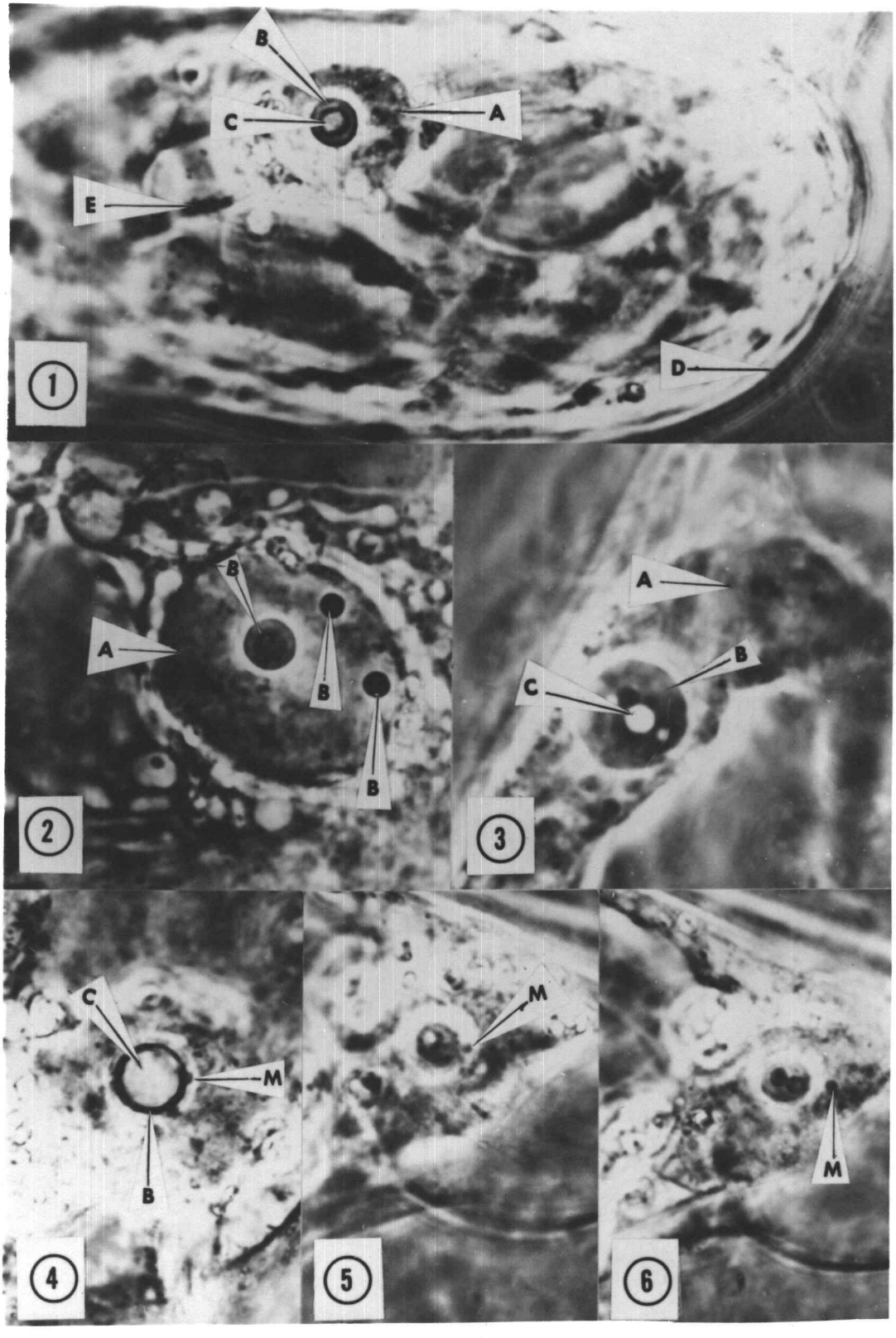
Figure 5

Same nucleolus as shown in Figure 4 but two hours later. Nucleolar vacuole (C) has now nearly disappeared and the protuberance (M) is breaking free from the nucleolus. (1300x)

Figure 6

The same nucleolus as shown in Figures 4 and 5. This photograph taken five minutes later than Figure 5 showing the nucleolar extrusion (M) moving out into the nucleus. (1300x)

A = nucleus
B = nucleolus
C = nucleolar vacuole
D = cell wall
E = cytoplasmic strand


B
C
A
E
D
1
B
A
B
B
2
A
B
C
3
C
M
B
4
M
5
M
6

the same day. Other nuclei in recently divided cells also contained several small nucleoli, and over a period of from one to five days these fused to form either a few larger nucleoli or only one large nucleolus. The observation that nucleoli fuse during interphase is in agreement with the findings reported by Swift (74, p. 270) for nucleoli in the cells of onion root tips. He reported that the nucleolar number is usually higher in onion root tip cells in early interphase than in late interphase, because of the tendency of the nucleoli to fuse as interphase progressed.

Observations of living tobacco cells in microcultures revealed that the number of nucleoli increased in some tobacco cells as they aged and became senescent. One senescent cell, in which the nucleus initially contained 14 nucleoli, had 30 nucleoli after an additional 48 days in microculture. Other increases in the number of nucleoli from one to two, two to four, and from four to six were observed within time periods of from one to four days. In two instances fragmentation of the nucleolus took place one to three days prior to the death of the senescent cells. In the first instance the nucleolus fragmented into three parts, and in the second the nucleolus separated into six parts.

An additional phenomenon, which was noted occasionally in tobacco nucleoli, was the apparent budding of material from the periphery of the nucleolus (Figures 4-6). In nucleoli in which this budding was observed, a small protuberance formed at the periphery, and it appeared to evaginate and then pinch off to float free

within the nucleus. This release of peripheral nucleolar material was observed in only a few nucleoli, and the process was not studied in detail. The release of solid material from the periphery of the nucleolus, however, has been reported by González-Ramírez, et al. (27, p. 51-57) in living HeLa cells. Related literature is summarized in a review by Gates (26, p. 342) concerning the release of nucleolar material and budding from nucleoli.

Studies were also undertaken on the nucleoli of living tobacco cells to determine changes in nucleolar volume with age of the cell. Individual tobacco cells and their nucleoli were observed, and the diameters of nucleoli and vacuoles were measured periodically from the date of initial observations until the death of the cells concerned. The problem of finding the same cell for subsequent observations was easily resolved; living tobacco cells are non-motile, and cell enlargement and elongation caused only minor positional displacement. Each cell was located by recording the coordinates on the mechanical stage of the microscope and by topographical sketches of the cells in a group. Cells were arbitrarily selected for measurements of nucleoli on each of ten microcultures of the tobacco cells from single-cell clone H-239, prepared on 7/15/63 and single-cell clone H-196, made on 7/15/63. Microcultures of H-239 were especially favorable for measurements and observations. Cells were arbitrarily selected in which only one nucleolus was seen within the nucleus; this eliminated the problem of determining which nucleolus within a given nucleus was being measured,

and it eliminated the possibility that one nucleolus might mask the other. It was not possible to obtain complete data for all nucleoli and cells which were initially recorded. Some cells were later hidden by other cells growing over them; in others the nucleolus and nucleus became masked by plastids and masses of other organelles. Some cells, in which nucleoli were being measured, divided. In such cases measurements were continued only in the end cell or in that cell nearest to the end of the filament. The values shown in Figures 7-12 represent net nucleolar volumes; i. e., volume of the nucleolus minus the volume of any nucleolar vacuoles. Instances in which a cell was followed from shortly after division to the death of the cell are illustrated in Figures 8 and 9.

As can be seen from Figures 7-12 and Appendix Table I, the nucleolar volume in all cases became markedly smaller two to four days prior to the death of each cell. Also, it is significant to note that in nearly all cases observed, vacuoles were not seen within a nucleolus one to eight days prior to the death of that cell.

The above results are in agreement with the findings of Dermen (23, p. 306) in cells of stamen hairs of Callisia, which he kept alive in water cultures. He noted that one nucleolus progressively decreased in size from a diameter of 9 microns on the first day to 1.8 microns on the sixth day, and then the cell died. Dermen also observed that as the nucleolus decreased in diameter the nucleolar vacuole disappeared, and the nucleolar vacuole did not reappear before death of this cell.

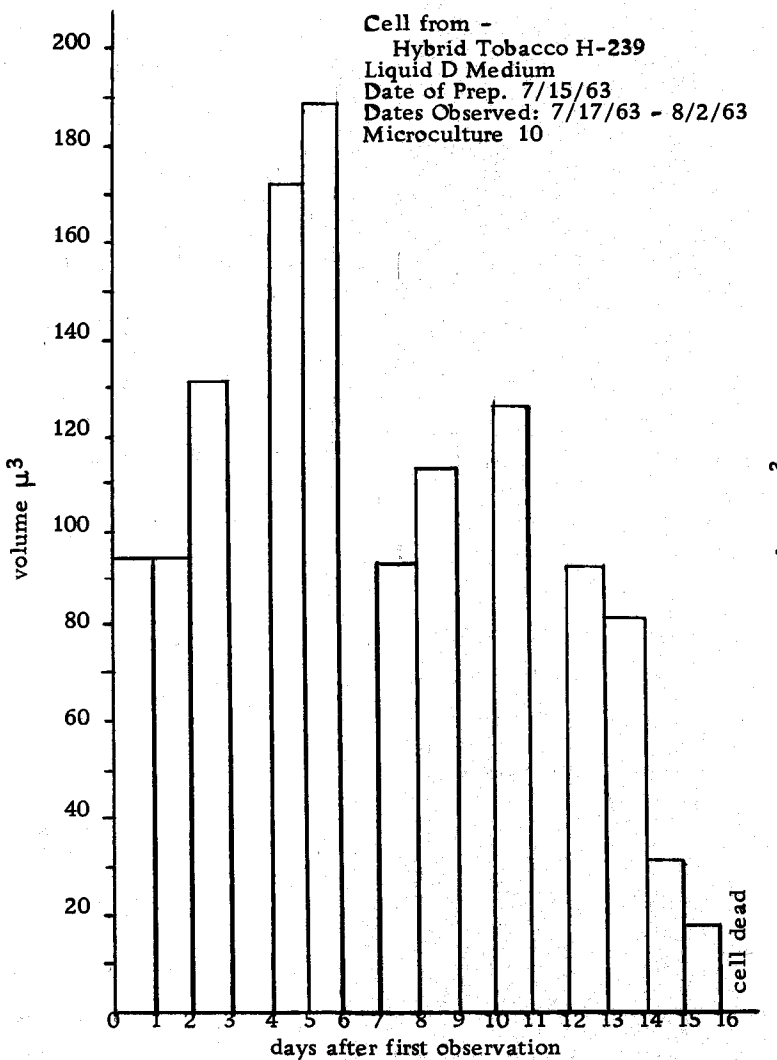


Figure 7. Changes of Volume of Nucleolus with Age

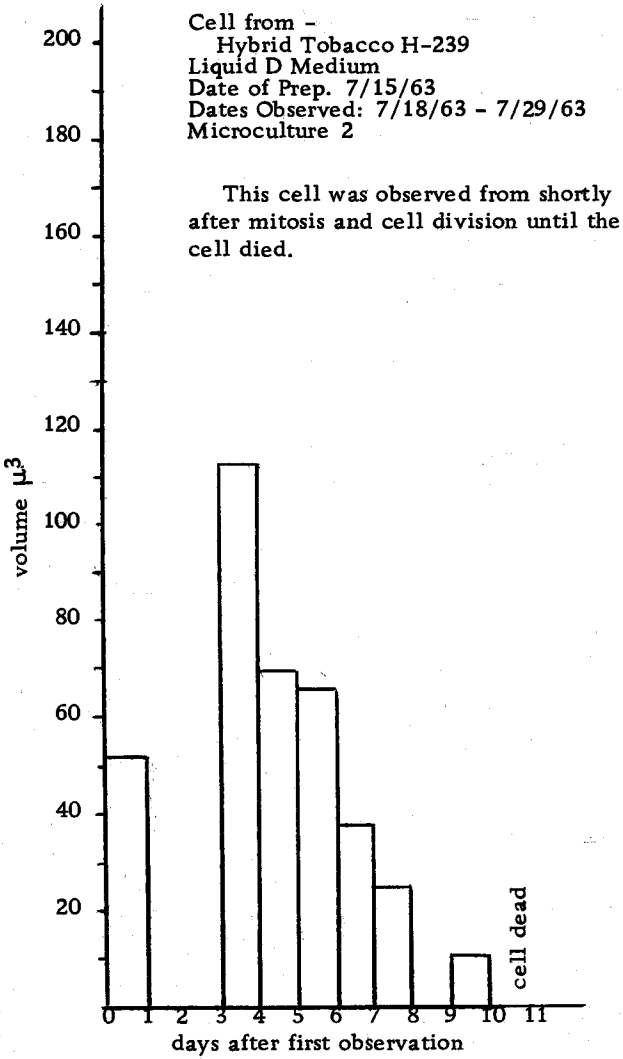


Figure 8. Changes of Volume of Nucleolus with Age

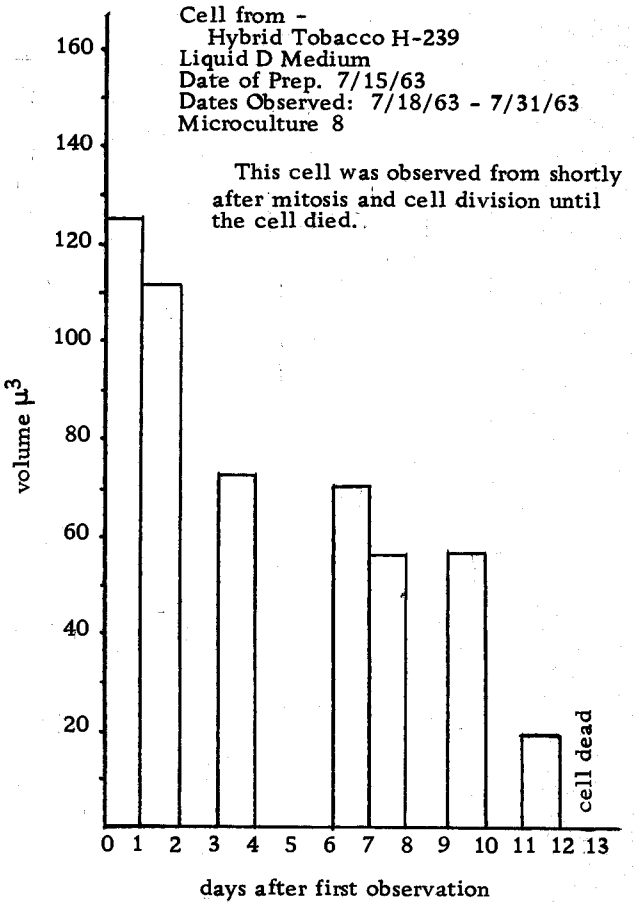


Figure 9. Changes in Volume of Nucleolus with Age

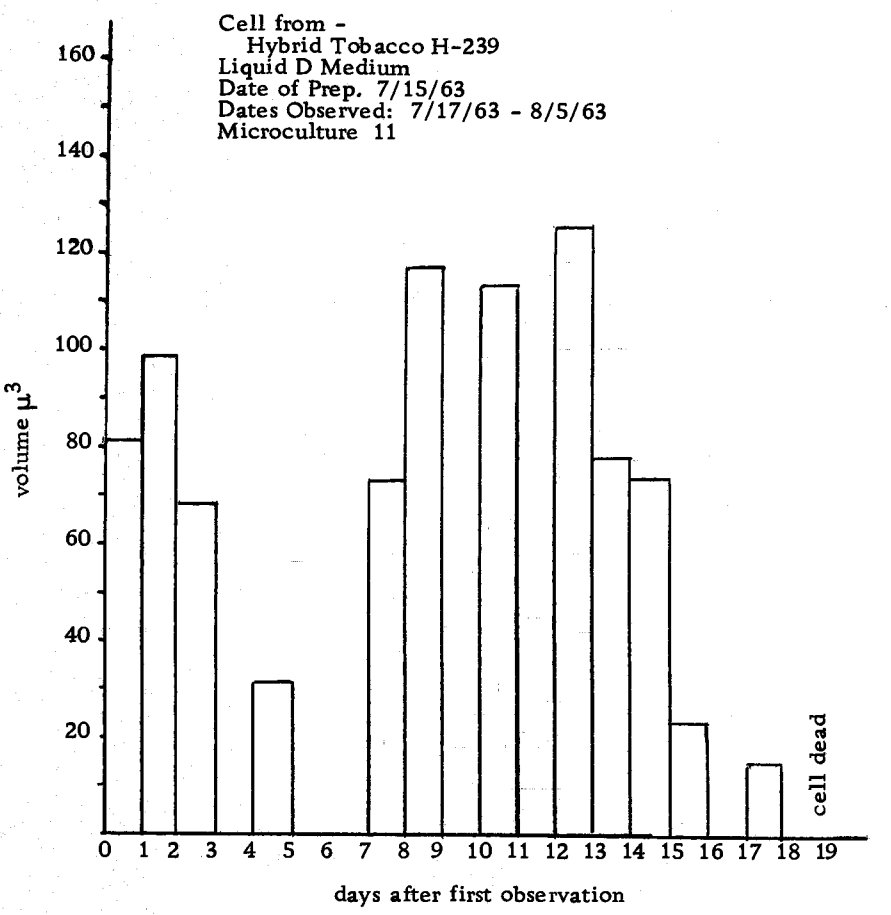


Figure 10. Changes in Volume of Nucleolus with Age

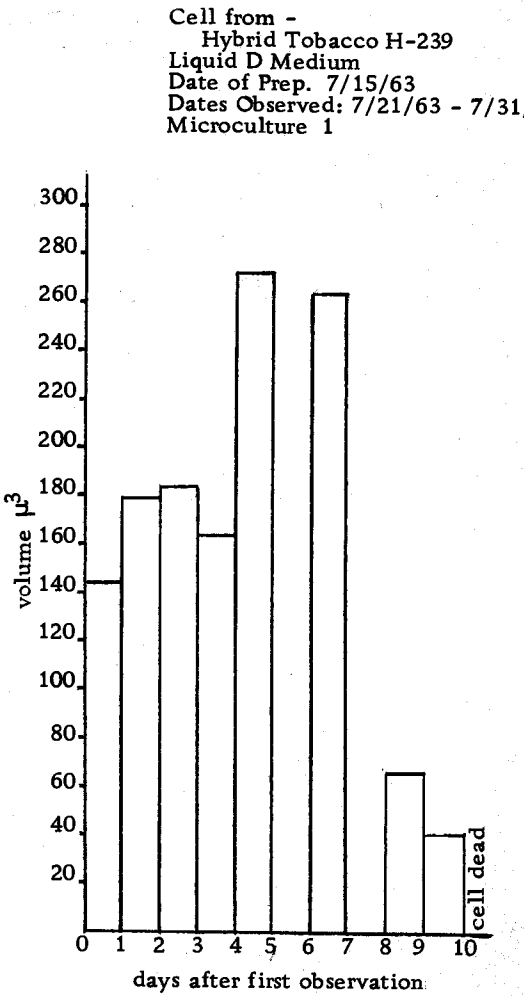


Figure 11. Changes in Volume of Nucleolus with Age

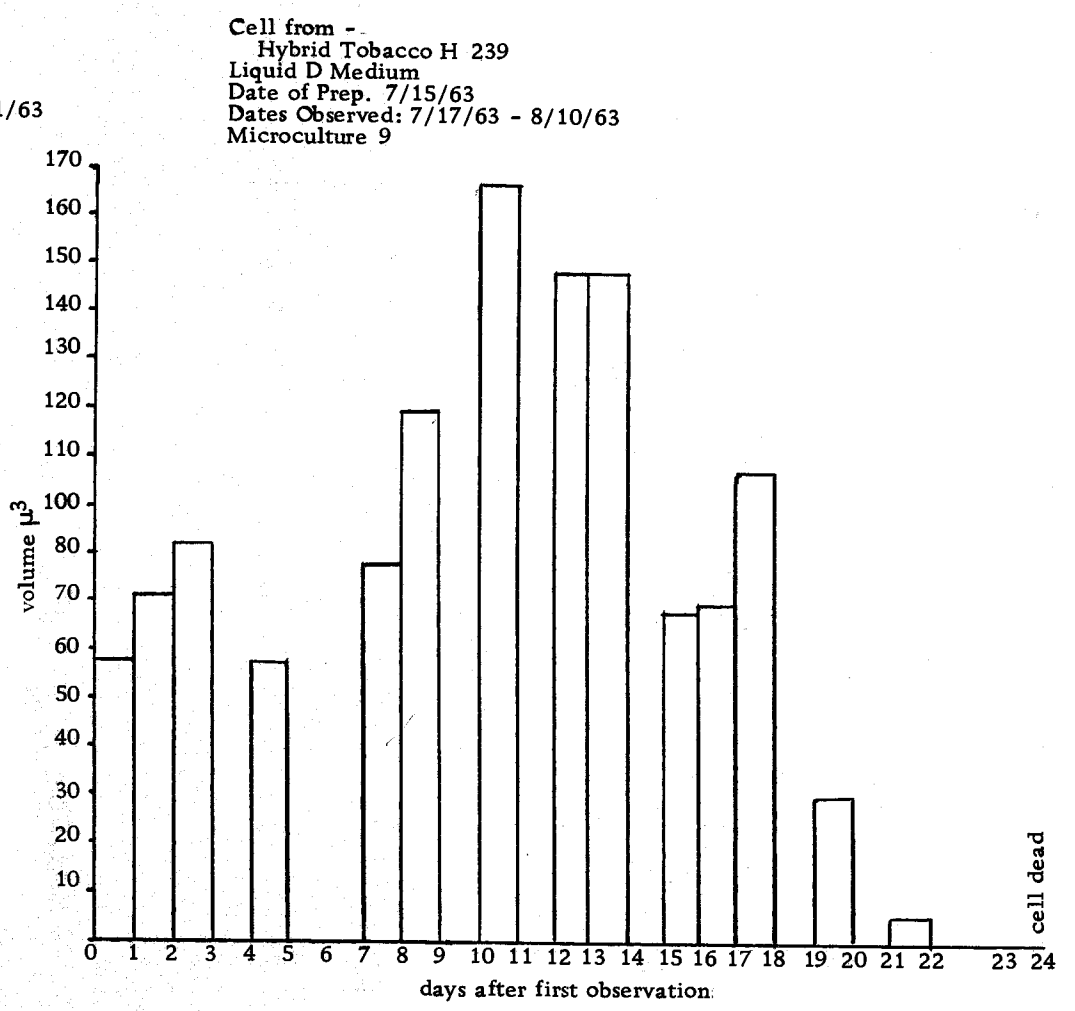


Figure 12. Changes in Volume of Nucleolus with Age

The results obtained in this study with tobacco cells are in general agreement with studies carried out by Lesher (46, p. 586-588), on salivary gland tissue from larvae of Drosophilia, in which he recorded changes in nuclear and nucleolar volumes through time. He found that the nucleolar volume began to decrease shortly after cessation of active secretion by the gland. He recorded a decrease in nucleolar volume from 1,273 μ^3 at 170 hours after oviposition to 490 μ^3 ten hours later. He also mentioned that the decrease in nucleolar size is accompanied by a loss of the so-called "vacuoles" within the nucleolus. Lesher also correlated these measurements with the basophilia of the surrounding cytoplasm and suggested that a close relationship exists between the ribonucleic acid systems, the nucleolus, and the secretory function of the Drosophilia salivary gland cells.

Soudek and Stránská (68, p. 241-243) reported that the nucleolus was large and nucleolar vacuoles were prevalent in actively growing cells of the fungus, Basidiobolus ranarum. However, in cells that had ceased to grow the nucleolus decreased in size and no longer formed vacuoles.

NUCLEOLAR VACUOLES

The nucleoli and their enclosed nucleolar vacuoles appeared to be spherical structures within living cells of tobacco. The following observations are given to support these conclusions.

By gentle manipulation it is possible to rotate a living cell along its longitudinal axis and to observe the appearance of the nucleus, nucleolus and nucleolar vacuole during the rotation. When this was done, both nucleolus and the nucleolar vacuoles appeared spherical or nearly spherical in all views. Figures 13-16 illustrate face and side views of the nucleus, nucleolus and nucleolar vacuole of a tobacco cell which was slowly rotated.

Evidence that the vacuoles are within the nucleoli was also obtained from killed, fixed and stained tobacco cells. In most instances the nucleolar vacuoles appeared as round, stained or only lightly stained areas within the nucleoli (Figure 17). However, in a few instances nucleoli were observed in which the outer boundary of the nucleolus had collapsed into the vacuolar area (Figure 18). This gave the nucleolus the appearance of an invaginated hollow ball and indicated that the vacuole was a sphere within the nucleolus.

Behavior of the nucleoli and nucleolar vacuoles in living cells also indicated that the vacuoles were spherical and within the nucleoli. As illustrated by Figures 19-23, the nucleolar vacuole opened at the margin of the nucleolus and, when contracting, appeared to release its contents into the nucleus. Associated with the contraction of the vacuoles was a measurable decrease in the diameter of the nucleolus. Tables I and II show measured decreases in nucleolar diameter following contraction of nucleolar vacuoles.

Figure 13

A low power view of a living tobacco cell showing the nucleus and nucleolus as they appeared in face view. (450x)

Figure 14

A low power view of the same cell illustrated in Figure 13. The cell has now been rolled so that the nucleus is at right angles to the position seen in Figure 13. (450x)

Figure 15

A more highly magnified view of the same nucleus illustrated in Figure 13. Note the spherical shape of the nucleolus (B) and of the nucleolar vacuole (C). (1300x)

Figure 16

A more highly magnified view of the nucleus as illustrated in Figure 14. Note that in this view of the nucleus, the nucleolus and nucleolar vacuole still appear spherical. (1300x)

Figure 17

A killed and fixed tobacco cell stained with Trypan Blue and mounted in 10 percent Karo. The nucleolus (B) and nucleolar vacuole (C) appeared much the same as in the living cell. The vacuole was unstained. (1300x)

Figure 18

A nucleus and nucleolus from the same slide as the cell in Figure 17. The outer boundry of the nucleolus has collapsed into the vacuole forming the invagination labeled N. (1300x)

A = nucleus
B = nucleolus
C = nucleolar vacuole
D = cell wall
N = invaginated area of nucleolus

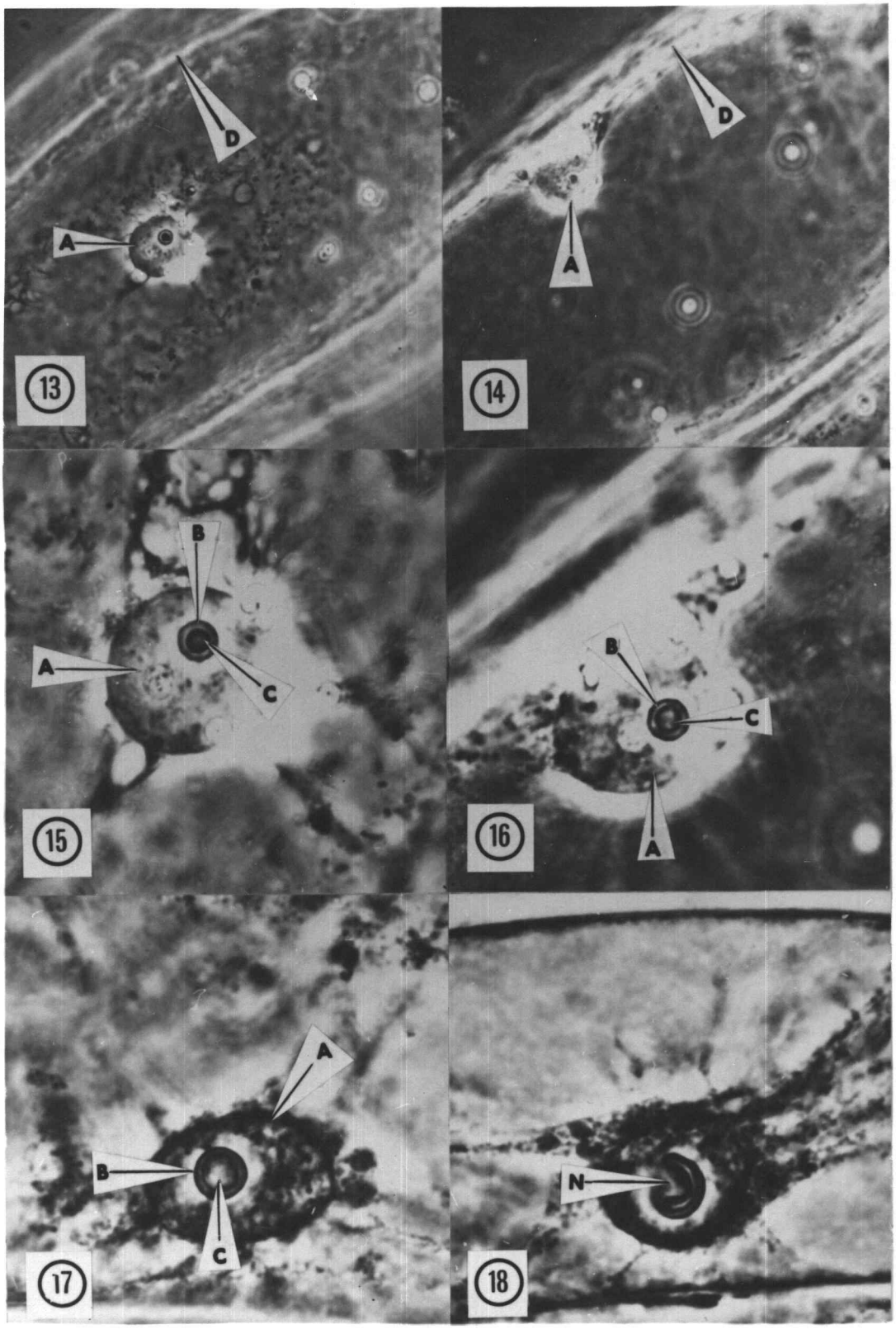

D
A
13
D
A
14
B
A
C
15
B
C
A
16
A
B
C
17
N
18

Figure 19
Nucleus, nucleolus and nucleolar vacuole in a living tobacco cell just prior to contraction of the nucleolar vacuole.

Figure 20
Beginning of the contraction of the nucleolar vacuole. This photograph taken one minute later than Figure 19. Note opening of nucleolar vacuole to the outside of the nucleolus.

Figure 21
Continuation of vacuolar contraction. This photograph taken about 15 seconds after Figure 20.

Figure 22
Nucleolar vacuole almost completely contracted. This photograph about 15 seconds later than Figure 21.

Figure 23
Nucleolar vacuole completely gone. Total time elapsed between Figure 19 and Figure 23 about two minutes.

Figure 24
Same nucleus and nucleolus as shown in Figures 19-23 one hour later. Large nucleolar vacuole (C) has reformed again within the nucleolus.

Figures 19-24 at 1300x.

A = nucleus
B = nucleolus
C = nucleolar vacuole

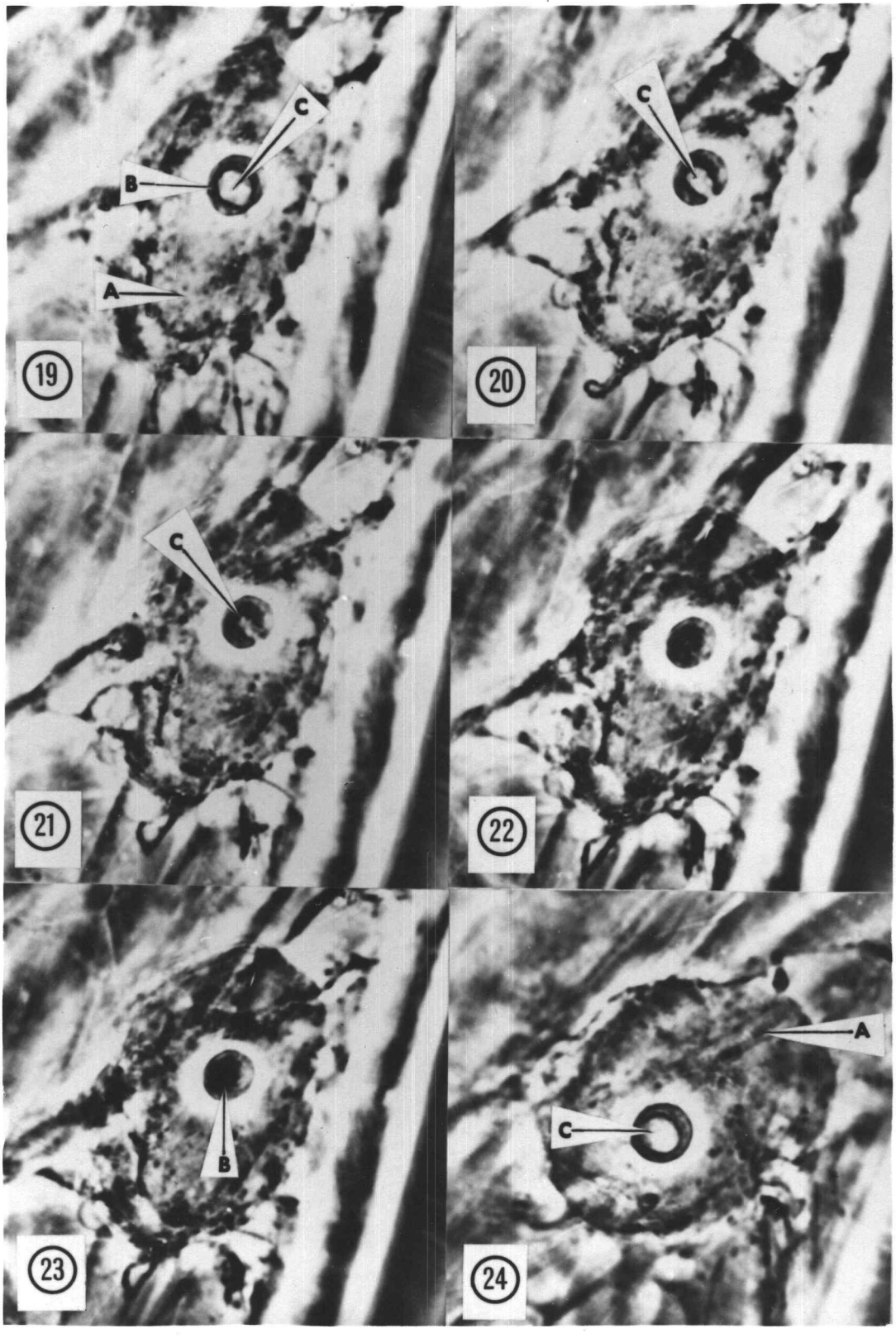

C
B
A
19
C
20
C
21
22
B
23
A
C
24

Table I

Decrease in Diameter of Nucleolus Following Contraction of Nucleolar Vacuole *

	Measured Diameter of Nucleolus			Measured Diameter of Vacuole	
	Before Contraction μ	After Contraction μ	Calculated terminal diameter of Nucleolus μ	Before Contraction μ	After Contraction μ
1	9	8.5	8.4	5.25	0
2	7.5	7	6.9	4.5	0
3	7.5	6.75	6.7	5	0
4	7.3	5.8	6.2	5.3	0
5	8.2	7.3	7.2	5.7	0
6	9.3	7.2	8	7.3	4.66
7	9.3	7	7.9	7	3.3
8	8.6	7.7	7.5	6.3	3.3
9	11.7	10.8	11	7	4
10	12.7	10.3	10.2	10.8	6.7
11	8.3	7.3	7.5	5.3	1.8
12	10.8	8	8.8	8	3

Table II

Decrease in Diameter of Nucleolus Following Contraction of Nucleolar Vacuole *

	Measured Diameter of Nucleolus			Measured Diameter of Vacuole	
	Before Contraction μ	After Contraction μ	Calculated terminal diameter of Nucleolus μ	Before Contraction μ	After Contraction μ
1	7.7	5.6	5.9	6.3	0
2	7	6.6	6.9	2.4	0
3	14	9.5	9.7	12.6	5.6
4	13.5	10.8	12.2	10	7.2
5	11.7	9	8.8	10	4.5
6	10	7.7	8.2	8	4
7	9	8	8.4	6.3	5
8	8.7	6.6	6.4	7.5	3
9	9.3	7.2	6.6	8.4	4.2
10	7.8	7	7	5.4	3.3
11	12	10.8	11.8	7.2	3.3

* Method for Calculations given on pages 17 and 18 of Methods and Materials

As can be seen in Tables I and II there was close agreement between the calculated values for diameters of the nucleoli after vacuolar contraction and the actual measured values. The close agreement between the measured and calculated values illustrates that the decrease in diameter of the nucleoli following the contraction of a nucleolar vacuole was in accordance with values that would be expected if the contents of a smaller sphere (the vacuole) were released external to the larger sphere (the enclosing nucleolus). The differences between the calculated and measured values, as shown in Tables I and II, were probably due to fluctuations in the focal plane of the nucleolus while taking the photographs and to limitations in the method used for obtaining measurements with the ocular micrometer.

The above observations and results lend support to the conclusion that the nucleoli and nucleolar vacuoles are generally spherical, that the nucleolar vacuoles are within the nucleoli, and that the contents of the nucleolar vacuoles are discharged into the nucleus.

Behavior of Nucleolar Vacuoles

Detailed observations of the behavior of the nucleolar vacuoles were made on living cells in microcultures and by analyses of four-second time-lapse cinemicrographs taken of the nucleoli and nucleolar vacuoles within cells growing in microcultures (35, p. 81). The most striking feature of the nucleolar vacuoles was

that they periodically contracted, expelling their contents into the nucleus. After contraction nucleolar vacuoles were observed to reform and contract again. Figures 19-24 illustrate this series of events. Two types of vacuolar contraction have been observed: (a) a complete contraction, as illustrated by Figures 19-23 or (b) an incomplete contraction, as illustrated by Figures 25-26. If the contraction was seen at right angles to the optical axis there was a channel from the nucleolar vacuole extending through the margin of the nucleolus (Figure 20). In the case of a complete contraction the total contents of the nucleolar vacuole seemed to be expelled into the nucleus. During an incomplete contraction, the channel retracted from the margin of the nucleolus; the contraction of the vacuole stopped, and the vacuole became round again. Because both the nucleoli and the nucleolar vacuoles are spheres, only rarely is the channel of the vacuole seen at the outer margin of the nucleolus, as in Figure 20. Therefore, during many contractions the vacuoles seem to disappear either toward the upper or lower surface of the nucleolus, or toward various eccentric positions on the periphery of the nucleolus.

The length of time required for the contraction of nucleolar vacuoles varied, depending upon the size of the vacuole and the extent of the contraction. Large nucleolar vacuoles (one-fifth the diameter of the nucleolus or larger) usually required from one to seven minutes to contract. The incomplete contraction of large vacuoles required from 30 seconds to 6 minutes depending upon the

Figure 25

Nucleus, nucleolus and nucleolar vacuole in a living tobacco cell just prior to a partial contraction of the large nucleolar vacuole (C). (2000x)

Figure 26

The same nucleus, nucleolus and nucleolar vacuole as seen in Figure 25 two minutes later, after an incomplete contraction of the nucleolar vacuole (C). Note the decrease in diameter of the nucleolus and of the nucleolar vacuole. (2000x)

Figure 27

Large nucleolus in a living tobacco cell. Note vacuole (C) at this time separate from the other vacuole within the nucleolus. (2000x)

Figure 28

Same nucleolus as illustrated in Figure 27 about six minutes later. The two vacuoles are just beginning to fuse. (2000x)

Figure 29

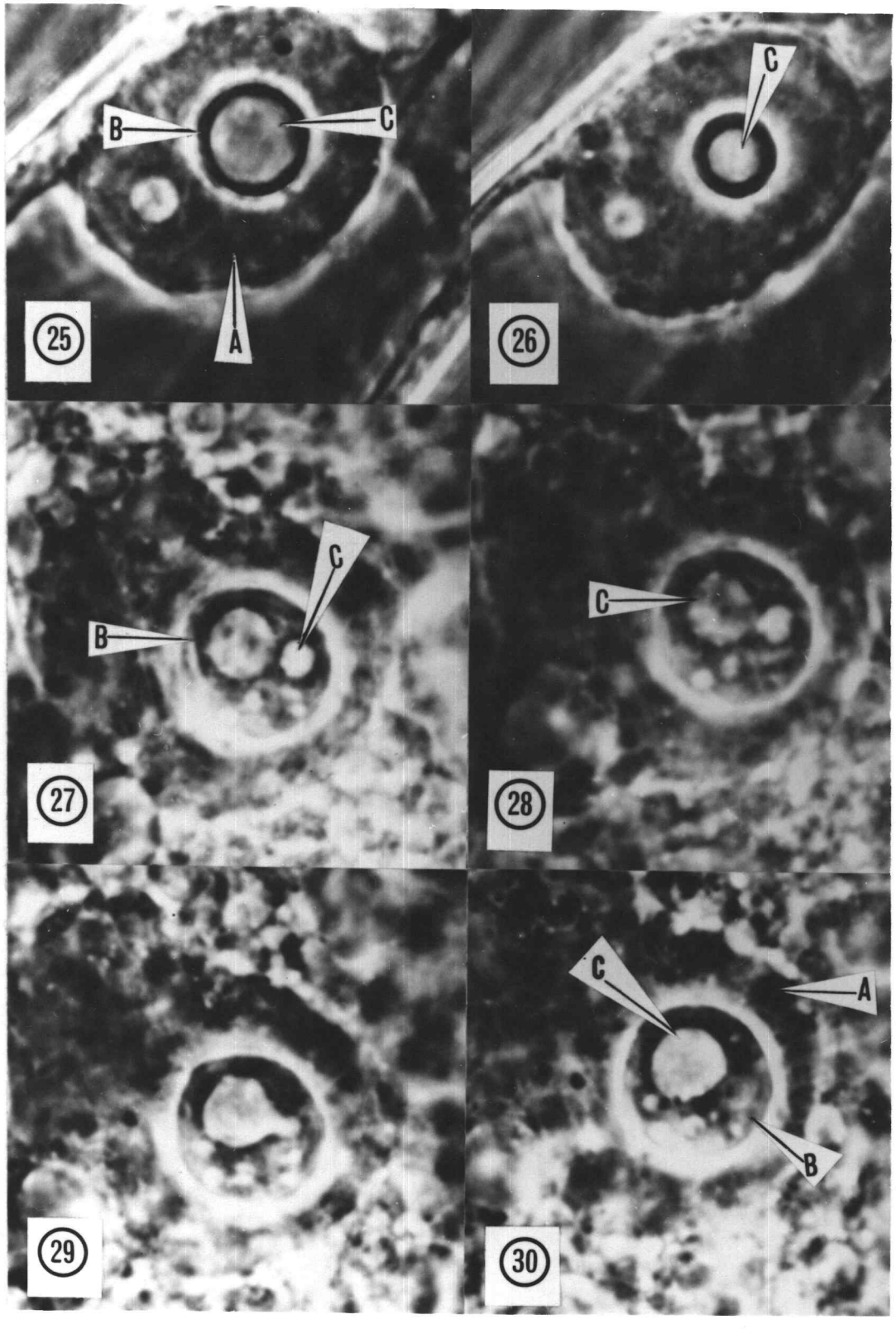
Further progress in the fusion of the two nucleolar vacuoles. One minute later than Figure 28. (2000x)

Figure 30

The two nucleolar vacuoles are now rounded up as one large vacuole. (2000x)

The sequence of events from Figure 27 to Figure 30 took place in eight minutes.

A = nucleus
B = nucleolus
C = nucleolar vacuole


B
C
A
25
C
26
C
B
27
C
28
C
A
B
29
30

amount of vacuolar material released. Small vacuoles (less than one-fifth the diameter of the nucleolus) often contracted within 10 to 30 seconds.

After a complete contraction, the nucleolus usually appeared as a homogeneous, dark gray body (Figure 23), when viewed with dark phase-contrast optics. Often within five minutes, small, indistinct, light vacuolar areas appeared at different focal planes within the nucleolus (Figures 3 and 27-30). As the small vacuoles formed they either fused into larger vacuoles (Figures 27-30), or moved to the edge of the nucleolus and contracted. Usually large vacuoles formed by the fusion of several small vacuoles. Occasionally, after the contraction of a large nucleolar vacuole, only one small vacuole would form which slowly increased in volume by the accumulation of more and more vacuolar material until the vacuole reached a maximum size. Small vacuoles usually did not form following the incomplete contraction of a large vacuole. Either a subsequent contraction of the residual vacuole occurred or there was a gradual increase in volume of the residual vacuole. A summary of the contractions and reformations of nucleolar vacuoles is presented in Figures 31-39. These data represent volume changes in nucleolar vacuoles of cells which were observed at 15 minute intervals over six to eight hours of time, and they show that each cell had its individual pattern for the formation and contraction of nucleolar vacuoles. This might be expected, since the tobacco callus cells were growing in a complete nutrient medium,

VOLUME OF NUCLEOLAR VACUOLES

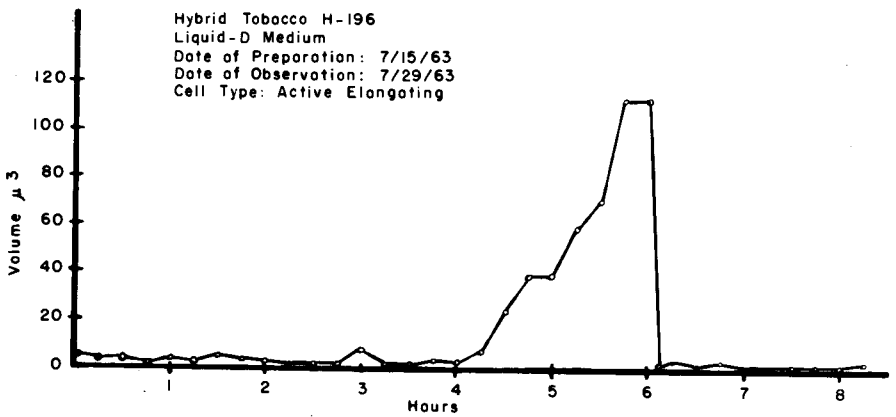


Figure: 31

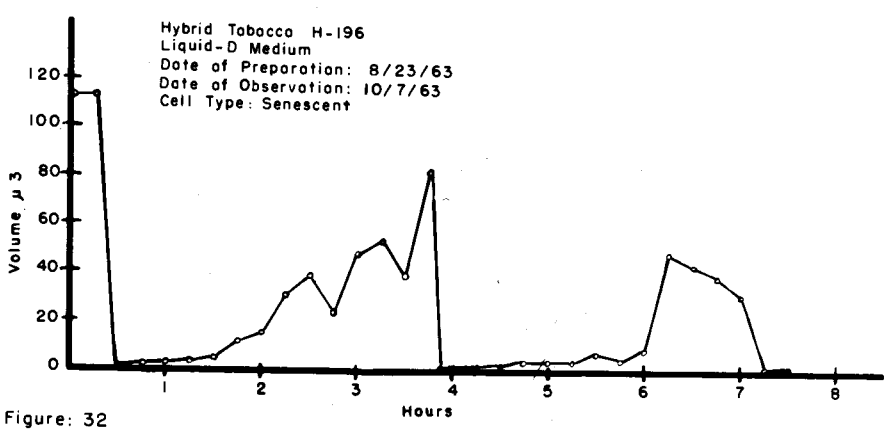


Figure: 32

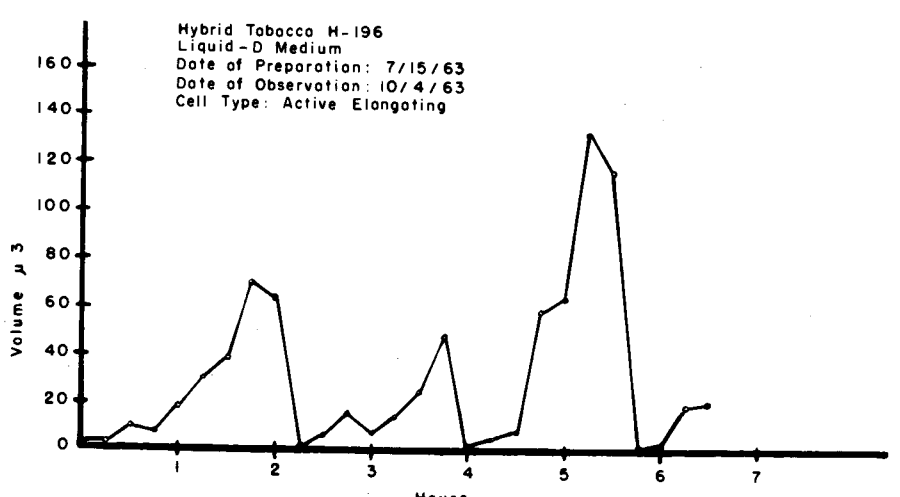


Figure: 33

VOLUME OF NUCLEOLAR VACUOLES

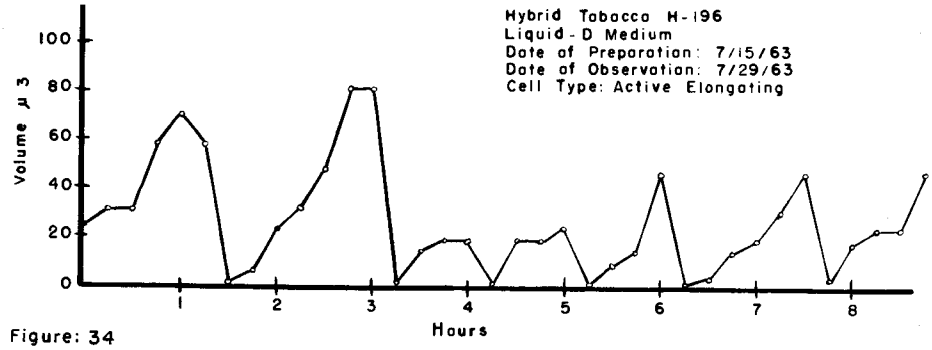


Figure: 34

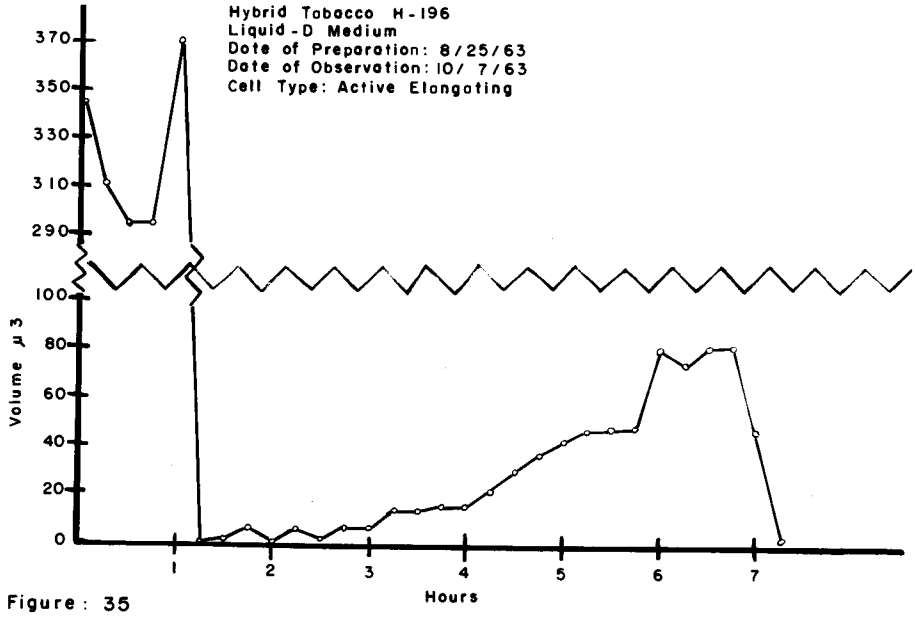


Figure: 35

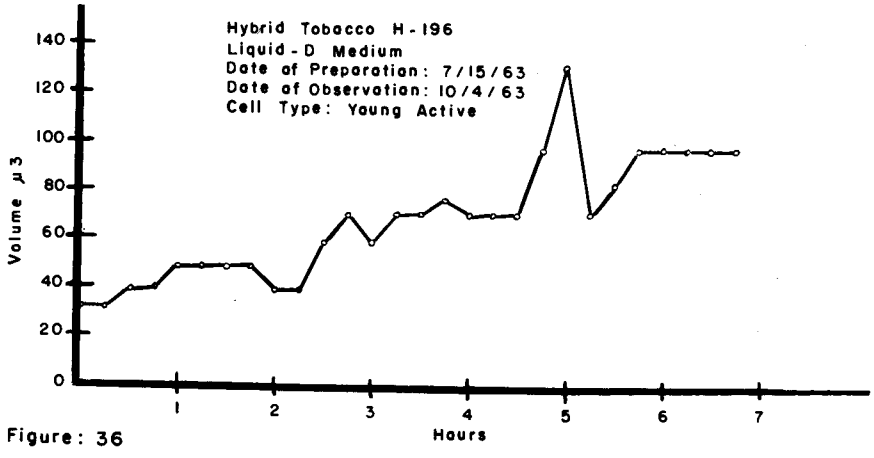


Figure: 36

VOLUME OF NUCLEOLAR VACUOLES

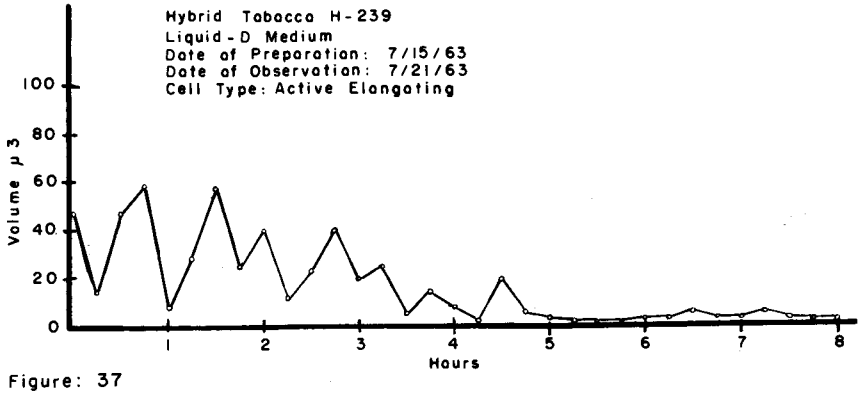


Figure: 37

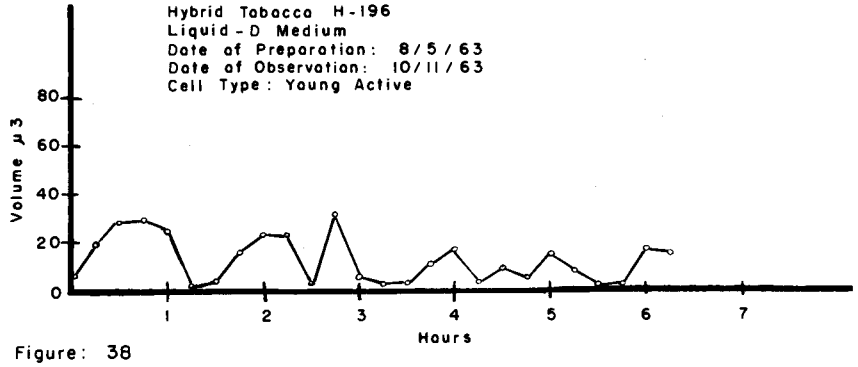


Figure: 38

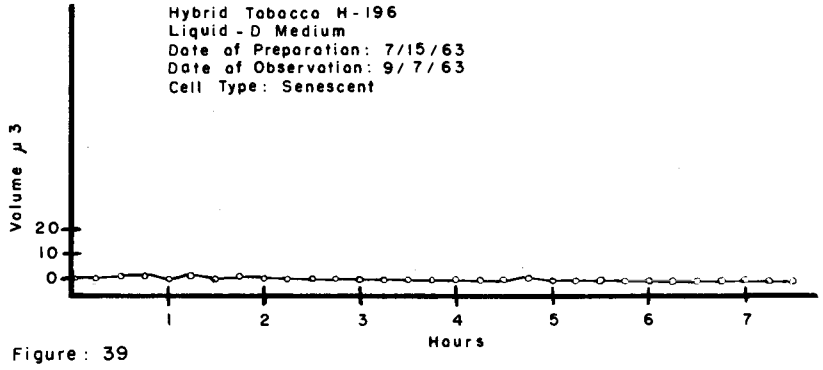


Figure: 39

and each cell apparently was physiologically independent of the others in the microculture. There is, however, no doubt that the nucleolar vacuoles do form and periodically contract within living tobacco cells.

Occurrence of Nucleolar Vacuoles

The correlation of nucleolar vacuoles to the morphogenic status of the cell was investigated. In these studies three visible categories, concerning the morphogenic status of the cells, were subjectively determined. These categories were: (a) young, active cells, (b) active, elongating cells, and (c) senescent or weakened cells. The young, active cells were distinguished by being of smaller size than the other cells and generally being of an isodiametric or slightly elongated shape. These cells had abundant cytoplasmic strands in which active cytoplasmic streaming and mass movement of organelles was obvious. Most of the visible organelles in the young, active cells were discrete, round units and very few filiform mitochondrial units were present. These young, active cells frequently divided (34, p. 4-5, 38, p. 4, 39, p. 82, and 40, p. 660-661). Active, elongating cells were larger in diameter and were longer than the young, active cells. The elongating cells showed active cytoplasmic streaming, and most of the cytoplasmic organelles were discrete, but some mitochondrial units were filiform. Elongating cells divided only occasionally; their usual growth pattern was further enlargement,

and eventually senescence and death. Senescent or weakened cells included a grouping of two kinds of cells which showed some similar internal characteristics. Senescent cells were generally greatly enlarged and most of the cytoplasm was parietal in position with very few strands crossing the vacuole. Cytoplasmic streaming was generally slow. Most of the mitochrondria in senescent cells were in long filiform units and another group of round organelles, the spherosomes, were often swollen. Also there generally were a great many thin filiform tubules or channels in the cytoplasm giving it a vesiculate appearance. Cells showing the above characteristics were senescent because they would usually die without undergoing further changes (38, p. 4, 39, p. 82, and 40, p. 660-661). Cells in a weakened condition due to injury or to some other reason may or may not be enlarged. The cytoplasm and visible organelles of weakened cells resembled those in senescent cells; in addition, there usually was much disorganization of the cytoplasm and a rapid Brownian movement of the visible organelles. A cell with these characteristics usually died within a few days.

Table III presents a compilation of data taken from five separate studies in which the morphogenic status of the cell and the occurrence of nucleolar vacuoles was recorded. Appendix Tables II, III, and IV contain the data of the five studies from which Table III was compiled. The percentage of cells in which nucleolar vacuoles were observed was much lower in senescent cells or cells in a weakened condition than in young, active or active, elongating cells.

Table III
Occurrence of Nucleolar Vacuoles in Cultures of Cells from
Hybrid Tobacco H-196 in Liquid D Medium*

Morphological condition of cells	Total Cells counted	Total Cells with vacuoles	Total Cells without vacuoles
Young. Active Cells. Total number based on four different microculture series and a total of 40 different microcultures. data taken 1 to 20 days after preparation of microcultures.	2006	1337 69% (25% Large) (44% Small)	629 31%
Active. Elongating Cells. Total number based on four different microculture series and a total of 40 different microcultures. data taken 1 to 34 days after preparation of microcultures.	4485	3151 70% (20% Large) (50% Small)	1334 30%
Senescent or Weakened Cells. Total number based on five different micro-culture series and a total of 59 different microcultures. data taken 1 to 49 days after preparation of microcultures.	2041	682 33% (9% Large) (24% Small)	1359 67%

*Appendix Tables II. III and IV give data from which this table was compiled

This apparent relationship between the morphogenic status of a cell and the occurrence of nucleolar vacuoles was consistent from one microculture series to another.

An effect of physical manipulation upon the nucleolar vacuoles was studied. There is an apparent sensitivity of the vacuoles to the physical manipulations involved in making the microcultures. This apparent shock response was observed in all new microcultures studied and was especially pronounced in young, active and active, elongating cells. On the day the microcultures were made a relatively low percentage of cells had nucleolar vacuoles; the percentage of cells showing vacuoles increased during the following days. Figures 40-41 illustrate this behavior in two microculture series; additional data from other microculture series are given in Appendix Tables II, III, and IV.

There appears to be a relationship between contracting nucleolar vacuoles and mitosis and cell division in that typically the nucleolar vacuoles contract completely just prior to the disappearance of the nucleolus in late prophase.

Cytochemical Tests

The chemical nature of the contents of the nucleolar vacuoles was investigated by using the Methyl Green and Pyronin (20, p. 166-167 and 32, p. 251) and Azure-B (20, p. 220 and 32, p. 251-252) methods to determine the presence of nucleic acids. These were used with killed and fixed hybrid tobacco cells, single-cell clones

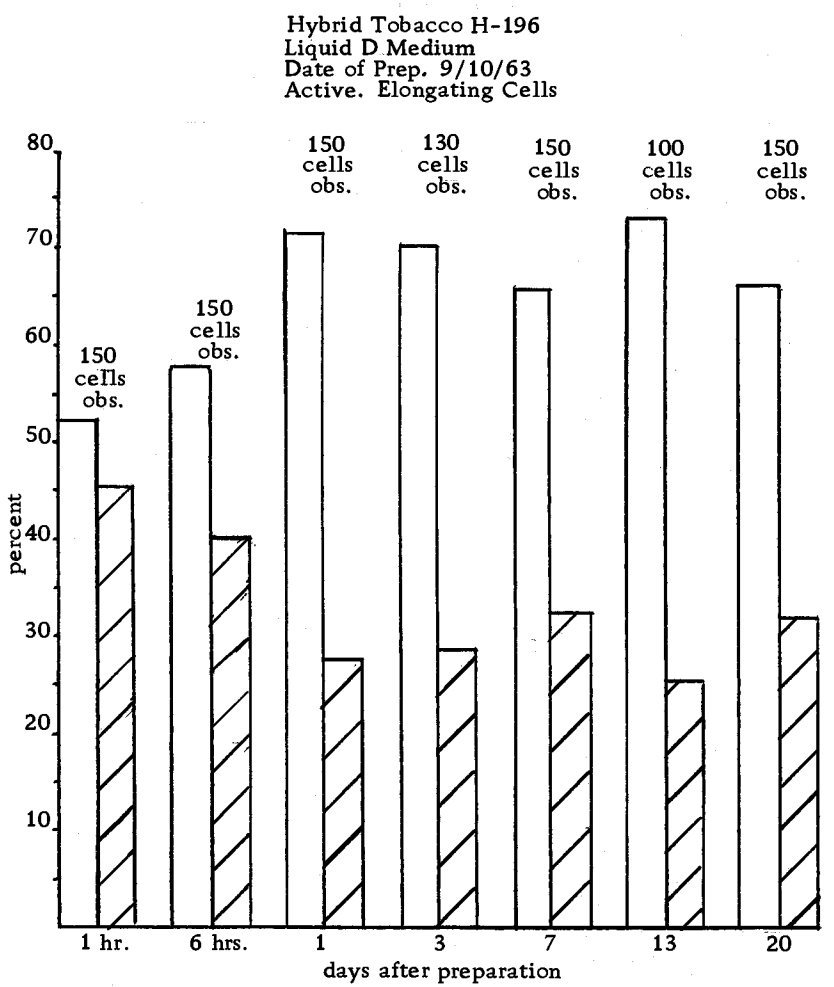


Figure 40. Response of Nucleolar Vacuoles to Physical Manipulations during Preparation of Microcultures

□ = cells with vacuoles

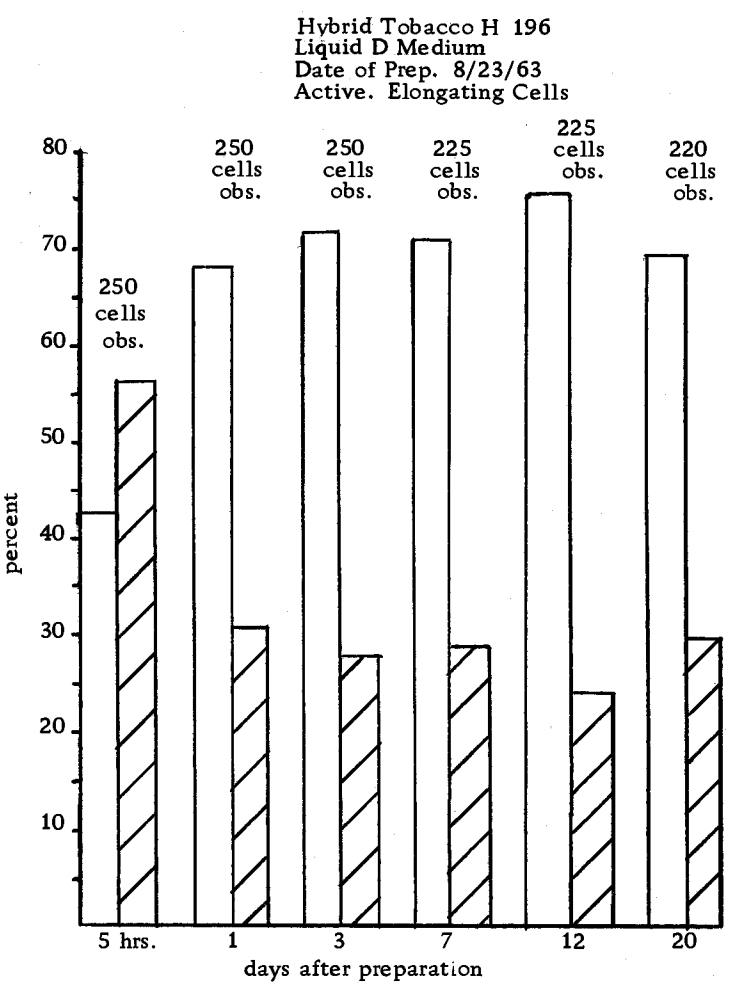


Figure 41. Response of Nucleolar Vacuoles to Physical Manipulations during Preparation of Microcultures

▨ = cells without vacuoles

H-196 and H-239, Vicia faba root tips, and with stem sections of Nicotiana glutinosa and Nicotiana tabacum. After these tissues were stained with Methyl Green and Pyronin (20, p. 166-167 and 32, p. 251), the chromatin was bluish green and the nucleoli appeared rose or pink. The nucleolar vacuoles generally appeared colorless or light pink in color. In most instances when a pink color appeared in the nucleolar vacuoles the coloration could be attributed to pink or rose-stained material present in the matrix of the nucleolus above and/or below the nucleolar vacuole. Ribonuclease (RNase) digestion (3, p. 6) was used to determine the specificity of Pyronin and Azure-B stains for Ribonucleic Acid (RNA). After digestion with RNase, the cells showed only a faint pink pyronin stain or no stain at all in the nucleoli, while the chromatin was still stained a bluish green. These results indicated that the rose or pink stained pyronin-positive areas did contain RNA. After the callus and paraffin sections of root tips and stem tissues were stained with Azure-B (20, p. 220 and 32, p. 251-252), the nuclei were blue and the nucleoli purple to purplish blue. With the Azure-B method a rather wide range in stain intensity of nucleoli was noted in both tobacco cells and Vicia faba root tips. The nucleolar vacuoles generally appeared as a light bluish purple color which generally was lighter in color than the surrounding matrix of the nucleolus. In some instances what appeared to be a darkly stained boundary surrounded the nucleolar vacuole. This boundary was darker than either the nucleolar matrix or the

vacuole. The apparent color seen in the nucleolar vacuole was possibly due to color in the nucleolar matrix above and below the vacuole, but this determination was difficult to establish with certainity. RNase digested cells generally showed, after staining with Azure-B, no stain or, at most, a faint purple stain in the nucleoli. These results with Azure-B stain again indicated that the purple portions of the nucleoli contained RNA. From the results described above with both the Methyl Green and Pyronin and Azure-B staining methods, the conclusion was drawn that the nucleolar vacuoles did not contain an accumulation of RNA that was held in position during the recommended killing, fixing and staining procedures.

The protein content of the nucleolar vacuoles was determined by using the Mercuric Bromphenol Blue technique (51, p. 57-67). After staining by this method, the nucleoli appeared blue, indicating the presence of protein in the matrix of the nucleoli; however, the nucleolar vacuoles were colorless or protein-negative.

The lipid content of the nucleolar vacuoles was determined by staining with Sudan IV (33, p. 191). The nucleoli were unstained or only lightly stained an orange color by Sudan IV; the nucleolar vacuoles were not stained. Small spherical globules in the cytoplasm were stained a bright orange, indicating the presence of lipid droplets in the cytoplasm and that the staining method was properly applied.

Although the results obtained with the above cytochemical tests were negative in that they failed to show the presence of nucleic acids, proteins or lipids within the nucleolar vacuoles; they are consistent with the reports of others. Soudek and Stránská (68, p. 241) reported that no definite information could be given concerning the contents of nucleolar vacuoles in fungi. They stated that according to Stich critical cytochemical proof of the nature of the contents of the nucleolar vacuoles cannot be made because the materials within the nucleolar vacuoles are either very dilute or are water soluble; if water soluble, the contents of the vacuoles cannot be permanently fixed and held in position by the usual procedures. Brachet (7, p. 127) also reported that the nucleolar vacuoles do not respond positively to cytochemical tests for nucleic acids or proteins. He suggested that the nucleolar vacuoles contained soluble products of metabolic activity. Mulnard (59, p. 488) reported that the vacuoles within nucleoli of Drosophila salivary gland cells stained negatively for proteins and/or nucleic acids. Sirlin (64, p. 448-450) showed, in cells of Xenopus somites, vacuolar areas in the nucleoli which did not stain with the Methyl Green and Pyronin method. Swift (74, p. 270) also reported small non-staining vacuoles in the nucleoli of onion root tip cells.

Ribonuclease Experiments

Experiments with ribonuclease (RNase) were carried out to determine the morphological effect of this enzyme upon living

tobacco cells and particularly its effect upon nucleoli and the nucleolar vacuoles. Both microcultures and standard liquid cultures were used in these experiments. The RNase-D medium was prepared as described in Methods and Materials. In the first experiment ten microcultures of tobacco cells from the single-cell clone H-196 were grown in RNase-D medium (RNase concentration 0.1 mg/ml), as well as ten in the standard D medium. When the RNase was used at this concentration, no specific morphological effects were found in the nucleolus, nucleolar vacuoles or other portions of the cells when compared with the cells grown in the control medium. Even after 20 days in the RNase-D medium, cells were observed to be dividing and nucleolar vacuoles were observed to contract and reform. Figures 42-45 show a comparison between the occurrence of nucleolar vacuoles in the RNase treated cells and the cells grown in the control medium. There is no marked change in the percentage of cells showing nucleolar vacuoles in the RNase treated microcultures. Possible explanations for these results are that concentration of enzyme was too low or that the cells inactivated the RNase in the microcultures.

In a second experiment, H-196 tobacco cells were grown in RNase-D medium containing 1.0 mg/ml of ribonuclease and compared with cells growing in standard D medium. No apparent morphological differences were noted between the RNase treated cells and the controls during the first seven days in the microcultures. After 13 days, many of the cells in these RNase-D

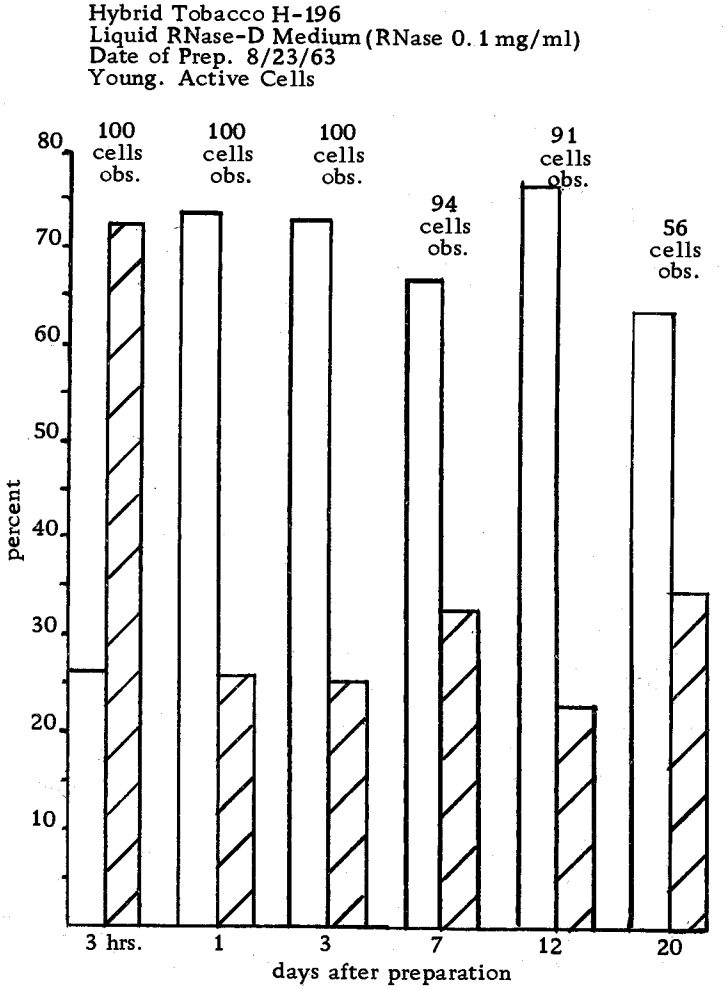


Figure 42. Occurrence of Nucleolar Vacuoles in RNase-D Medium

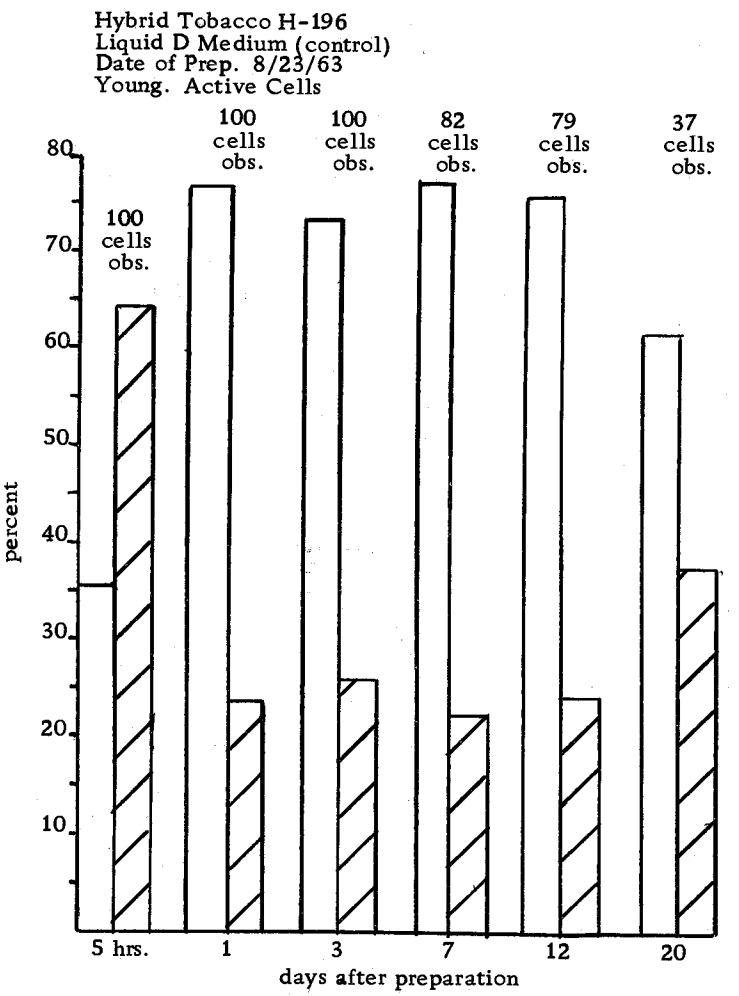


Figure 43. Occurrence of Nucleolar Vacuoles in D Medium

□ = cells with nucleolar vacuoles

▨ = cells without nucleolar vacuoles

The number of observations when possible was based on the first ten cells of this type observed per microculture in 10 different microcultures for both RNase treated cells and the control cells.

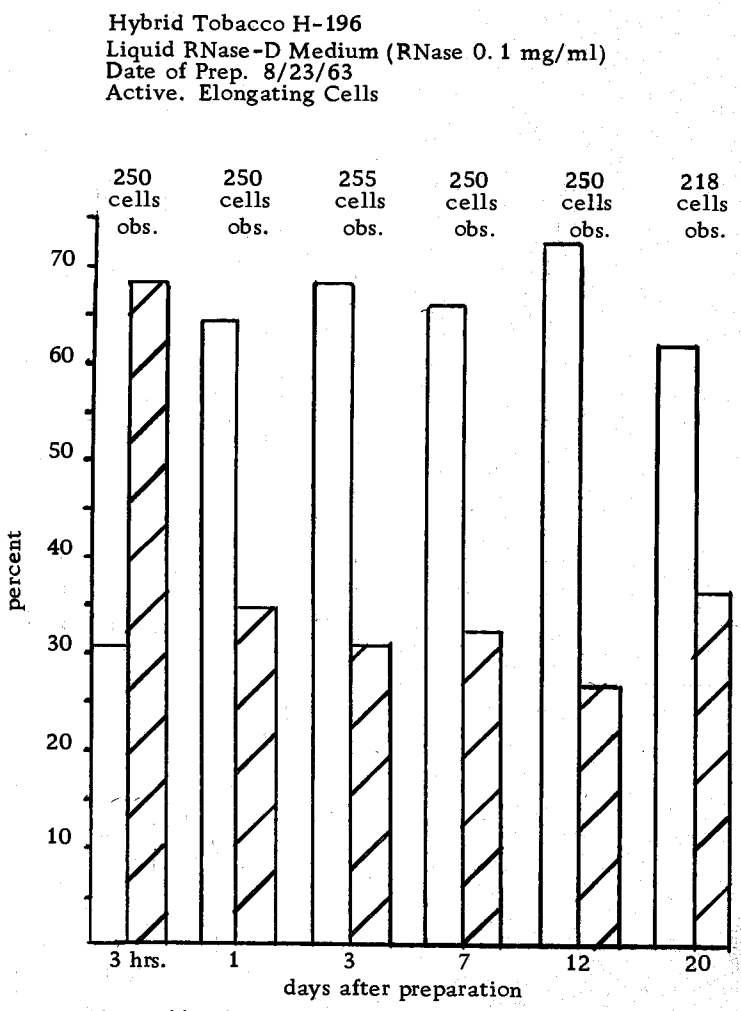


Figure 44. Occurrence of Nucleolar Vacuoles in RNase-D Medium

□= cells with nucleolar vacuoles

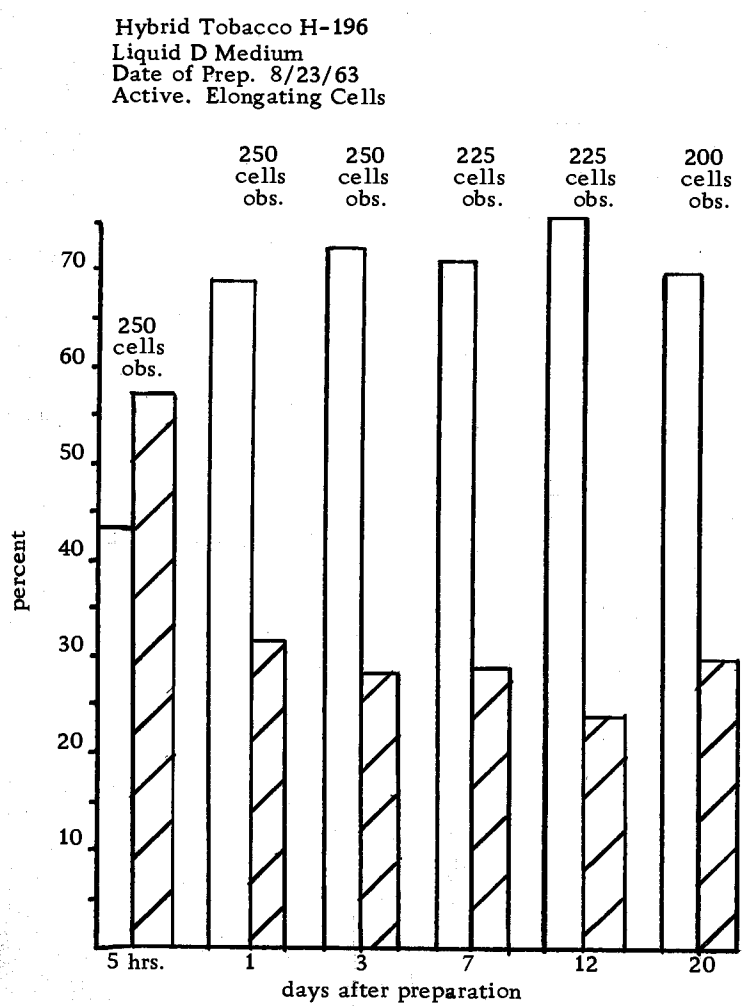


Figure 45. Occurrence of Nucleolar Vacuoles in D Medium

▨= cells without nucleolar vacuoles

The number of observations when possible was based on the first 25 cells of this type observed per microculture in 10 different microcultures for both the RNase treated cells and the control cells.

microcultures were dead, and none of the living cells were dividing. After 13 days in the standard D medium, most of the cells were alive, and six cells in the ten microcultures were seen in the process of mitosis and cell division. After 20 days only 94 cells were alive in the ten RNase treated microcultures; whereas, 684 cells were alive and growing in the ten control microcultures. At the end of 34 days only 29 cells remained alive in the ten RNase treated microcultures; whereas, in the ten control microcultures approximately 730 cells were growing. Still, no marked morphological effect induced by the RNase was noted within the nucleoli or the nucleolar vacuoles when compared with the cells in the control microcultures. In addition there was no marked change in the percentage of cells containing nucleolar vacuoles in the RNase treated microcultures when compared with the controls in standard D medium (Figures 46-47). One minor RNase effect was that RNase treated cells required a longer period to develop a high percentage of nucleolar vacuoles than did the cells in standard D medium.

A supplementary experiment was carried out in conjunction with the above experiment. Tests were made to determine the RNase activity in the RNase-D medium (1.0 mg/ml RNase) used in the above experiment. The RNase-D medium was stored under sterile conditions at room temperature along with the microcultures. Samples of this medium were tested for RNase activity periodically by using the absorption of ultraviolet light as described

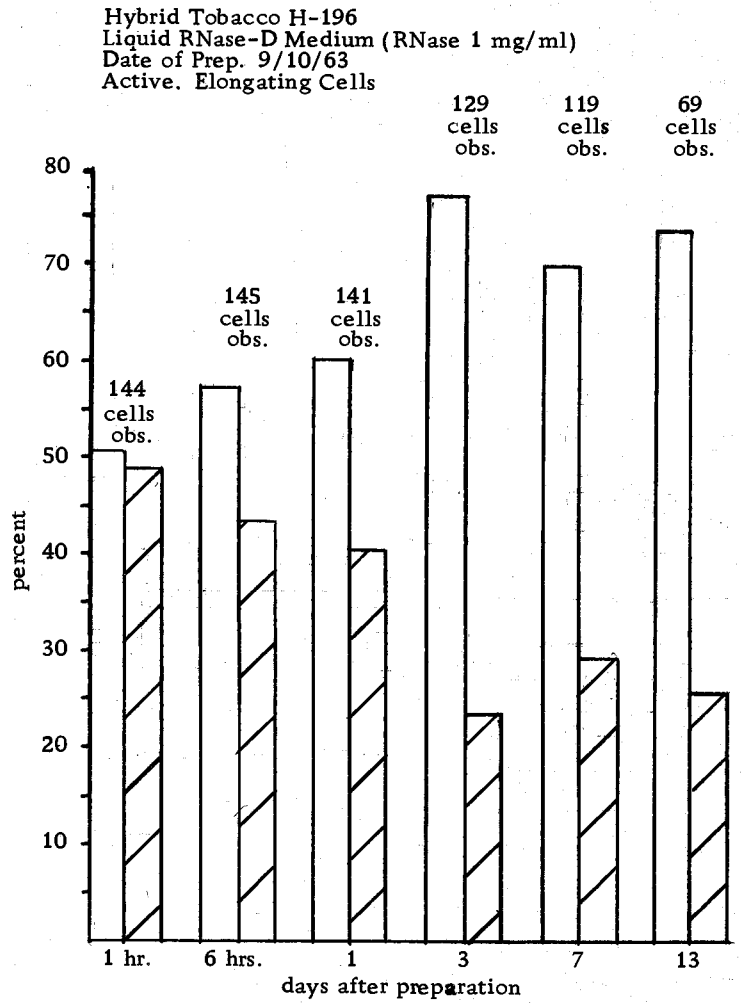


Figure 46. Occurrence of Nucleolar Vacuoles in RNase-D Medium

□ = cells with nucleolar vacuoles

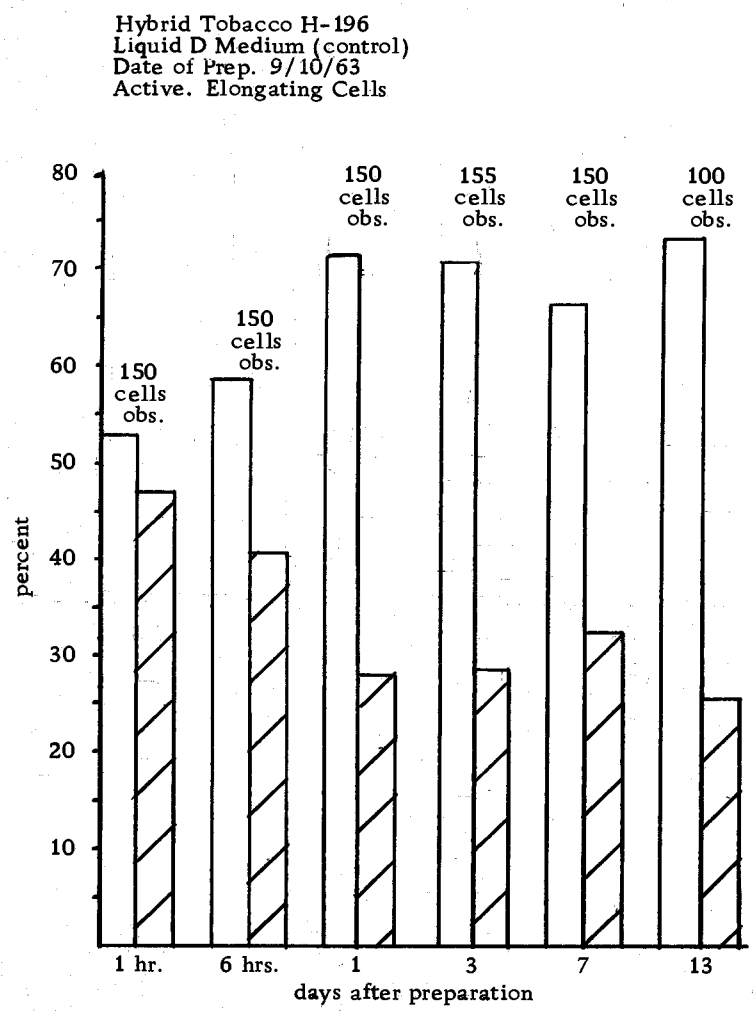


Figure 47. Occurrence of Nucleolar Vacuoles in D Medium

▨ = cells without nucleolar vacuoles

The number of observations when possible was based on the first 15 cells of this type observed per microculture in 10 different microcultures for both the RNase treated and control cells.

by McDonald (52, p. 228). After 14 days there was little or no decrease in the activity of the RNase stored under the conditions described (Figure 48).

Further RNase experiments were conducted with tobacco cells. In these experiments H-196 tobacco cells were placed in prescription bottles containing 25 ml of liquid medium. These cultures were then grown on a reciprocating shaker. Three categories were used in this experiment: (a) RNase-D (1.0 mg/ml RNase) with tobacco cells, (b) RNase-D (1.0 mg/ml RNase) without tobacco cells, and (c) standard D medium with tobacco cells as a control. The bottles of RNase-D medium without cells were used as a check to see if the cells inactivated the RNase. Periodically, these different types of samples were checked for living cells, and each medium was tested for RNase activity. The results from these experiments were similar to those obtained from the microculture experiments. After seven days many living cells were found in samples from the RNase treated material; one cell was observed to be dividing. Nucleolar vacuoles and nucleoli showed no marked morphological differences when compared with those in cells growing in the standard D medium. Nucleolar vacuoles within cells from the RNase treated material were observed to contract. By 13 days, however, no living cells could be found in samples taken from the RNase-D medium. At this time a comparison of dry weights was made between one bottle of RNase-D treated material and a comparable sample growing in D medium. The RNase treated material had a

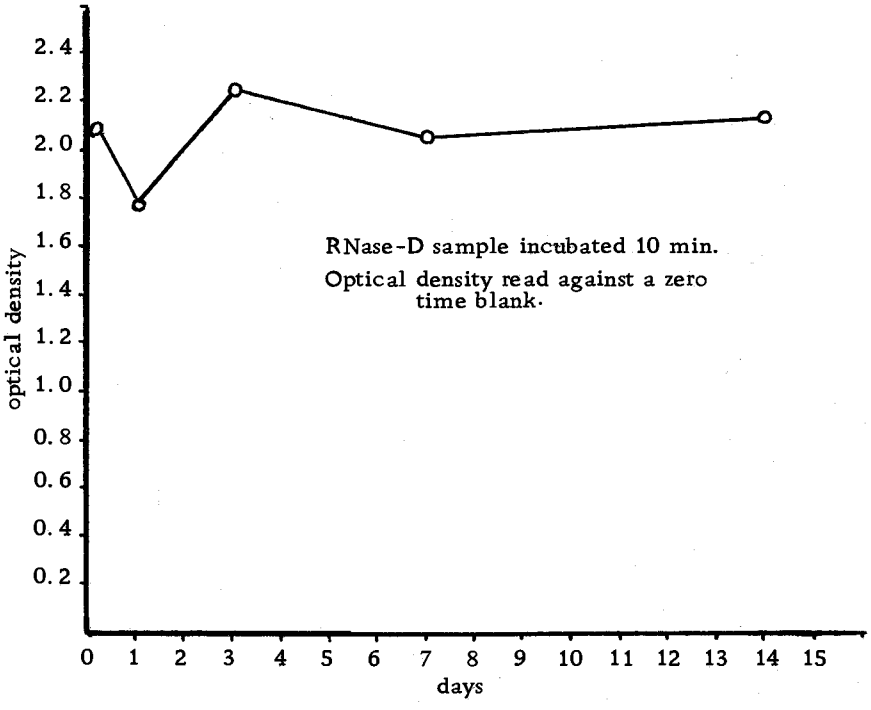


Figure 48. RNase Activity of RNase-D Medium

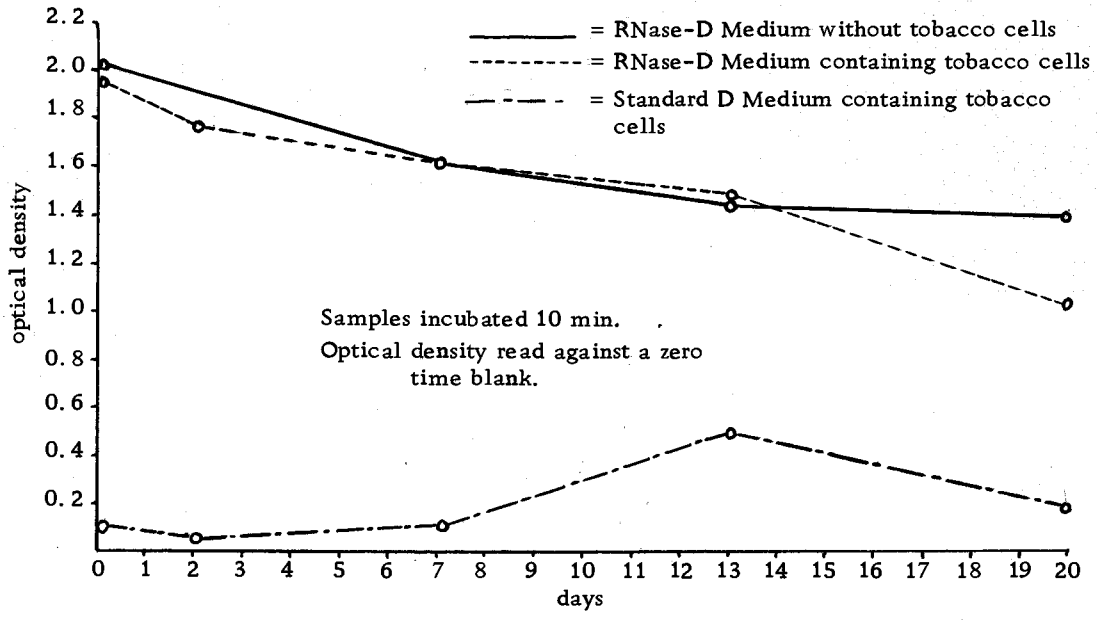


Figure 49. Comparison of RNase Activity in RNase-D Medium with tobacco cells, RNase-D Medium without tobacco cells and Standard D Medium containing tobacco cells.

dry weight of 66.5 mg; whereas, the cells growing in standard D medium had a dry weight of 259.5 mg. The results of periodic tests for RNase activity, during the 20 days in which the cells were grown on the reciprocating shaker, are shown in Figure 49, based on readings obtained from one bottle in each of the three categories. Similar results were obtained from three other sets of bottles sampled. The results in Figure 49 indicate that the cells have little effect on the RNase activity of the RNase-D medium. The RNase remained relatively active for up to 20 days and there was only a very slight amount of RNase activity in the standard D medium containing growing tobacco cells.

RNase experiments show that RNase (1.0 mg/ml) does not kill tobacco cells rapidly but may cause death of the treated cells within 13 to 20 days. Of some help in interpreting the results of these experiments are a series of papers by Brachet dealing with the effect of RNase on living onion root tips. Brachet (4, p. 876) found that crystalline RNase at 1.0 mg/ml inhibited the incorporation of glycine and phenylalanine by the onion roots. An inhibition of 50 percent was observed after one hour RNase treatment, and almost 90 percent after three hours of treatment. Brachet also reported that oxygen consumption was not effected by the RNase treatment, even after 15 hours of treatment. Organic phosphate, presumed to be adenosine triphosphate, showed a 30 percent increase during the first three hours of RNase treatment and thereafter remained constant. RNA content dropped 20 percent after

three hours of RNase treatment and remained at this level even after 18 hours. Soluble nucleotides increased, reaching a value 40-50 percent higher than the controls after 18 hours. In a 1955 paper, Brachet (5, p. 611-613) reported that the growth rate of living onion root tips was strongly inhibited by RNase (1.0 mg/ml). There was a 35 percent inhibition of growth after the first hour, a 77 percent for the second hour, and 88 percent for the third hour. He also noted that inhibition of growth produced by RNase could be overcome partially and temporarily by the addition of ribonucleic acid from yeast. In 1956 Brachet (6, p. 583) suggested that an RNA-RNase complex was formed when cells were first treated with RNase, followed by destruction of the RNA. In 1959 Brachet and Six (8, p. 580-581) stated that after 30 minutes of RNase treatment (1.0 mg/ml) 40 percent of the soluble RNA in living onion root cells was destroyed without exerting any significant effect upon the RNA of the other cell fractions.

The fact that cells did remain alive in the RNase-D medium (1.0 mg/ml) for 13 to 20 days may possibly be accounted for by assuming that the cells are capable of most normal processes except synthesis of proteins due to destruction of RNA by the RNase. Thus, the tobacco cells could remain alive until proteins essential for normal metabolism were broken down. Some support for this suggestion is gained from Brachet (4, p. 876) who showed that RNase treatment did not effect oxygen consumption in onion root tips, at least for the 15 hour duration of his study.

It was observed in the microcultures that cells injured while preparing the microcultures were among the first to die in the RNase-D medium. In microcultures with standard D medium many of the injured cells apparently recovered. In the RNase treated microcultures (1.0 mg/ml) no cells were seen either to divide or to enlarge and elongate; however, one cell in RNase-D treated bottle cultures was observed to divide. In the standard D microcultures mitosis and cell division, cell enlargement and cell elongation took place. These results agree with the findings of Brachet (5 p. 611-613 and 6, p. 583) in which he observed that growth and elongation were strongly inhibited in RNase treated root tips. The lack of morphological changes in the nucleolus and the nucleolar vacuoles in cells in the RNase-D medium are not readily explained.

DISCUSSION

The ability to grow tobacco cells in microcultures has presented a unique opportunity to study the behavior of nucleoli in living, growing plant cells. It was possible to watch a given cell for hours at a time, as well as to make intermittent observations over weeks of time. This study has revealed that contracting nucleolar vacuoles are a general and consistent feature of somatic tobacco cells of two single-cell clones, H-196 and H-239, of hybrid tobacco (Nicotiana tabacum X N. glutinosa). In actively growing tobacco cells nucleolar vacuoles were observed in about 70 percent of the cells examined; whereas, nucleolar vacuoles were present in only about 33 percent of the senescent or weakened cells (Table III). This difference in the occurrence of nucleolar vacuoles indicates a probable relationship between the occurrence of nucleolar vacuoles and the metabolic activity of the cells. These observations in tobacco cells are in agreement with the findings of Soudek and Stránská (68, p. 241-243) and Soudek (69, p. 447-452) in the fungus Basidiobolus ranarum, in which they showed a close correlation between growth rate of the cells in this fungus and the presence of nucleolar vacuoles.

The nucleolar vacuoles in tobacco cells periodically contracted either completely or incompletely, and, apparently, released a fluid material into the nucleus. Associated with the contraction of the nucleolar vacuole is a decrease in the diameter of the nucleolus (Tables I-II). This decrease during contraction indicated that material moved from the nucleolar vacuole into the nucleus. The absence of a stainable material within the nucleolar vacuoles after recommended killing and fixing procedures, the finding of collapsed vacuoles in killed and fixed cells (Figure 18), and the fact that no solid material could be seen to move from the nucleolar vacuole

into the nucleus indicated the fluid and soluble nature of the vacuolar contents. The presence of nucleolar vacuoles is not an indication of damage or physiological abnormality, because many tobacco cells containing nucleolar vacuoles were observed to undergo mitosis and cell division.

Nucleolar vacuoles were also observed in living cells from microcultures of incense cedar (Libocedrus decurrens), horse bean (Vicia faba), annual bristleweed (Haplopappus gracilis), carrot (Daucus carota var. sativa), potato (Solanum tuberosum), tomato (Lycopersicon esculentum), hollyhock (Althaea rosea), wheat (Triticum aestivum), and in living root hairs of lettuce (Lactuca sativa), as well as in germinating pollen grains of Douglas fir (Pseudotsuga menziesii). The contraction of nucleolar vacuoles was observed in cells of hollyhock, tomato, horse bean, and tobacco, Nicotiana glutinosa single-cell clone G-252, as well as in the two single-cell clones of hybrid tobacco H-196 and H-239, all growing in microcultures. It has not as yet been established whether the vacuoles in the nucleoli of other species also contract. Many reports of nucleolar vacuoles within the nucleoli of other organisms, both plant and animal, are to be found in the literature.

At present no definite function can be assigned to the contracting nucleolar vacuoles; however, circumstantial evidence from the literature suggests a possible function. The nucleolus by cytochemical and other chemical analyses has been shown to contain RNA (ribonucleic acid) (67 p. 31-32 and 77, p. 281-283). Sirlin (67, p. 32) stated that the total nucleolar RNA concentration was usually about five percent on a dry weight basis, but the RNA concentration of nucleoli has been reported by Brown and Ris (10, p. 398) to be as high as 23.83 percent in amphibian oocytes. Recently the

significance of RNA in protein synthesis has been established (63, p. 333-368, 53, p. 110-116, and 54, p. 69-106).

Autoradiographic studies have shown that radioactive RNA precursors first accumulate in the nucleolus and later move rapidly from the nucleolus into the cytoplasm (82, p. 309-318, 83, p. 133-134, 65, p. 35-38, and 75, p. 108). These workers reported the following general time-pattern of movement of the label. Five minutes to one hour after the addition of radioactive nucleotide, most of the label was found associated with the nucleolus while little label was associated with the chromosomes or cytoplasm. Forty-five minutes to two hours after label was added, most of the radioactivity usually was still associated with the nucleolus, some was associated with the chromosomes, and a trace of label was found in the cytoplasm. Heavy radioactivity in the cytoplasm did not appear until 1.5 to 8 hours after the addition of the labeled nucleotide. Perry (60, p. 216-220) and Perry and Errera (61, p. 24-29) also were able to show the dependence of cytoplasmic RNA upon the nucleolar RNA of HeLa cells in which the nucleoli were inactivated by an ultraviolet microbeam.

Vincent and Baltus (79, p. 19) presented evidence that labeled cytidine was added to preexisting RNA in the nucleolus of starfish oocytes, and they suggested that this labeled RNA might be soluble or transfer RNA. Birnsteil, et al. (1, p. 1578) have recently reported the establishment of the nucleolus as the primary site within pea nuclei for the methylation of transfer RNA. They also

presented some evidence to support the view that the nucleolus is the site of synthesis of transfer RNA. Sirlin (67, p. 39) summarized a discussion of nucleolar RNA as follows:

> To summarize, autoradiography and chemical studies show first that the rapid nRNA(1) turnover is mainly due to continuous turnover of nuRNA(2). Secondly, it is shown chemically that part of this nuRNA turnover represents terminal turnover of tRNA(3). Thirdly, autoradiography studies show, when the intracellular pools are small and permit rapid access of tracer, that nucleoli (and chromosomes) have slow and fast turnover components, the latter with a half life of less than two hours; two nuRNA's with different rates of turnover were also chemically demonstrated.

From the literature presented above there appears to be little doubt that the nucleolus is an active site of RNA metabolism and that RNA moves from the nucleolus into the cytoplasm. The mechanism, however, for the release of the RNA from the nucleoli was not established. Woods (83, p. 134-136) suggested that RNA diffused from the nucleolus to the cytoplasm, but he gave no further explanation. Soudek and Stránská (68, p. 242-243) and Soudek (69, p. 447-452) have shown that the contracting nucleolar vacuoles of Basidiobolus ranarum are closely correlated to growth of the cells and, therefore, to protein synthesis. Lesher (45, p. 577-585 and 46, p. 586-588) also noted in Drosophila salivary gland tissue that, with cessation of active secretion by the gland, there

(1) nRNA -- nuclear ribonucleic acid
(2) nuRNA -- nucleolar ribonucleic acid
(3) tRNA -- transfer ribonucleic acid

was an associated decrease in nucleolar size and a loss of the vacuoles within the nucleoli.

The present study revealed that there is a higher percentage of vacuoles in the nucleoli of young, active cells and in active, elongating cells than in senescent or weakened tobacco cells (Table III). The former types of cells would be expected to have a higher rate of protein synthesis than the latter. It has also been shown that the nucleolar vacuoles expand and contract repeatedly in the same tobacco cell, so there is an apparent release of some fluid material that has been accumulated in the nucleolar vacuoles. Figures 31-39 show these repeating processes. It is, therefore, suggested that the contracting nucleolar vacuoles may be involved in the controlled movement from the nucleolus into the cytoplasm of a soluble metabolic intermediate; this could be soluble or transfer RNA.

It is true that no autoradiographic or cytochemical evidence is available to prove that the above mechanism is operational in tobacco cells. One thing that could have caused the negative cytochemical reactions for RNA within the nucleolar vacuoles is that both transfer and messenger RNA are water soluble; hence, if either or both types of RNA were present in the nucleolar vacuoles, they would be lost during the preparation of standard cytochemical tests for RNA in killed and fixed samples. Woods (83, p. 133-134) pointed out that the processing of Vicia faba roots for autoradiographs caused the loss of more than one-third of the total

radioactivity of roots when the sections were passed through the lower alcohols to water. Swift (75, p. 110), when speaking of RNA localization in the cell, stated that small molecular-weight fractions (soluble RNA) are undoubtedly lost from tissues during autoradiographic and cytochemical determinations. He also suggested that methods to fix their position within cells during cytochemical studies are obviously needed.

Most indirect evidence, as well as observations reported in this study, make it reasonable to assume that RNA could be released from the nucleolus into the nucleus by the contracting nucleolar vacuoles. This RNA could then move from the nucleus into the cytoplasm through the pores in the nuclear envelope. Such pores have been demonstrated in cells of corn root tips (81, p. 425) and in Bradysia mycorum (Diptera) (31, p. 452).

If the nucleolar vacuoles do contain a soluble metabolic intermediate, such as transfer RNA, they could, by releasing a large quantity of soluble RNA, induce sudden changes in growth patterns. Such changes are characteristic of growing, differentiating plant cells. Thus, the contracting nucleolar vacuoles could serve as a timing mechanism for differentiation of cells during growth.

Other points concerning the nucleoli of living tobacco cells were also established in this study. In many instances the volume of the nucleolus decreased as the tobacco cells aged, became senescent and approached death. These results are in general agreement with the findings of Dermen (23, p. 306), who found

that the diameter of nucleoli in stamen hairs of Callisia decreased prior to death of the cell. Lesher (46, p. 586-588) reported a decrease in nucleolar volume when cells ceased to function in the salivary glands of Drosophila, and Soudek and Stránská (68, p. 241-243) showed that nucleoli in the fungus Basiodobolus ranarum decreased in size when the cells stopped growing.

Occasionally protuberances of solid nucleolar material were formed at the periphery of the nucleolus and released into the nucleus of tobacco cells. These observations were similar to those of González-Ramírez, et al. (27, p. 51-56) who reported the movement of solid material from the surface of nucleoli in living HeLa cells.

The number of nucleoli within the nuclei of living tobacco cells varied from 1 to as many as 30, but the usual number was from 1 to 6. In recently divided tobacco cells nucleoli were seen to fuse after telophase to form either one large nucleolus or a few nucleoli of intermediate size. These results are in agreement with the results reported by Swift (74, p. 270) for the cells of onion root tips. He reported that the nucleoli in onion root tip cells showed a tendency to fuse as interphase progressed.

In this study of living tobacco cells the number of nucleoli increased in some aging cells. Apparent fragmentation of nucleoli took place one to three days prior to the death of two cells observed.

SUMMARY

Single-cell clones H-196 and H-239 of hybrid tobacco (Nicotiana tabacum X N. glutinosa) were studied in microcultures. The following points were established concerning the nucleoli of these cells:

(a) The periodic formation and contraction of nucleolar vacuoles were found to be consistent and general features within the nucleoli of growing tobacco cells.

(b) Either complete and/or partial contraction of nucleolar vacuoles were observed in the majority of growing tobacco cells that were studied.

(c) The contents of nucleolar vacuoles appeared to be discharged into the nucleus when the vacuoles contracted. The inability to fix, hold and stain any substance within the nucleolar vacuoles in killed, fixed and stained preparations, and the fact that solid material was not observed to move from the vacuoles into the nucleus during contractions are evidence that the nucleolar vacuoles are filled with soluble, fluid materials.

(d) A decrease in the diameter of the entire nucleolus was associated with the contraction of the nucleolar vacuoles. This decrease in nucleolar diameter indicated that fluid material moved from the contracting nucleolar vacuole into the nucleus.

(e) A higher percentage of nucleolar vacuoles was found in actively growing cells than in senescent or weakened cells.

(f) The presence of nucleolar vacuoles was not an indication of damage or physiological abnormality, because many tobacco cells containing nucleolar vacuoles were observed to undergo mitosis and cell division.

(g) In many instances there was a decrease in total nucleolar volume as tobacco cells aged, became senescent and approached death.

(h) Occasionally, protuberances of solid material formed at the periphery of the nucleolus; these moved into the nucleus.

(i) The number of nucleoli within the nuclei of the tobacco cells varied from 1 to as many as 30, but usually there were from 1 to 6.

(j) In recently divided tobacco cells, numerous, small nucleoli were seen to fuse following telophase to form either one large nucleolus or a few nucleoli of intermediate size.

(k) In some instances the number of nucleoli increased as the cells became senescent. Apparent fragmentation of nucleoli just prior to the death of some cells was observed.

Evidence from this study and from reports in the literature indicate that nucleolar vacuoles are a normal component of nucleoli in cells of many organisms, both plant and animal. Evidence from the literature also indicates that the nucleolus is active in RNA (ribonucleic acid) metabolism and specifically in the metabolism of transfer RNA. On the basis of evidence obtained in this study

and from the indirect evidence in the literature, it is suggested that the contracting nucleolar vacuoles found in the living tobacco cells may be a mechanism for the controlled movement of a soluble metabolic intermediate, perhaps transfer RNA, from the nucleolus into the cytoplasm.

BIBLIOGRAPHY

1. Birnstiel, Max L., Erwin Fleissner and Ernest Borek. Nucleolus: a center of RNA methylation. Science 142:1577-1580. 1963.

2. Brachet, Jean. Étude histochimique des protéines au cours du développement embryonnaire des poissons des amphibiens et des oiseaux. Archives de Biologie 51:167-202. 1940.

3. _____ The use of basic dyes and ribonuclease for the cytochemical detection of ribonucleic acid. The Quarterly Journal of Microscopical Science, ser. 3, 94:1-10. 1953.

4. _____ Effects of ribonuclease on the metabolism of living root-tip cells. Nature 174:876-877. 1954.

5. _____ Further observations on the effects of ribonuclease on living root-tip cells. Biochimica et Biophysica Acta 16:611-613. 1955.

6. _____ The mode of action of ribonuclease on living root-tips. Biochimica et Biophysica Acta 19:583. 1956.

7. _____ Biochemical cytology. New York, Academic Press, 1957. 516 p.

8. Brachet, Jean and N. Six. New observations on the mode of action of ribonuclease on living root-tips. Biochimica et Biophysica Acta 35:580-581. 1959.

9. Brachet, Jean. Nucleocytoplasmic interactions in unicellular organisms. In: The cell, ed. by Jean Brachet and Alfred E. Mirsky. Vol. 2. New York, Academic Press, 1961. p. 771-841.

10. Brown, Charles A. and Hans Ris. Amphibian oocyte nucleoli. Journal of Morphology 104:377-402. 1959.

11. Busch, Harris, Paul Byvoet and Karel Smetana. The nucleolus of the cancer cell: a review. Cancer Research 23:313-339. 1963.

12. Caspersson, Torbjörn and Jack Schultz. Pentose nucleotides in the cytoplasm of growing tissues. Nature 143:602-603. 1939.

13. _____ Ribonucleic acids in both nucleus and cytoplasm, and the function of the nucleolus. Proceedings of the National Academy of Sciences 26:507-515. 1940.

14. Caspersson, Torbjörn. The relations between nucleic acid and protein synthesis. In: Nucleic acid. New York, Academic Press, 1947. p. 127-151. (Symposia of the Society for Experimental Biology, No. 1).

15. _____ Cell growth and cell function, a cytochemical study. New York, W. W. Norton, 1950. 185 p.

16. _____ Nucleo-cytoplasmic relations in normal and malignant cells. In: The molecular control of cellular activity, ed. by John M. Allen. New York, McGraw-Hill, 1962. p. 127-141.

17. Chayen, J. The structure of the root meristem cells of Vicia faba. In: Structural aspects of cell physiology. New York, Academic Press, 1952. p. 290-305. (Symposia of the Society for Experimental Biology, No. 6).

18. Chayen, J., H. G. Davies and Ursula J. Miles. Observations on some plant interphase nuclei. Proceedings of the Royal Society of London, ser. B, 141:190-199. 1953.

19. Cleland, Ralph E. The reduction divisions in the pollen mother cells of Oenothera franciscana. American Journal of Botany 9:391-413. 1922.

20. Conn, Harold Joel, Mary A. Darrow and Victor M. Emmel. Staining procedures used by the Biological Stain Commission. 2d ed. Baltimore, Williams and Wilkins, 1960. 289 p.

21. Coons, Albert H. The cytology of antibody formation. Journal of Cellular and Comparative Physiology 52 (suppl. 1):55-67. 1958.

22. Dalcq, A. La localisation cytochimique de l'adénosinetriphosphatase dans les oeufs des mammifères et sa relation avec leur organisation morphogénétique. Bulletin de l'Académie Royale de Medécine de Belgique 24:825-898. 1959.

23. Dermen, Haig. Origin and behavior of the nucleolus in plants. Journal of the Arnold Arboretum, Harvard University 14:282-323. 1933.

24. De Robertis, E. D. P., W. W. Nowinski and Francisco A. Saez. General cytology. Philadelphia, W. B. Saunders, 1960. 555 p.

25. Dutta, Chitta R., Kenneth A. Siegesmund and Clement A. Fox. Light and electron microscopic observations of an intranucleolar body in nerve cells. Journal of Ultrastructure Research 8:542-551. 1963.

26. Gates, R. Ruggles. Nucleoli and related nuclear structures. The Botanical Review 8:337-409. 1942.

27. González-Ramírez, Jorge, M. Zúñiga and B. A. Núñez. Nucleolar physiology. I. Passage of two different nucleolar components towards the cytoplasm in living cells. Boletín del Instituto de Estudios Médicos y Biológicos 17:51-57. 1959.

28. González-Ramírez, Jorge, Cesar Wong-Chia and Angelina Núñez-Galvan. Nucleolar physiology. II. Electron microscopy of nucleolar materials in HeLa cells. Boletín del Instituto de Estudios Médicos y Biológicos 18:153-161. 1960.

29. Hildebrandt, A. C., A. J. Riker and B. M. Duggar. The influence of the composition of the medium on growth in vitro of excised tobacco and sunflower tissue cultures. American Journal of Botany 33:591-597. 1946.

30. Höpker, W. Über den Nucleolus der Nervenzelle. Zeitschrift für Zellforschung und Mikroskopische Anatomie 38:218-229. 1953.

31. Jacob, J. and A. Jurand. Electron microscope studies on salivary gland cells. II. The nuclear envelope of Bradysia mycorum Frey (Sciaridae). Chromosoma 14:451-458. 1963.

32. Jensen, William August. Botanical histochemistry: principles and practice. San Francisco, W. H. Freeman, 1962. 408 p.

33. Johansen, Donald Alexander. Plant microtechnique. New York, McGraw-Hill, 1940. 523 p.

34. Johnson, J. Morris and Leo E. Jones. Cinemicrographic studies of mitosis and cell division in living somatic cells of tobacco in microcultures. (Abstract) Abstracts of Papers, Oregon Academy of Science, March, 1962. p. 4-5.

35. _____ Cinemicrographs from microcultures of living cells of seed plants. II. Contractile nucleolar vesicles and nucleolar budding; the changing status of the nucleus with age. (Abstract) Proceedings of the American Society of Cell Biologists, San Francisco, Nov. 5-7, 1962. p. 81.

36. _____ Phase-contrast observations of the nucleus and nucleoli during growth and aging of tobacco cells in microculture. (Abstract) American Journal of Botany 49(6, pt. 2):660. 1962.

37. Jones, L. E., A. C. Hildebrandt, A. J. Riker and J. H. Wu. Growth of somatic tobacco cells in microculture. American Journal of Botany 47:468-475. 1960.

38. Jones, Leo E. and J. Morris Johnson. Phase-contrast cinemicrographs of living protoplasm during the growth of somatic tobacco cells in microcultures. (Abstract) Abstracts of Papers, Oregon Academy of Science, March, 1962. p. 4.

39. _____ Cinemicrographs from microcultures of living cells of seed plants. I. Cytokinesis and changes in organelles and cytoplasmic vesicles from the meristematic condition, through senescence, to death. (Abstract) Proceedings of the American Society of Cell Biologists, San Francisco, Nov. 5-7, 1962. p. 82.

40. Jones, L. E., J. M. Johnson and Ruth M. Pickering. Phase-contrast motion pictures of the cytoplasm and organelles during growth and aging of tobacco cells in microculture. (Abstract) American Journal of Botany 49(6, pt. 2):660-661. 1962.

41. Jörgensen, Max. Zellenstudien. I. Morphologische Beiträge zum Problem des Eiwachstums. Archiv für Zellforschung 10:1-126. 1913.

42. Lafontaine, J. G. Structure and mode of formation of the nucleolus in meristematic cells of Vicia faba and Allium cepa. Journal of Biophysical and Biochemical Cytology 4:777-784. 1958.

43. Lafontaine, J. G. and L. A. Chouinard. A correlated light and electron microscope study of nucleolar material during mitosis in Vicia faba. The Journal of Cell Biology 17:167-201. 1963.

44. Latter, Joan. The pollen development of Lathyrus odoratus. Annals of Botany 40:277-314. 1926.

45. Lesher, S. Studies on the larval salivary gland of Drosophila. I. The nucleic acids. Experimental Cell Research 2:577-585. 1951.

46. _____ Studies on the salivary gland of Drosophila. II. Changes in nuclear and nucleolar volumes and their possible significance. Experimental Cell Research 2:586-588. 1951.

47. Lewis, Warren H. Nucleolar vacuoles in living normal and malignant fibroblasts. Cancer Research 3:521-536. 1943.

48. Lowman, F. G. Electron-microscope studies of Drosophila salivary-gland chromosomes. Chromosoma 8:30-52. 1956.

49. Ludford, Reginald James. The morphology and physiology of the nucleolus. Journal of the Royal Microscopical Society, 1922, p. 113-150.

50. Maggio, Rachele, Philip Siekevitz and George E. Palade. Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic subfractions. The Journal of Cell Biology 18:293-312. 1963.

51. Mazia, Daniel, Philip A. Brewer and Max Alfert. The cytochemical staining and measurement of protein with mercuric bromphenol blue. The Biological Bulletin 104:57-67. 1953.

52. McDonald, Margaret R. Ribonucleases. In: Methods in enzymology, ed. by Sydney Paul Colowick. Vol. 2. New York, Academic Press, 1955. p. 427-436.

53. McElroy, William D. Cellular physiology and biochemistry. Englewood Cliffs, New Jersey, Prentice-Hall, 1961. 120 p.

54. McQuillen, Kenneth. Ribosomes and the synthesis of proteins. In: Progress in biophysics and biophysical chemistry, ed. by J. A. V. Butler, H. E. Huxley and R. E. Zirkle. Vol. 12. New York, Pergamon Press, 1962. p. 69-106.

55. Mensinkai, S. W. The conception of the satellite and the nucleolus and the behavior of these bodies in cell division. Annals of Botany, new ser., 3:763-794. 1939.

56. Montgomery, Thomas H., Jr. Comparative cytological studies, with especial regard to the morphology of the nucleolus. Journal of Morphology 15:265-582. 1898.

57. Muir, W. H., A. C. Hildebrandt and A. J. Riker. Plant tissue cultures produced from single isolated cells. Science 119:877-878. 1954.

58. _____ The preparation, isolation, and growth in culture of single cells from higher plants. American Journal of Botany 45:589-597. 1958.

59. Mulnard, Jacques. Présence d'inclusions feulgen-positives dans les nucléoles larvaires de quelques Diptères. Archives de Biologie 67:485-498. 1956.

60. Perry, R. P. On the nucleolar and nuclear dependence of cytoplasmic RNA synthesis in HeLa cells. Experimental Cell Research 20:216-220. 1960.

61. Perry, R. P. and M. Errera. The influence of nucleolar ribonucleic acid metabolism on that of the nucleus and cytoplams. In: The cell nucleus, ed. by J. S. Mitchell. New York, Academic Press, 1960. p. 24-29.

62. Raven, Chr. P. Morphogenesis: the analysis of molluscan development. New York, Pergamon Press, 1958. 311 p.

63. Simpson, M. V. Protein biosynthesis. Annual Review of Biochemistry 31:333-368. 1962.

64. Sirlin, J. L. Autoradiography of nucleolar contents. Experimental Cell Research 14:447-453. 1958.

65. _____ Facts and speculation on the function of nuclear components. In: The cell nucleus, ed. by J. S. Mitchell. New York, Academic Press, 1960. p. 35-48.

66. _____ The nucleolus of the cell nucleus. Endeavor 20:146-153. 1961.

67. _____ The nucleolus. In: Progress in biophysics and biophysical chemistry, ed. by J. A. V. Butler, H. E. Huxley and R. E. Zirkle. Vol. 12. New York, Pergamon Press, 1962. p. 25-66.

68. Soudek, D. and E. Stránská. Manifestations of growth activity in the nucleolus of Basidiobolus ranarum Eidam. Folia Biologica 4:241-243. 1958.

69. Soudek, D. The correlation between the growth of the cell and nucleolar secretion in Basidiobolus ranarum Eidam. Experimental Cell Research 20:447-452. 1960.

70. Stich, Hans. Experimentelle karyologische und cytochemische Untersuchungen an Acetabularia mediterranea. Zeitschrift für Naturforschung, ser. B, 6:319-326. 1951.

71. _____ Bau und Funktion der Nukleolen. Experientia 12:7-14. 1956.

72. Stockinger, L. Das Kernkörperchen. Protoplasma 42:365-413. 1953.

73. Swift, Hewson. Cytochemical techniques for nucleic acids. In: The nucleic acids, ed. by Erwin Chargaff and J. N. Davidson. Vol. 2. New York, Academic Press, 1955. p. 51-92.

74. _____ Studies on nucleolar function. In: A symposium on molecular biology, ed. by Raymond E. Zirkle. Chicago, University of Chicago Press, 1959. p. 266-303.

75. _____ Nucleic acids and cell morphology in Dipteran salivary glands. In: The molecular control of cellular activity, ed. by John M. Allen. New York, McGraw-Hill, 1962. p. 73-125.

76. Vincent, W. S. and A. H. Huxley. The dry matter content of starfish oocyte nucleoli. (Abstract) The Biological Bulletin 107:290. 1954.

77. Vincent, W. S. Structure and chemistry of nucleoli. International Review of Cytology 4:269-298. 1955.

78. Vincent, W. S. and Elyane Baltus. A function for the nucleolus. (Abstract) The Biological Bulletin 119:299-300. 1960.

79. _____ The ribonucleic acids of nucleoli. In: The cell nucleus, ed. by J. S. Mitchell. New York, Academic Press, 1960. p. 18-23.

80. Vogt, Cécile and Oskar Vogt. Eine neurohistologische Beleuchtung der Nucleusfunktion. Biologisches Zentralblatt 65:61-69. 1946.

81. Whaley, W. G., H. H. Mollenhauer and J. H. Leech. The ultrastructure of the meristematic cell. American Journal of Botany 47:401-449. 1960.

82. Woods, Philip S. and J. Herbert Taylor. Studies of ribonucleic acid metabolism with tritium-labeled cytidine. Laboratory Investigation 8:309-318. 1959.

83. Woods, Philip S. Autoradiographic studies of ribonucleic acid metabolism with tritium-labeled cytidine. In: The cell nucleus, ed. by J. S. Mitchell. New York, Academic Press, 1960. p. 127-137.

84. Zirkle, Conway. Nucleoli of the root tip and cambium of Pinus strobus. Cytologia 2:85-105. 1931.

APPENDIX

Appendix Table I
Changes in Volume of Nucleoli and Vacuoles until Death of Cell

Material
Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/21/63 - 7/31/63
Microculture: 1

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	151	8	143
1	195	19	177
2	195	14	181
3	221	58	163
4	310	39	272
6	345	82	262
8	82	19	64
9	39	0	39
10	CELL IS DEAD		

Material
Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/18/63 - 7/29/63
Microculture: 2

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	82	31	51
3	113	0.61	112
4	69	0.0	69
5	69	5	65
6	39	0.9	38
7	24	0.0	24
9	10	0.0	10
11	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/16/63 - 8/2/63
Microculture: 7

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	310	172	138
2	508	221	287
3	905	195	710
5	24	0.0	24
8	39	14	25
9	39	14	25
11	69	39	30
13	48	7	40
14	48	3	45
15	39	5	34
16	14	0.0	14
17	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/18/63 - 7/31/63
Microculture: 8

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	151	24	126
1	113	0.0	113
3	131	58	73
6	82	11	71
7	58	0.0	58
9	58	0.0	58
11	19	0.0	19
13	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/17/63 - 8/10/63
Microculture: 9

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	58	0.0	58
1	82	10	72
2	113	31	82
4	58	0.113	58
7	82	5	77
8	113	3	110
10	195	39	157
12	195	58	138
13	195	58	138
15	82	14	68
16	69	0.0	69
17	97	0.0	97
19	31	0.0	31
21	5	0.0	5
24	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/17/63 - 8/2/63
Microculture: 10

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	113	19	94
1	113	19	94
2	131	0.0	131
4	195	24	171
5	195	7	188
7	151	58	93
8	113	0.0	113
10	195	69	126
12	131	39	92

13	82	0.0	82
14	31	0.0	31
15	18	0.0	18
16	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/17/63 - 8/5/63
Microculture: 11

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	113	31	82
1	113	14	99
2	69	0.113	69
4	39	7	32
7	131	58	73
8	131	14	117
10	195	82	113
12	151	24	126
13	97	19	78
14	113	39	74
15	24	0.0	24
17	14	0.0	14
19	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 6/21/63
Dates Observed: 7/5/63 - 7/17/63
Microculture: 14

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0	524	268	256
	239	34	206
	382	82	300
1	382	82	300
2	382	82	300

3	195	10	185
4	382	131	251
5	524	268	266
6	131	3	128
7	131	10	121
12	CELL IS DEAD		

Material

Cell from: Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Dates Observed: 7/19/63 - 7/31/63
Microculture: 10

days after first observation	volume nucleolus μ^3	volume vacuole μ^3	net. volume nucleolus μ^3
0 (2 nucleoli)	69	24	45
	58	39	19 = 64
1 (1 nucleolus)*	716	310	406
2	195	58	137
5	113	0.0	113
6	97	0.0	97
7	69	0.0	69
10	69	0.0	69
12	CELL IS DEAD		

* original nucleoli fused

Appendix Table II
Occurrence of Nucleolar Vacuoles
(Young. Active Cells)

	Large Vacuoles		Small Vacuoles		Total Large and Small Vacuoles		Without Vacuoles		Total No. of Cells	Date of Observation
	no.	%	no.	%	no.	%	no.	%		1963
	8	13	11	19	19	32	40	68	59	8/5"
H-196	5	4	61	55	66	59	46	41	112	8/6
D Medium	47	32	58	39	105	71	42	29	147	8/8
8/5/63	49	34	54	37	103	71	42	29	145	8/12
Series B	44	34	56	44	100	78	28	22	128	8/17
	33	27	43	36	76	63	45	37	121	8/25
	9	6	36	26	45	32	94	68	139	8/5"
H-196	13	10	53	40	66	50	67	50	133	8/6
D Medium	56	27	84	41	140	69	64	31	204	8/8
8/5/63	51	37	48	35	99	72	38	28	137	8/12
Series C	41	32	53	43	94	75	32	25	126	8/17
	32	23	61	45	93	68	43	32	136	8/25
	6	6	30	30	36	36	64	64	100	8/23"'
H-196	19	19	58	58	77	77	23	23	100	8/24
D Medium	22	22	52	52	74	74	26	26	100	8/26
8/23/63	21	26	43	52	64	78	18	22	82	8/30
	20	25	40	51	60	76	19	24	79	9/4
	7	19	16	43	23	62	14	38	37	9/12
H-196	9	6	78	52	87	58	63	42	150	9/10'
D Medium	7	5	79	53	86	57	64	43	150	9/10"'
9/10/63	19	14	69	50	88	63	51	37	139	9/11
	12	15	37	46	49	61	31	39	80	9/13
Totals	530		1120		1650		954		2604	
Average %		20		43		63		37		

' 1 hr. after preparation
" 2-4 hrs. after preparation
"' 5-6 hrs. after preparation

Appendix Table III
Occurrence of Nucleolar Vacuoles
(Active. Elongating Cells)

	Large Vacuoles		Small Vacuoles		Total Large and Small Vacuoles		Without Vacuoles		Total No. of Cells	Date of Observation 1963
	no.	%	no.	%	no.	%	no.	%		
	39	10	72	18	111	28	283	72	394	8/5
H-196	47	10	241	50	288	59	197	41	485	8/6
D Medium	30	19	81	51	111	69	49	31	160	8/8
8/5/63	44	28	74	46	118	74	42	26	160	8/12
Series B	48	30	74	46	122	76	38	24	160	8/17
	36	23	79	49	115	72	45	28	160	8/25
	17	9	48	25	65	34	124	66	189	8/5
H-196	20	10	81	42	101	52	93	48	194	8/6
D Medium	51	22	116	50	167	72	66	28	233	8/8
8/5/63	68	25	149	56	217	81	50	19	267	8/12
Series C	71	23	165	53	236	76	76	24	312	8/17
	59	30	91	46	150	76	48	24	198	8/25
	16	6	92	37	108	43	142	57	250	8/23
H-196	42	17	131	52	173	69	77	31	250	8/24
D Medium	36	14	145	58	181	72	69	28	250	8/26
8/23/63	52	23	107	48	159	71	66	29	225	8/30
	61	27	109	48	170	76	55	24	225	9/4
	49	22	106	48	155	70	65	30	220	9/12
	12	8	68	45	80	53	70	47	150	9/10'
	4	3	85	57	89	59	61	41	150	9/10'''
	22	15	86	57	108	72	42	28	150	9/11
H-196	33	21	77	50	110	71	45	29	155	9/13
D Medium	27	18	74	49	101	67	49	33	150	9/17
9/10/63	22	22	52	52	74	74	26	26	100	9/23
	26	17	75	50	101	67	49	33	150	9/30
	24	17	72	51	96	69	44	31	140	10/7
	21	15	77	55	98	70	43	30	141	10/14
Totals	977		2627		3604		2014		5618	
Average %		17		47		64		36		

' 1 hr. after preparation
''' 5-6 hrs. after preparation

Appendix Table IV
Occurrence of Nucleolar Vacuoles
(Senescent or Cells in Weakened Condition)

	Large Vacuoles		Small Vacuoles		Total Large and Small Vacuoles		Without Vacuoles		Total No. of Cells	Date of Observation
	no.	%	no.	%	no.	%	no.	%		1963
H-196 D Medium 6/20/63	20	11	47	25	67	36	121	64	188	8/7
	20	10	35	18	55	28	140	72	195	8/5
H-196	17	11	80	51	97	62	60	38	157	8/6
D Medium	13	15	20	22	33	37	56	63	89	8/8
8/5/63	6	6	31	31	37	37	63	63	100	8/12
Series B	11	11	24	24	35	35	65	65	100	8/17
	11	11	19	19	30	31	68	69	98	8/25
	0	--	11	37	11	37	19	63	30	8/5
H-196	5	6	4	5	9	11	76	89	85	8/6
D Medium	9	6	24	16	33	22	114	78	147	8/8
8/5/63	16	10	40	26	56	36	99	64	155	8/12
Series C	29	14	61	29	90	43	119	57	209	8/17
	6	9	14	22	20	31	45	69	65	8/25
H-196	14	16	13	14	27	30	63	70	90	9/4
D Medium 8/23/63	6	9	9	14	15	23	50	77	65	9/12
	2	4	6	13	8	17	39	83	47	9/10'
	0	--	8	13	8	13	56	87	64	9/10"'
H-196	4	4	20	20	24	24	74	76	98	9/11
D Medium	2	3	12	16	14	19	59	81	73	9/13
9/10/63	14	8	40	24	54	32	113	68	167	9/17
	6	7	21	24	27	30	62	70	89	9/23
	3	4	11	17	14	21	52	79	66	9/30
Totals	214		550		764		1613		2377	
Average %		9		23		32		68		

' 1 hr. after preparation
"' 5-6 hrs. after preparation

Appendix Table V
Changes in Diameter and Volume of Nucleoli and
Nucleolar Vacuoles as Observed at 15 min. Intervals

Material
Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 7/21/63
Cell Type: Young. Active
Microculture: 2 (cell A)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
6.0	113	4.2	39	
6.3	131	4.5	48	15 min.
5.1	70	2.4	7	30 min.
5.7	97	3.3	19	45 min.
6.0	113	4.5	48	1 hr.
4.8	58	0.9	0.4	15 min.
		0.6 (2)	0.2	
4.8	58	0.9	0.4	30 min.
		0.6 (4)	0.45	
4.8	58	0.6	0.11	45 min.
4.8	58	0.0	0.0	2 hr. to 6 hr.
4.8	58	0.0	0.0	15 min.
4.8	58	0.0	0.0	30 min.
4.5	48	0.0	0.0	45 min.
4.5	48	0.0	0.0	7 hr. to 8 hr.
4.5	48	0.0	0.0	45 min.

Material

Hybrid Tobacco H-239
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 7/21/63
Cell Type: Active, Elongating
Microculture: 2 (cell B)

nucleolus diameter μ	nucleolus volume μ3	vacuole diameter μ	vacuole volume μ3	time of observation
7.8	249	4.5	48	
6.9	172	3.0	14	15 min.
6.9	172	4.5	48	30 min.
7.2	195	4.8	58	45 min.
6.3	131	2.4	7	1 hr.
6.9	172	3.8	28	15 min.
7.2	195	4.8	58	30 min.
6.9	172	3.6	24	45 min.
7.0	180	4.2	39	2 hr.
6.5	144	2.7	10	15 min.
6.6	150	3.5	22	30 min.
6.9	172	4.2	39	45 min.
6.3	131	3.3	19	3 hr.
6.6	150	3.6	24	15 min.
6.3	131	1.8	3	19 min.
6.3	131	3.0	14	30 min.
6.1	118	2.4	7	45 min.
6.3	131	0.6 (6)	0.7	4 hr.
6.6	150	3.3	19	15 min.
6.3	131	2.1	5	30 min.
6.0	113	1.5		45 min.
		0.6	1.9	
6.3	131	1.2		5 hr.
		0.6	1.0	
6.0	113	0.0	0.0	15 min.
6.0	113	0.0	0.0	30 min.
6.3	131	1.2		45 min.
		0.6	1.0	
6.3	131	1.2	0.9	6 hr.
6.3	131	1.5		15 min.
		1.8		
		0.9	5	
6.3	131	1.2		30 min.
		0.6	1.0	
6.0	113	1.2		45 min.
		0.6	1.0	
6.3	131	2.8	3	7 hr.

6.3	131	1.2		15 min.
		0.9		
		0.6	1.4	
6.3	131	1.2 (2)	1.6	30 min.
6.3	131	1.5	1.8	45 min.
6.3	131	1.2	0.9	8 hr.
6.3	131	0.6	0.11	15 min.
6.3	131	0.6	0.11	30 min.
6.3	131	0.9	0.4	45 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 7/29/63
Cell Type: Active, Elongating
Microculture: 6 (cell A)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
6.6	150	3.6	24	
6.6	150	3.9	31	15 min.
6.6	150	3.9	31	30 min.
7.0	180	4.8	58	45 min.
7.5	221	5.1	70	1 hr.
7.65	235	4.8	58	15 min.
6.9	172	0.6 (2)		30 min.
		0.9	0.4	
6.9	172	2.1		45 min.
		0.6		
		1.2	6	
6.9	172	3.6	24	2 hr.
7.65	235	3.9	31	15 min.
7.5	221	4.5	48	30 min.
7.0	180	5.4	82	45 min.
7.65	235	5.4	82	3 hr.
7.0	180	1.2		15 min.
		0.6	1.0	
6.9	172	3.0	14	30 min.
7.2	195	3.3	19	45 min.
7.0	180	3.3	19	4 hr.
6.75	161	0.6	0.11	15 min.
6.9	172	3.3	19	30 min.
7.5	221	3.3	19	45 min.
6.3	131	3.6	24	5 hr.
6.9	172	3.6	24	15 min.
6.3	131	0.0	0.0	16 min.

6.75	161	2.4	7	30 min.
7.2	195	3.0	14	45 min.
7.0	180	0.9		6 hr.
		3.6	48	
6.0	113	0.0	0.0	1 min.
6.0	113	1.8		15 min.
		0.6 (2)	3	
6.9	172	3.0	14	30 min.
7.0	180	3.3	19	45 min.
6.9	172	3.9	31	7 hr.
6.9	172	4.5	48	15 min.
5.7	97	2.1	5	30 min.
6.3	131	3.3	19	45 min.
6.6	146	3.6	24	8 hr.
6.9	172	3.6	24	15 min.
7.0	180	4.5	48	30 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 7/29/63
Cell Type: Young. Active
Microculture: 6 (cell B)

nucleolus diameter μ	nucleolus volume μ3	vacuole diameter μ	vacuole volume μ3	time of observation
3.9	31	2.7	10	
4.5	48	3.0	14	15 min.
4.2	39	3.0	14	30 min.
4.2	39	3.3	19	45 min.
3.9	31	1.8	3	1 hr.
4.2	39	2.7	10	15 min.
3.9	31	1.5	2	30 min.
3.3	19	0.0	0.0	45 min.
3.6	24.5	1.2	0.89	2 hr.
3.6	24.5	0.6		15 min.
		0.9	0.5	
3.6	24.5	0.0	0.0	30 min.
3.6	24.5	0.0	0.0	45 min.
3.6	24.5	0.0	0.0	3 hr.
3.6	24.5	0.0	0.0	15 min.
3.3	19	0.0	0.0	30 min.
3.6	24.5	0.0	0.0	45 min.
3.3	19	0.0	0.0	4 hr.
3.6	24.5	0.0	0.0	15 min.
3.6	24.5	0.0	0.0	30 min.

3.6	24.5	0.0	0.0	45 min.
3.6	24.5	0.0	0.0	5 hr.
3.6	24.5	0.6	0.11	15 min.
3.6	24.5	0.0	0.0	30 min.
3.6	24.5	0.6	0.11	45 min.
3.3	19	0.0	0.0	6 hr.
3.3	19	0.0	0.0	15 min.
not visible				30 min.
3.3	19	0.0	0.0	45 min.
3.3	19	0.0	0.0	7 hr.
3.3	19	0.0	0.0	15 min.
not visible				30 min.
3.3	19	0.0	0.0	45 min.
3.0	14	0.0	0.0	8 hr.
3.0	14	0.0	0.0	15 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 7/29/63
Cell Type: Active. Elongating
Microculture: 6 (cell C)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
6.6	150	1.2		
		0.6		
		1.8	4	
6.0	113	1.2		15 min.
		1.5	2.7	
6.6	150	1.2 (3)	2.7	30 min.
6.3	131	0.9	0.4	45 min.
6.3	131	0.6 (3)		1 hr.
		1.2		
		1.5	3	
6.3	131	0.6 (2)		15 min.
		1.2 (2)	1.8	
6.3	131	0.6		30 min.
		0.9		
		1.8	3.6	
6.0	113	0.6		45 min.
		1.2 (3)	2.8	
6.0	113	0.6 (2)		2 hr.
		1.5	2	
6.0	113	0.9	0.4	15 min.

6.0	113	0.6		30 min.
		1.2	1.0	
6.0	113	0.6		45 min.
		1.2	1.0	
6.0	113	0.9		55 min.
		1.2	1.3	
6.3	131	0.6 (2)		3 hr.
		2.4	7.5	
6.0	113	0.6 (2)		15 min.
		1.2	1.1	
6.3	131	1.2	0.9	30 min.
6.3	131	0.9		45 min.
		1.8	3.5	
6.3	131	1.8	3	4 hr.
6.3	131	2.4	7	15 min.
7.2	195	3.6	24	30 min.
7.2	195	4.2	39	45 min.
6.9	172	4.2	39	5 hr.
6.9	172	4.8	58	15 min.
6.9	172	5.1	70	30 min.
6.9	172	6.0	113	45 min.
7.2	195	6.0	113	6 hr.
6.0	113	1.2	0.9	13 min.
6.0	113	1.8	3	15 min.
6.0	113	1.2	0.9	30 min.
6.6	150	1.8	3	45 min.
6.3	131	0.0	0.0	7 hr.
6.3	131	0.9	0.4	15 min.
6.3	131	0.9		30 min.
		0.6	0.5	
6.0	113	0.6 (2)	0.2	45 min.
6.3	131	0.6		8 hr.
		1.2	1.0	
5.85	105	1.8	3	15 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 9/7/63
Cell Type: Senescent
Microculture: 10 (cell A)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
5.7	97	0.0	0.0	
5.55	89.5	0.0	0.0	15 min.
6.0	113	0.6	0.11	30 min.

6.0	113	0.6	0.11	45 min.
6.0	113	0.0	0.0	1 hr.
5.7	97	0.6 (2)	0.22	15 min.
5.7	97	0.0	0.0	30 min.
6.0	113	0.9	0.38	45 min.
6.0	113	0.0	0.0	2 hr.
6.0	113	0.0	0.0	15 min.
6.0	113	0.0	0.0	30 min.
6.0	113	0.0	0.0	45 min.
5.85	105	0.0	0.0	3 hr.
5.85	105	0.0	0.0	15 min.
5.7	97	0.0	0.0	30 min.
5.7	97	0.0	0.0	45 min.
6.0	113	0.0	0.0	4 hr.
6.0	113	0.0	0.0	15 min.
6.0	113	0.0	0.0	30 min.
5.55	89.5	0.6	0.11	45 min.
5.55	89.5	0.0	0.0	5 hr.
5.7	97	0.0	0.0	15 min.
5.55	89.5	0.0	0.0	30 min.
5.55	89.5	0.0	0.0	45 min.
5.55	89.5	0.0	0.0	6 hr.
5.55	89.5	0.0	0.0	15 min.
5.55	89.5	0.0	0.0	30 min.
5.7	97	0.0	0.0	45 min.
5.7	97	0.0	0.0	7 hr.
5.7	97	0.0	0.0	15 min.
5.85	105	0.0	0.0	30 min.
5.55	89.5	0.0	0.0	45 min.
6.0	113	0.6	0.11	8 hr.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 9/7/63
Cell Type: Young. Active
Microculture: 10 (cell B)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
10.2	555.5	8.7	345	
7.95	263	3.6	24	15 min.
7.8	249	0.9		30 min.
		3.6	25	
7.8	249	1.2 (2)		45 min.
		1.5		
		4.35	50	

8.1	278	2.4		1 hr.
		4.5	55	
8.4	310.5	5.4	82	15 min.
8.4	310.5	5.4	82	30 min.
8.1	278	5.85	105	45 min.
8.55	327	6.0	113	2 hr.
8.7	345	6.3	131	15 min.
9.0	382	6.9	172	30 min.
9.3	421	7.0	180	45 min.
9.0	382	7.2	195	3 hr.
9.3	421	7.2	195	15 min.
9.0	382	7.2	195	30 min.
9.6	463	7.8	249	45 min.
9.6	463	7.8	249	4 hr.
10.2	555.5	8.1	278	15 min.
9.9	508	7.2	195	30 min.
9.9	508	7.5	221	45 min.
9.9	508	7.5	221	5 hr.
9.9	508	7.8	249	15 min.
9.6	463	7.5	221	30 min.
9.9	508	7.8	249	45 min.
9.45	442	7.65	235	6 hr.
9.6	463	8.1	278	15 min.
9.6	463	8.1	278	30 min.
9.9	508	8.1	278	45 min.
10.2	555.5	9.0	382	7 hr.
10.2	555.5	8.7	345	15 min.
10.2	555.5	8.7	345	30 min.
10.2	555.5	8.7	345	45 min.
10.2	555.5	8.4	310	8 hr.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 10/4/63
Cell Type: Young
Microculture: 3 (cell A)

nucleolus diameter μ	nucleolus volume μ³	vacuole diameter μ	vacuole volume μ³	time of observation
6.0	113	3.9	31	
6.0	113	3.9	31	15 min.
6.6	150	4.2	39	30 min.
6.15	122	4.2	39	45 min.
6.45	140	4.5	48	1 hr.
6.45	140	4.5	48	15 min.

6.9	172	4.5	48	30 min.
6.9	172	4.5	48	45 min.
6.9	172	4.2	39	2 hr.
6.9	172	4.2	39	15 min.
6.75	161	4.8	58	30 min.
6.6	150	5.1	70	45 min.
6.9	172	4.8	58	3 hr.
6.9	172	5.1	70	15 min.
6.9	172	5.1	70	30 min.
6.9	172	5.25	76	45 min.
6.9	172	5.1	70	4 hr.
6.9	172	5.1	70	15 min.
6.9	172	5.1	70	30 min.
7.5	221	5.7	97	45 min.
7.5	221	6.3	131	5 hr.
6.9	172	5.1	70	15 min.
7.05	180	5.4	82	30 min.
7.2	195	5.7	97	45 min.
7.8	249	5.7	97	6 hr.
7.5	221	5.7	97	15 min.
7.8	249	5.7	97	30 min.
7.5	221	5.7	97	45 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 10/4/63
Cell Type: Active. Elongating
Microculture: 3 (cell B)

nucleolus diameter μ	nucleolus volume μ3	vacuole diameter μ	vacuole volume μ3	time of observation
7.65	234.5	0.6 (5)		
		0.9 (5)	2.5	
7.95	263	0.6		15 min.
		0.9 (2)		
		1.2 (2)	3	
7.8	249	0.6		30 min.
		0.9 (2)		
		1.2		
		1.5		
		1.8 (2)	10	
7.8	249	0.6 (2)		45 min.
		1.2		
		2.4	8	
7.8	249	3.3		1 hr.
		0.9	19	

8.1	278	3.9		15 min.
		0.6 (2)	31	
8.1	278	4.2	39	30 min.
8.7	345	5.1	70	45 min.
9.0	382	4.95	63	2 hr.
7.5	221	0.6 (4)		15 min.
		1.2	1	
7.65	234.5	0.6 (2)		30 min.
		1.2		
		2.1	6	
8.1	278	0.9		45 min.
		1.5		
		3.0	16	
8.1	278	0.6 (2)		3 hr.
		2.4	7.5	
8.1	278	0.6 (2)		15 min.
		3.0	14	
8.1	278	3.6	24.5	30 min.
8.4	310.5	4.5	48	45 min.
7.8	249	0.9	0.38	4 hr.
7.8	249	0.6 (5)		15 min.
		1.5 (2)	4	
8.4	310.5	0.6 (3)		30 min.
		1.5		
		2.1	7	
8.4	310.5	4.8	58	45 min.
7.65	234.5	4.95	63	5 hr.
11.4	776	6.3	131	15 min.
9.0	382	6.0	113	30 min.
8.1	278	0.0	0.0	45 min.
8.25	294	0.6 (2)		6 hr.
		0.9 (2)		
		1.5	3	
8.1	278	3.3	19	15 min.
8.25	294	0.6 (2)		30 min.
		3.3	19	

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 7/15/63
Date of Observation: 10/4/63
Cell Type: Senescent
Microculture: 3 (cell C)

nucleolus diameter μ	nucleolus volume μ3	vacuole diameter μ	vacuole volume μ3	time of observation
6.6	150	1.8	3	
6.6	150	1.8	3	15 min.
6.6	150	1.8	3	30 min.
6.9	172	3.0	14	45 min.
6.9	172	3.9	31	1 hr.
6.9	172	3.9	31	15 min.
6.9	172	4.5	48	30 min.
7.8	249	5.4	82	45 min.
7.5	221	4.8	58	2 hr.
7.5	221	5.1	70	15 min.
7.5	221	5.1	70	30 min.
7.5	221	5.1	70	45 min.
7.5	221	5.1	70	3 hr.
7.5	221	5.4	82	15 min.
7.65	234.5	5.4	82	30 min.
7.8	249	5.4	82	45 min.
7.95	263	5.7	97	4 hr.
8.1	278	6.0	113	15 min.
8.1	278	6.0	113	30 min.
8.4	310.5	6.6	150	45 min.
8.1	278	6.0	113	5 hr.
8.1	278	6.3	131	15 min.
8.7	345	6.9	172	30 min.
8.7	345	6.9	172	45 min.
8.7	345	6.9	172	6 hr.
8.4	310.5	6.9	172	15 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/23/63
Date of Observation: 10/7/63
Cell Type: Senescent
Microculture: 10 (cell A)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
7.8	249	6.0	113	
7.8	249	6.0	113	15 min.
6.9	172	0.0	0.0	30 min.
6.6	150	1.2		45 min.
		0.6	1	
6.9	172	0.6		1 hr.
		0.9		
		1.5	2	
7.05	180	0.6 (3)		15 min.
		0.9		
		1.5	2	
6.6	150	0.6		30 min.
		0.9		
		1.8	3.5	
6.9	172	1.2 (2)		45 min.
		2.7	11	
7.2	195	0.6		2 hr.
		2.1		
		2.7	15	
7.5	221	3.9	31	15 min.
7.5	221	4.2	39	30 min.
7.2	195	3.6	24.5	45 min.
7.8	249	4.5	48	3 hr.
8.1	278	4.65	53	15 min.
7.8	249	4.2	39	30 min.
8.1	278	5.4	82	45 min.
6.3	131	0.0	0.0	52 min.
6.6	150	0.6 (3)	0.4	4 hr.
6.6	150	0.6 (3)	0.4	15 min.
6.6	150	0.6 (5)	0.6	30 min.
7.2	195	0.6		45 min.
		0.9 (2)		
		1.2 (2)	3	
6.9	172	0.6		5 hr.
		0.9 (2)		
		1.5	3	

6.9	172	0.9 (3)		15 min.
		1.2 (2)	3	
6.9	172	1.8 (2)	6	30 min.
6.9	172	1.95 (2)	4	45 min.
7.2	195	1.5		6 hr.
		1.8 (2)	8	
7.2	195	4.5	48	15 min.
7.2	195	4.35	43	30 min.
7.2	195	4.2	39	45 min.
7.2	195	3.9	31	7 hr.
6.0	113	0.0	0.0	15 min.
6.0	113	0.6 (2)	0.2	30 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/23/63
Date of Observation: 10/7/63
Cell Type: Young. Active
Microculture: 10 (cell B)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
10.2	555.5	8.7	345	
10.2	555.5	8.7	345	15 min.
10.5	606	9.0	382	30 min.
10.0	524	9.0	382	45 min.
10.2	555.5	8.7	345	1 hr.
10.2	555.5	8.7	345	15 min.
10.2	555.5	9.0	382	30 min.
10.2	555.5	9.0	382	45 min.
10.8	660	9.0	382	2 hr.
8.1	278	4.2	39	15 min.
8.1	278	3.9	31	20 min.
8.1	278	4.2	39	35 min.
8.7	345	4.8	58	50 min.
8.7	345	4.8	58	3 hr.
9.0	382	5.1	70	15 min.
9.0	382	5.1	70	30 min.
8.1	278	3.9	31	45 min.
8.25	294	4.5	48	4 hr.
8.4	310.5	4.8	58	15 min.
8.4	310.5	4.8	58	30 min.
8.4	310.5	4.8	58	45 min.
8.7	345	5.4	82	5 hr.
8.4	310.5	4.8	58	15 min.
8.85	363	5.7	97	30 min.
8.7	345	5.4	82	45 min.

8.7	345	5.4	82	6 hr.
8.7	345	5.4	82	15 min.
8.7	345	5.4	82	30 min.
9.0	382	6.0	113	45 min.
9.0	382	6.0	113	7 hr.
9.0	382	5.85	105	15 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/23/63
Date of Observation: 10/7/63
Cell Type: Active. Elongating
Microculture: 10 (cell C)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
11.4	776	8.7	345	
11.4	776	8.4	310.5	15 min.
11.4	776	8.25	294	30 min.
11.4	776	8.25	294	45 min.
11.7	839	9.0	382	1 hr.
9.0	382	0.0	0.0	15 min.
9.0	382	0.9		30 min.
		1.2	1	
9.0	382	0.6 (2)		45 min.
		2.4	7.5	
9.3	421	0.6 (6)	0.7	2 hr.
9.3	421	0.6 (8)		15 min.
		2.1	6	
9.6	463	0.6 (9)		30 min.
		1.2	2	
10.2	555.5	0.6 (2)		45 min.
		0.9 (2)		
		1.2		
		1.5 (3)	7	
9.9	508	0.6		3 hr.
		0.9		
		1.2 (2)		
		2.1	7	
9.6	463	0.9 (2)		15 min.
		2.4 (2)	15	
9.15	401	1.2 (2)		30 min.
		2.4 (2)	16	
9.15	401	0.6		45 min.
		1.2 (2)		
		1.5		
		1.8		
		2.7	17	

9.9	508	0.9		4 hr.
		1.2		
		1.5		
		1.8		
		2.7	16	
9.9	508	0.9		15 min.
		1.2		
		1.8		
		2.4		
		2.7	22	
10.05	524	1.2 (2)		30 min.
		2.7		
		3.3	30	
9.9	508	3.0		45 min.
		3.6	38.5	
9.9	508	3.3		5 hr.
		3.6	43	
9.75	485	3.6 (2)	49	15 min.
9.75	485	3.6 (2)	49	30 min.
9.9	508	0.6 (2)		45 min.
		3.6 (2)	49	
9.6	463	0.6 (2)		6 hr.
		5.4	82.5	
10.2	555.5	5.25	76	15 min.
10.2	555.5	0.6 (3)		30 min.
		5.4	82.5	
9.0	382	0.9 (3)		45 min.
		5.4	83	
9.9	508	4.5	48	7 hr.
9.0	382	0.9 (3)		15 min.
		1.2 (2)	3	

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/5/63
Date of Observation: 10/11/63
Cell Type: Active. Elongating
Microculture: 8-C (cell B)

nucleolus diameter μ	nucleolus volume μ3	vacuole diameter μ	vacuole volume μ3	time of observation
9.0	382	6.0	113	
9.0	382	6.0	113	15 min.
9.0	382	6.0	113	30 min.
9.0	382	6.0	113	45 min.
9.0	382	6.0	113	1 hr.

9.0	382	6.0	113	15 min.
9.3	421	6.3	131	30 min.
9.3	421	6.6	150	45 min.
9.3	421	6.3	131	2 hr.
9.3	421	6.3	131	15 min.
9.3	421	6.6	150	30 min.
9.3	421	6.6	150	45 min.
9.15		7.2	195	3 hr.
9.6	463	6.9	172	15 min.
9.9	508	6.9	172	30 min.
9.6	463	7.05	180	45 min.
9.9	508	6.9	172	4 hr.
9.9	508	6.9	172	15 min.
9.9	508	6.9	172	30 min.
9.6	463	6.6	150	45 min.
9.3	421	6.9	172	5 hr.
9.0	382	6.9	172	15 min.
9.3	421	6.9	172	30 min.
9.3	421	6.9	172	45 min.
9.3	421	6.9	172	6 hr.
9.3	421	6.9	172	15 min.
9.6	463	7.05	180	30 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/5/63
Date of Observation: 10/11/63
Cell Type: Active. Elongating
Microculture: 8-C (cell C)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
7.35	208	0.0	0.0	
7.2	195	0.6 (4)		15 min.
		0.9	0.5	
7.5	221	0.6 (5)		30 min.
		1.2	1.0	
7.5	221	1.87	3	45 min.
7.5	221	1.8	3	1 hr.
7.2	195	0.0	0.0	15 min.
7.2	195	0.6 (4)	0.45	30 min.
7.2	195	1.2	0.89	45 min.
7.35	208	0.6 (5)		2 hr.
		1.2	1	
7.5	221	0.6 (3)		15 min.
		1.5	2	

7.35	208	1.65		30 min.
7.5	221	0.0	0.0	45 min.
6.75	161	0.0	0.0	3 hr.
6.9	172			15 min.
7.2	195	1.5	2	30 min.
7.35	208	1.8	3	45 min.
7.5	221	0.6 (2)		4 hr.
		1.5	2	
7.5	221			15 min.
7.5	221	0.6 (8)	0.8	30 min.
7.2	195	0.6 (5)		45 min.
		0.9	0.95	
7.2	195	0.6 (3)	0.34	5 hr.
6.9	172	0.0	0.0	15 min.
6.9	172	0.0	0.0	30 min.
7.2	195	0.6 (4)	0.45	45 min.
7.2	195			6 hr.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/5/63
Date of Observation: 10/16/63
Cell Type: Active. Elongating
Microculture: 6-C (cell A)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
11.52	800	0.96 (2)		
		1.44		
		1.92	6	
11.52	800	0.96 (3)		15 min.
		1.44	3	
12.0	905	0.48 (4)		30 min.
		2.4 (4)	30	
12.0	905	0.48 (2)		45 min.
		1.92 (2)		
		2.4	15	
11.76	852	0.48		1 hr.
		0.96		
		1.92 (2)		
		2.88	20	
12.0	905	1.92		15 min.
		2.4	11	
11.76	852	0.48 (2)		30 min.
		2.4	7	
11.76	852	0.48 (7)	0.4	45 min.

11.76	852			2 hr.
12.0	905	0.96 (6)	3	15 min.
12.0	905	0.48 (3)		30 min.
		1.44 (3)		
		1.92		
		2.4	16	
11.52	800	0.96		45 min.
		1.44 (2)		
		1.92	7	
11.52	800	0.48 (2)		3 hr.
		1.92		
		2.4	11	
11.52	800	0.48 (2)		15 min.
		0.96 (2)		
		2.4 (2)	15.5	
11.76	852	0.96 (3)		30 min.
		2.4		
		2.88	21	
11.52	800	1.44		45 min.
		2.4		
		2.88	21	
11.28	751	1.92		4 hr.
		2.88		
		3.84	46	
11.28	751	2.88		15 min.
		3.84	42	

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/5/63
Date of Observation: 10/16/63
Cell Type: Young. Active
Microculture: 6-C (cell B)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
7.68	237	3.84	30	
7.68	237	3.84	30	15 min.
8.16	284	4.8	58	30 min.
8.16	284	4.56	50	45 min.
8.16	284	5.28	77	1 hr.
6.72	159	0.48 (2)		15 min.
		1.44	2	
6.72	159	2.4		30 min.
		0.48 (5)	7.5	
7.2	195	0.96 (3)		45 min.
		2.88	14	

7.68	237	0.96		2 hr.
		1.44		
		1.92		
		3.36	26	
7.68	237	0.96 (3)		15 min.
		3.84	31	
7.2	195	0.96 (2)		30 min.
		4.32	43	
6.72	159	1.44		45 min.
		1.92	5	
6.72	159	1.44 (2)		3 hr.
		1.92	7	
7.2	195	1.92 (2)		15 min.
		2.4	14.5	
7.44	216	1.92		30 min.
		2.88		
		3.36	36	
7.68	237	4.8	58	45 min.
7.68	237	0.96		4 hr.
		4.8	59	
7.68	237	4.8	58	15 min.

Material

Hybrid Tobacco H-196
Liquid D Medium
Date of Preparation: 8/5/63
Date of Observation: 10/16/63
Cell Type: Active. Elongating
Microculture: 6-C (cell C)

nucleolus diameter μ	nucleolus volume μ^3	vacuole diameter μ	vacuole volume μ^3	time of observation
12.0	905	9.6	463	
12.0	905	9.12	397	15 min.
contraction - no measurement				30 min.
9.12	397	4.8	58	34 min.
9.12	397	5.28	77	45 min.
9.12	397	5.28	77	1 hr.
9.37	431	5.76	100	15 min.
9.37	431	5.28	77	30 min.
9.12	397	5.28	77	45 min.
9.6	463	5.76	100	2 hr.

9.6	463	5.76	100	15 min.
9.6	463	5.76	100	30 min.
9.6	463	5.76	100	45 min.
10.08	524	6.24	127	3 hr.
10.56	616	6.72	159	15 min.
10.56	616	6.72	159	30 min.
10,08	524	6.72	159	45 min.
10.08	524	6.24	127	4 hr.